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PERFIL PROTEÔMICO DA URINA PARA DETECÇÃO DE POTENCIAIS BIOMARCADORES PARA A DOENÇA RENAL CRÔNICA

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UBERLÂNDIA

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"Quem caminha sozinho pode até chegar mais rápido, mas aquele que vai acompanhado com certeza vai mais longe" - Autor desconhecido

“Viva como se você fosse morrer amanhã.

Aprenda como se você fosse viver para sempre.”

- Mahatma Gandhi

RESUMO

Introdução: O número crescente de portadores de doença renal crônica (DRC), causado principalmente por mudanças no estilo de vida e pelo envelhecimento da população, demonstra a necessidade da identificação de novos biomarcadores que possibilitem o monitoramento da progressão da DRC e, consequentemente, a predição para a doença renal em estágio terminal (DRET). **Objetivo:** Analisar o perfil proteômico em amostras de urina de indivíduos saudáveis e no estágio final da doença para identificação de potenciais biomarcadores para a DRC. **Metodologia:** Foram coletadas amostras de urina de 10 indivíduos saudáveis e 10 portadores de DRC em estágio final. Estas amostras foram analisadas de acordo com as seguintes etapas da metodologia shotgun: precipitação das proteínas, detecção colorimétrica e dosagem das proteínas, digestão em solução e dessalinização dos peptídeos. Em seguida, os peptídeos foram analisados em um equipamento de cromatografia líquida acoplado a um espectrômetro de massas em tandem e, por fim, realizou-se pesquisas de bioinformática, ontologia gênica e de interação das proteínas. **Resultados:** Foram identificadas 416 proteínas no perfil proteômico dos grupos analisados e 19 proteínas apresentaram diferenças estatisticamente significativas entre os grupos. Destas, cinco proteínas (hemopexina, beta-2-microglobulina, proteína de ligação ao retinol 4, transtirretina e fator D) foram consideradas potenciais biomarcadores para a DRC. **Conclusão:** As proteínas encontradas foram capazes de caracterizar e diferenciar os perfis proteômicos urinários dos dois grupos. Também, as cinco proteínas selecionadas podem ser vistas como potenciais candidatas a biomarcadores da DRC.

Palavras-chave: Biomarcadores. Doença renal crônica. Progressão da doença. Proteômica.

ABSTRACT

Introduction: The growing number of people with chronic kidney disease (CKD), caused mainly by changes in lifestyle and the aging of the population, demonstrates the need to identify new biomarkers that enable the monitoring of the progression of CKD and, consequently, the prediction for end-stage renal disease (ESRD). **Objective:** To analyze the proteomic profile in urine samples from healthy individuals and those in the final stage of the disease to identify potential biomarkers for CKD. **Methodology:** Urine samples were collected from 10 healthy individuals and 10 with end-stage CKD. These samples were analyzed according to the following steps of the shotgun methodology: protein precipitation, colorimetric detection and protein measurement, in-solution digestion and peptide desalting. Next, the peptides were analyzed using liquid chromatography equipment coupled to a tandem mass spectrometer and, finally, bioinformatics, gene ontology and protein interaction research was carried out. **Results:** 416 proteins were identified in the proteomic profile of the analyzed groups and 19 proteins showed statistically significant differences between the groups. Of these, five proteins (hemopexin, beta-2-microglobulin, retinol-binding protein 4, transthyretin and factor D) were considered potential biomarkers for CKD. **Conclusion:** The proteins found were able to characterize and differentiate the urinary proteomic profiles of the two groups. Also, the five selected proteins can be seen as potential candidates for CKD biomarkers.

Key words: Biomarkers. Chronic kidney disease. Disease progression. Proteomics.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS

ACN	*Acetonitrile*
AUC	Área sob a curva
BCA	*Bicinchoninic acid*
CH_2Cl_2	*Dichloromethane*
CH_3OH	*Methanol*
CKD-EPI	*Chronic Kidney Disease Epidemiology Collaboration*
CVD	*Cardiovascular disease*
DCNT	Doenças crônicas não transmissíveis
DM	Diabetes melito
DP	Diálise peritoneal
DRC	Doença renal crônica
DRET	Doença renal em estágio terminal
DTT	*Dithiothreitol*
FDR	*False Discovery Rate*
GO	*Gene ontology*
HA	Hipertensão arterial
HD	Hemodiálise
IAA	*Iodoacetamide*
IMC	Índice de massa corporal
IPAQ	*International Physical Activity Questionnaire*
KDIGO	*Kidney Disease: Improving Global Outcomes*
LC	Cromatografia líquida
LC-MS/MS	Cromatografia líquida acoplada à espectrometria de massas em tandem
MDRD	*The Modification of Diet in Renal Disease*
MHC	Complexo principal de histocompatibilidade
MS	Espectrometria de massas
NHANES	*National Health and Nutrition Examination Survey*
OMS	Organização Mundial da Saúde

OPLS-DA	*Orthogonal discriminant analysis by partial least squares*
PANTHER	*Protein Analysis Through Evolutionary Relationships*
ROC curve	*Receiver operating characteristic curve*
SBN	Sociedade Brasileira de Nefrologia
STRING	*Search Tool for the Retrieval of Interacting Genes/Proteins*
SUS	Sistema Único de Saúde
TFA	*Trifluoroacetic acid*
TFG	Taxa de filtração glomerular
TRS	Terapia renal substitutiva
Uniprot	*The Universal Protein Resource*
VIP	*Importance of the variable in the projection*

SUMÁRIO

1 - Introdução

As doenças crônicas não transmissíveis (DCNT) são consideradas um importante problema de saúde pública pois, são responsáveis por aproximadamente 38 milhões de mortes por ano em todo o mundo (Carvalho *et al*., 2021). Estas doenças são caracterizadas pela longa duração e origem não-infecciosa, sendo o resultado de fatores genéticos, ambientais, fisiológicos e comportamentais (Silva *et al.,* 2022). Dentre as DCNT, destaca-se a doença renal crônica (DRC) (Brasil, 2014).

A DRC é definida como uma perda progressiva da função renal associada a consequente redução da capacidade de filtração do sangue e da manutenção da homeostase (Aguiar *et al.,* 2020). Esta doença pode ser determinada pela taxa de filtração glomerular (TFG) inferior a 60mL/min/1,73m² ou pela TFG superior a 60mL/min/1,73m² associada a pelo menos um marcador de dano renal – como a albuminúria – por um período de três meses ou mais, independentemente da causa (KDIGO, 2013).

Com relação à prevalência global, estima-se que aproximadamente 843,6 milhões de pessoas foram acometidas por esta doença (Kovesdy, 2022) no ano de 2017. Já no Brasil, presume-se que mais de dez milhões de pessoas possuam a DRC (Brasil, 2019). Assim, esta doença está associada com taxas elevadas de morbidade e mortalidade e alto impacto socioeconômico em todo o mundo (Aguiar *et al.,* 2020).

O diagnóstico precoce da DRC é essencial para diminuir a morbidade e mortalidade dos pacientes por meio da adoção de medidas capazes de reduzir a progressão da DRC para o estágio terminal (Dumont *et al.,* 2021). Contudo, nos estágios iniciais desta doença, são percebidos poucos sinais e/ou sintomas no indivíduo, o que dificulta a detecção precoce (Lee *et al.,* 2020). Soma-se a isso o fato de que os parâmetros utilizados atualmente não são considerados sensíveis para detecção desta doença nos primeiros estágios (Dumont *et al.,* 2021).

Diante do exposto, percebe-se a necessidade de identificar novos biomarcadores para a realização do diagnóstico e do monitoramento da progressão da DRC para o estágio terminal (Hocher & Adamski, 2017). A abordagem ômica é considerada um meio para a descoberta destes biomarcadores, que podem ser medidos em amostras de tecido, saliva, urina, sangue, entre outros (Hocher & Adamski, 2017). Dentre as técnicas utilizadas, destaca-se a análise

proteômica de amostras de urina visto que, a coleta deste material biológico é feita de forma simples e não invasiva (Good *et al.,* 2010).

Dessa forma, o presente estudo se justifica pela proposta de comparar o perfil proteômico de dois grupos – sendo um grupo de indivíduos saudáveis e o outro formado por pacientes no estágio final da DRC – por meio da análise de amostras de urina em um equipamento de cromatografia líquida associado à espectrometria de massas em tandem (LC-MS/MS). Até o momento, existem poucos estudos publicados envolvendo a proteômica urinária e a DRC em estágio final (Nkuipou-Kenfach *et al.,* 2014; Zhao *et al.,* 2021), portanto, este estudo pode contribuir com novos achados a respeito deste tema.

2 - Fundamentação teórica

2.1 – Doença renal crônica

A DRC é uma condição progressiva que envolve mudanças funcionais e estruturais nos rins (Kalantar-Zadeh *et al.,* 2021), como: perda irreversível de néfrons, redução da capacidade de regeneração, danos microvasculares, estresse oxidativo, inflamação e fibrose (Ruiz-Ortega *et al*., 2020).

Os principais fatores de risco para o desenvolvimento desta doença são: hipertensão arterial (HA), diabetes melito (DM), dislipidemia, obesidade, sedentarismo, tabagismo, entre outros (Lo *et al*., 2023). De acordo com o Censo Brasileiro de Diálise realizado no ano de 2021, a HA se destaca como a doença de base predominante nos portadores em terapia renal substitutiva (TRS), o que corresponde a 32% do total de casos (Nerbass *et al.,* 2022).

A idade avançada também é um fator que pode contribuir para o aumento do risco de desenvolvimento da DRC, visto que, o envelhecimento fisiológico leva à redução do fluxo sanguíneo renal e ao aumento da permeabilidade da membrana dos glomérulos renais (Malta *et al*., 2019). Em concordância, dados do inquérito *National Health and Nutrition Examination Survey* (NHANES) – realizado com a população dos Estados Unidos da América no ano de 2018 – evidenciaram um aumento da prevalência de DRC à medida em que a idade progredia, sendo de 6,6% em indivíduos de 20 a 39 anos, 10,6% na faixa etária de 40 a 59 anos e 32,6% em pessoas com 60 anos ou mais de idade (Centers for Disease Control and Prevention, 2018).

Uma revisão sistemática encontrou a prevalência de 11-13% de DRC em todo o mundo, sendo que as maiores taxas foram observadas no sexo feminino e em países desenvolvidos (Hill *et al*., 2016). Também, estima-se que 1,2 milhões de pessoas morreram no ano de 2017 devido a esta doença (GBD, 2020).

2.2 – Métodos diagnósticos da DRC

A DRC pode ser classificada por meio da TFG, que é definida como o volume de líquido filtrado dos capilares glomerulares para a cápsula de Bowman por unidade de tempo (López-Giacoman, Madero, 2015). Entretanto, a medição direta desta taxa é inviável pois, o processo de filtração ocorre simultaneamente em milhões de glomérulos e dessa forma, há alteração no volume e na composição do filtrado. Sendo assim, a TFG pode ser medida indiretamente por meio da eliminação de marcadores de filtração glomerular, como a inulina. A inulina é derivada de um polímero de frutose e é uma substância fisiologicamente inerte, tornando-a ideal para a

medição da TFG. Porém, devido à necessidade de infusões contínuas e várias coletas de amostras de sangue e urina, este método apresenta um alto custo e é considerado invasivo (López-Giacoman, Madero, 2015).

A TFG também pode ser estimada por meio de marcadores endógenos, como a cistatina C e a creatinina sérica. Na prática clínica, a creatinina é a mais utilizada em função da disponibilidade e do baixo custo, porém, não é considerada um parâmetro sensível nos estágios iniciais da doença visto que, a alteração nos níveis deste marcador ocorre após a redução de 50 a 60% da TFG, ou seja, a sua utilização pode contribuir para o subdiagnóstico precoce da DRC (Malta *et al*., 2019). Também, esta medida pode ser influenciada por diversos fatores, como: quantidade de massa muscular, secreção dos túbulos renais, dieta hiperproteica, atividade física, entre outros (Zou *et al.,* 2020). Com relação à cistatina C, vale ressaltar que esta também possui algumas limitações para a medição, isto é, os níveis deste marcador podem estar alterados nas seguintes situações: inflamação, tratamento com uso de medicamentos esteroides e disfunção na tireoide (Cañadas-Garre *et al.,* 2018a).

Quanto ao cálculo da estimativa da TFG, algumas fórmulas podem ser utilizadas. Dentre elas, as mais conhecidas são: Cockcroft Gault (1976*), The Modification of Diet in Renal Disease* (MDRD) (Levey *et al*., 1999) e *The Chronic Kidney Disease Epidemiology Collaboration* (CKD-EPI) (Levey *et al.,* 2009). Atualmente, a fórmula CKD-EPI é recomendada pelo Ministério da Saúde do Brasil e pelo *Kidney Disease: Improving Global Outcomes* (KDIGO) (KDIGO, 2013; Brasil, 2014).

De acordo com KDIGO (2013), a DRC pode ser categorizada em 6 estágios por meio do valor da TFG. Esta classificação está representada no quadro a seguir:

Quadro 1. Classificação da DRC segundo o valor da TFG.

Estágio da DRC	Definição	Valor da TFG
1	Lesão renal com TFG normal ou aumentada	≥ 90
2	Lesão renal com TFG ligeiramente diminuída	60-89,9
3A	Lesão renal com TFG moderadamente diminuída	45-59,9
3B	Lesão renal com TFG moderada a severamente diminuída	30-44,9
4	Lesão renal com TFG severamente diminuída	15-29,9
5	Falência renal	< 15

Fonte: Adaptado de KDIGO (2013)

O dano renal pode ser avaliado por meio da medida da albuminúria ou proteinúria, que são marcadores associados a rápida evolução da doença. Entretanto, estes marcadores nem sempre estão presentes no indivíduo com DRC ou podem variar de acordo com o estágio da doença (KDIGO, 2013).

Na medida em que a lesão renal progride, podem aparecer diversas complicações no organismo, como: anemia, distúrbio mineral e ósseo, doenças cardiovasculares, anorexia, fadiga, náuseas, desordens plaquetárias, acidose metabólica, entre outras (Bello *et al.,* 2017).

2.3 – Tipos de tratamento da DRC

O tratamento conservador possui o intuito de desacelerar a progressão da DRC para os estágios mais avançados, reduzir os sintomas e prevenir as complicações associadas a esta doença. Assim, recomenda-se o controle da glicemia, da pressão arterial e da dislipidemia; interrupção do tabagismo; uso de medicamentos para reduzir a proteinúria; realização de atividade física e adesão a uma alimentação saudável (BRASIL, 2014; SBN, 2023a). De acordo com a Sociedade Brasileira de Nefrologia (SBN), a alimentação na fase conservadora baseia-se na restrição de proteínas (principalmente de origem animal) e na moderação do consumo de alimentos ricos em fósforo e potássio (SBN, 2023b).

Quando o indivíduo se encontra no estágio de falência renal, faz-se necessária a utilização da TRS, que pode ser categorizada em três modalidades: hemodiálise (HD), diálise peritoneal (DP) e transplante renal. A HD é um procedimento realizado por meio de uma máquina que possui um cateter com a finalidade de filtrar o sangue e, consequentemente, eliminar o excesso de toxinas, sais minerais e líquidos do organismo (Ribeiro, Jorge, Queiroz, 2020). Já a DP é um tipo de diálise que utiliza o peritônio no abdome do paciente como uma membrana por meio da qual ocorre a troca entre o líquido infundido e o sangue (Htay *et al.,* 2021). Por fim, o transplante renal é um procedimento cirúrgico que tem como intuito a transferência de um rim saudável de um doador vivo ou falecido para o paciente com doença renal em estágio terminal (DRET) (SBN, 2023c). Em relação à alimentação, destaca-se o aumento na ingestão de proteínas para compensar a perda durante o processo de diálise, além do controle do consumo de alimentos fonte de fósforo e de potássio (SBN, 2023b).

No Brasil, o Sistema Único de Saúde (SUS) foi a principal fonte de financiamento de clínicas de diálise no ano de 2021, sendo responsável por 81,8% do total (Nerbass *et al.,* 2022). Além disso, entre os anos de 2011 e 2021, o número estimado de pacientes em diálise crônica

ampliou de 91.314 para 148.363 (Nerbass *et al.,* 2022). Este aumento pode estar relacionado a mudanças no estilo de vida e ao envelhecimento da população (Silva *et al*., 2016).

2.4 – Abordagens ômicas

As abordagens ômicas são definidas como análises coletivas de células, tecidos, órgãos ou todo o organismo em nível molecular (Govender *et al*., 2021). Estas possuem o objetivo de compreender o funcionamento celular e as alterações biológicas que ocorrem em um organismo. Dentre as abordagens mais utilizadas, pode-se citar a genômica (estudo da alteração dos genes), a transcriptômica (estudo da alteração dos transcritos), a metabolômica (estudo das alterações dos metabólitos) e a proteômica (estudo das alterações das proteínas) (Canuto *et al*., 2018).

Também, as abordagens ômicas são consideradas um meio para a descoberta de biomarcadores, que são medidos em amostras de tecido, saliva, urina, sangue, entre outros (Hocher & Adamski, 2017). Estes biomarcadores podem ser usados em diversas doenças para diagnóstico, estratificação de risco, predição de resposta ao tratamento, entre outras aplicações (Zabetian, Coca, 2021).

A proteômica é descrita como o estudo do proteoma (conjunto completo de proteínas expressas por um organismo) (Cañadas-Garre *et al*., 2018b). Esta técnica engloba a análise de peptídeos derivados de digestão enzimática e a análise das proteínas intactas (Provenzano *et al.,* 2021). Diferentemente do genoma, o proteoma é uma entidade dinâmica que pode sofrer alterações em decorrência dos processos celulares. Desse modo, a pesquisa do proteoma se torna uma oportunidade para identificar e caracterizar moléculas inerentes a processos patológicos específicos (Vieira, 2021).

2.5 – Análise proteômica da urina

Em condições fisiológicas normais, a urina pode conter baixos níveis de proteínas de pequeno peso molecular, porém, em situações patológicas, a excreção das proteínas pela urina pode ser intensificada (Cañadas-Garre *et al*., 2018b). Assim, a urina tem sido um dos fluidos biológicos mais utilizados para investigação a nível proteômico, visto que esta é considerada uma fonte importante de biomarcadores para várias doenças devido a alterações específicas no proteoma (Good *et al*., 2010). Também, a coleta deste material biológico é realizada de forma simples e não invasiva (Provenzano *et al.,* 2021).

Ademais, sabe-se que a análise proteômica de amostras de urina apresenta um potencial para o diagnóstico e/ou monitoramento da progressão da doença renal diabética (Fan *et al.,* 2021), nefropatia membranosa primária (Pang *et al.,* 2018), doença cardiovascular associada a DRC (Verbeke *et al*., 2021), entre outras.

Quanto às técnicas que podem ser utilizadas para separação das proteínas, destaca-se a eletroforese capilar, a espectrometria de massas (MS) e a cromatografia líquida (LC). A eletroforese capilar é uma técnica que utiliza corrente elétrica com o objetivo de fragmentar as proteínas por meio da migração de partículas de acordo com os pesos moleculares ou por diferença de carga elétrica (Oliveira *et al*., 2015). A MS consiste na ionização de moléculas seguida pela separação dos íons de acordo com as razões massa/carga para subsequente detecção, o que resulta no espectro de massas (Lyrio *et al*., 2022). Por fim, a LC é a técnica que utiliza uma fase móvel líquida para realizar a separação dos componentes de uma mistura (Akash, Reman, 2020).

A LC-MS/MS é uma tecnologia que apresenta alta resolução para identificação e quantificação de proteínas com elevada precisão e sensibilidade para detecção do proteoma urinário (Fang *et al.,* 2020).

3 - Objetivos

3.1 – Geral

O objetivo geral deste estudo foi analisar o perfil proteômico em amostras de urina de indivíduos saudáveis e com DRC em hemodiálise para identificação de potenciais biomarcadores para a doença.

3.2 – Específicos

- Descrever as variáveis clínicas, sociodemográficas e de estilo de vida dos participantes da amostra;
- Investigar o perfil de proteínas, por espectrometria de massas, em amostras de urina nos indivíduos saudáveis e portadores de DRC em hemodiálise;
- Identificar potenciais candidatos a biomarcadores a partir da diferença no perfil das proteínas entre os grupos analisados;
- Realizar a análise de ontologia gênica e de interação das proteínas com diferença estatística entre os grupos;
- Descrever o mecanismo de ação das proteínas candidatas a biomarcadores.

4 - Artigo

Urine proteomic profile for detection of potential biomarkers for chronic kidney disease

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Abstract: The growing number of people with chronic kidney disease (CKD), caused mainly by changes in lifestyle and the aging of the population, demonstrates the need to identify new biomarkers that enable the monitoring of the progression of CKD and, consequently, the prediction for end-stage renal disease (ESRD). The aim of this study was to analyze the proteomic profile in urine samples from healthy individuals and those in the final stage of the disease to identify potential biomarkers for CKD. Urine samples were collected from 10 healthy individuals and 10 with end-stage CKD. These samples were analyzed according to the following steps of the shotgun methodology: protein precipitation, colorimetric detection and protein measurement, in-solution digestion and peptide desalting. Next, the peptides were analyzed using liquid chromatography equipment coupled to a tandem mass spectrometer and, finally, bioinformatics, gene ontology and protein interaction research was carried out. 416 proteins were identified in the proteomic profile of the analyzed groups and 19 proteins showed statistically significant differences between the groups. Of these, five proteins (hemopexin, beta-2-microglobulin, retinol-binding protein 4, transthyretin and factor D) were considered potential biomarkers for CKD. It is concluded that the proteins found were able to characterize and differentiate the urinary proteomic profiles of the two groups. Also, the five selected proteins can be seen as potential candidates for CKD biomarkers.

Key words: Biomarkers. Chronic kidney disease. Disease progression. Proteomics.

Introduction

Chronic kidney disease (CKD) is characterized by a progressive loss of renal function associated with the consequent reduction of blood filtration capacity and maintenance of homeostasis [1, 2]. CKD can be determined by glomerular filtration rate (GFR) less than 60ml/min/1.73m², associated with at least one marker of kidney damage - such as albuminuria - for a period of three months or more, regardless of cause [2].

CKD is a serious public health problem. It is estimated that 843.6 million people were affected by this disease [3] and 1.2 million patients died in 2017 [4]. This disease is associated with high morbidity and mortality rates and high socioeconomic impact worldwide [1].

Early diagnosis of CKD is essential to reduce morbidity and mortality of patients through the adoption of measures capable of reducing the progression of CKD to the terminal stage [5]. However, in the initial stages of this disease, few signs and/or symptoms are perceived in the individual, which makes early detection difficult [6]. Added to this is the fact that the parameters currently used are not considered sensitive for detection of this disease in the initial stages [5]. The change in creatinine levels, for example, occurs after the decrease of about 50 to 60% of GFR, that is, the use of this test can contribute to the early sub diagnosis of CKD [7].

Given the above, it is necessary to identify new biomarkers for the diagnosis and monitoring of the progression of CKD [8]. The omics approach is considered a means for the discovery of these biomarkers, which can be measured in tissue samples, saliva, urine, blood, among others [8]. Among the techniques used, the proteomic analysis of urine samples stands out since the collection of this biological material is simple and non-invasive [9]. In addition, urine is considered an important source of biomarkers for various diseases due to specific changes in the proteome [9].

It is known that the proteomic analysis of urine samples has a potential for the diagnosis and/or monitoring of the progression of diabetic kidney disease (DKD) [10], primary membranous nephropathy [11], cardiovascular disease associated with CKD [12], among others.

Thus, the present study is justified by the proposal to compare the proteomic profile of two groups - one group of healthy individuals and the other formed by patients in the final stage of CKD - through the analysis of urine samples in a liquid chromatography equipment associated with tandem mass spectrometry (LC-MS/MS). It is worth noting that LC-MS/MS is a technology that presents high resolution for identification and quantification of proteins with high precision and sensitivity for detection of urinary proteome [13] and, so far, there are few published studies involving urinary proteomics and CKD in the final stage [14, 15].

Therefore, the aim of this study was to analyze the proteomic profile in urine samples of healthy individuals and with CKD in hemodialysis to identify potential biomarkers for the disease.

Materials and methods

Sample design and selection:

This is a study conducted with two groups, the control group was composed of 10 healthy individuals and the hemodialysis group was consisted of 10 patients with end-stage CKD.

The subjects of the control group were selected at the Clinical Analysis Laboratory of the Clinical Hospital of the Federal University of Uberlandia (HC-UFU). This group included those who were 18 or older and did not have diabetes mellitus (DM), arterial hypertension (AH), cardiovascular disease (CVD) and kidney disease (acute or chronic).

The subjects of the hemodialysis group were invited to the Hemodialysis Sector of HC-UFU. The inclusion criteria for this audience were: to be aged 18 or older, to present estimated GFR equal to or less than 15ml/min/1.73m² and to perform hemodialysis for more than one year.

In both groups, individuals with severe clinical conditions, as well as pregnant women and individuals with history of alcohol and/or drug abuse were excluded.

Data collection:

Sociodemographic (sex, age, race/color, schooling, and family income), behavioral (smoking, alcoholism, and sedentary lifestyle), clinical (presence of comorbidities, blood pressure and fasting glucose values), anthropometric (weight and height) and biochemistry (by collecting urine and blood samples) variables were collected. Anthropometric data and blood test results were obtained from the medical records of the participants.

For the collection of information regarding sociodemographic, behavioral, and clinical variables, a semi-structured interview script was used. To assess the level of physical activity, the short version of the International Physical Activity Questionnaire (IPAQ) proposed by the World Health Organization (WHO) and validated in Brazil was used [16]; after the application of this questionnaire, the participants were classified as "very active"/"active", "irregularly active"/"sedentary". The Body Mass Index (BMI) was calculated through the relationship between weight and height squared and classified according to the criteria of Lipschitz for the elderly [17] and WHO for adults [18]. In the case of patients in the hemodialysis group, the dry weight data was used to calculate the BMI. Also, for comparison purposes, the data of "overweight" and "obesity" were categorized as "overweight".

For the analysis of renal function, participants' GFR was estimated using the formula Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [2].

328 Urine samples from all participants were collected in sterile tubes of the brand FirstLab,
329 after clarification on the procedure and delivery of the containers for collection. These samples
330 were stored in the ultra-freezer of the brand Thermo Scientific Data Med at a temperature of -
331 81ºC.

332 *Ethical aspects:*

333 The study was approved by the Ethics Committee in Research with Human Beings
334 (CEP) of the Federal University of Uberlandia (UFU), under opinion number 4,430,315.

335 According to resolution Nº 466/2012 of the National Health Council, the participants
336 signed the Informed Consent Form (ICF), after the doubts were resolved and the guarantee that
337 they would not have the identities disclosed.

338 *Proteomic analysis:*

339 Regarding sample preparation, 300 microliters (µl) of urine, 300 µl of dichloromethane
340 (CH_2Cl_2) and 1200 µl of methanol (CH_3OH) were added. After that, this mixture was
341 homogenized by means of the vortex agitator and centrifuged at 9000 x g for 1 minute. It was
342 added 900 µl of Milli-Q (ultrapure) water on the supernatant and the contents of the aliquots
343 were homogenized and centrifuged at 9000 x g for 2 minutes. Then the supernatant was
344 discarded and 900 µl of CH_3OH was added to the pellet, which was shaken and centrifuged.
345 The supernatant was removed, and the pellet was dried in the vacuum concentrator.

346 After precipitation, the pellets contained in the microtubes were resuspended in 50 µl of
347 Milli-Q water to perform colorimetric detection and protein quantification, using Pierce ™
348 BCA Protein Assay Kit based on bicinchoninic acid ™ (BCA), according the manufacturer's
349 guidelines [19].

350 Soon after, the digestion step was performed in solution and 75 micrograms (µg) of
351 proteins in samples were used in final volume of 50 µl. The samples were treated with 1% of

surfactant (RapiGest SF – Waters: 186002123), 0.5 molar (M) of dithiothreitol (DTT) and 0.5M of iodoacetamide (IAA). [20]. The samples were digested by trypsin (20 ng/µl) at 37°C, overnight.

Subsequently, 400 µl of 0.5% trifluoroacetic acid (TFA) was added to the samples, which were kept at room temperature for 40 minutes. After that, they were centrifuged at 14.500 x g for 10 minutes and the supernatants were taken to a vacuum concentrator. The samples were resuspended in 100 µl of Milli-Q water.

For the desalination process of peptides, resin C18 (Omix, Agilent) was used according to the manufacturer's guidelines. Two washes were performed in acetonitrile (ACN) 50%, followed by two washes in TFA 0.1%. The samples were passed through the column, by the up-down movement. The resin was washed again with TFA 0.1% and eluted with 100 µl ACN 50% + TFA 0.1%. Soon after, the eluted peptides were processed with a vacuum concentrator and resuspended in TFA 0.1%.

Regarding the analyzes, these were performed in a liquid chromatograph (Agilent Infinity 1260), coupled to a high-resolution mass spectrometer with electrospray ionization source (Agilent 6520B Q-TOF). The chromatographic parameters were: column Agilent model AdvanceBio Peptide Mapping, 2.1 millimeters (mm) internal diameter, 10 centimeters (cm) long, particles of 2.7 micrometers (µm), mobile phase: water (A) and acetonitrile (B) both acidified with formic acid (0.1% vv-1), with gradient: 2% of B (0 min), 2% of B (10 min), 15% of B (40 min), 50% of B (150 min), 70% of B (200 min), 98% of B (220 min), 98% of B (300 min), 100% of B (301 min) and 100% of B (400 min) at a flow rate of 400 µL/min. The ionization parameters were: nebulizer pressure of 45 pound fource per square inch (psi), drying gas at 8L/min at a temperature of 325ºC and energy of 4 kilovolts (kV) in the capillary.

Finally, the raw data were analyzed by the software Spectrum Mill MS Proteomics (Agilent Technologies) to identify the peptides through the spectra that were generated by the LC-MS/MS approach. In this step, $p \leq 0.05$ was used to indicate a statistically significant difference between the groups, using the T-test, in addition to the FDR criterion (False Discovery Rate) to control false positive results [21].

Statistical analysis:

As for the description of sociodemographic, behavioral, and clinical variables, we used the mean and standard deviation for the quantitative variables and, absolute and relative frequency for the qualitative variables. The qualitative variables were compared using the chi-square test and the quantitative variables were analyzed using the T-student test. Statistical data were evaluated using the Stata package (version 14.2) and the level of significance established was $p \leq 0.05$.

Bioinformatics analyses were performed using the MetaboAnalyst Program (version 5.0). Also, the results were expressed on the logarithmic scale of base 10 considering the spectral count. For the analysis of the proteomic profile, a Venn diagram was presented, showing the absolute number of proteins identified in each group. When comparing the two groups, orthogonal discriminant analysis by partial least squares (OPLS-DA) was presented; the Volcano plot, which identifies proteins with a significant difference between the two groups; the variable importance score in the projection (VIP score ≥ 2.0) and the heatmap, which demonstrates the average intensity of proteins with significant differences between the two groups (considering the T-test significance level of $p \leq 0,05$ and fold-change > 2). Finally, analysis of the receiver operating characteristic curve (ROC curve) of the main proteins with significant differences was performed. The area under the curve (AUC) value, which is considered an estimate of behavior of the overall accuracy of the test, can be interpreted as

follows: between 0.5 and 0.6 = failed; between 0.6 and 0.7 = worthless; between 0.7 and 0.8 = poor; between 0.8 and 0.9 = good; above 0.9 = excellent [22].

Enrichment analysis:

Initially, the primary and secondary accesses of the proteins considered statistically significant were collected through the database The Universal Protein Resource (UniProt, https://www.uniprot.org, access on: March 5, 2023). Then, the accessions were inserted in the software Protein Analysis Through Evolutionary Relationships (PANTHER, version 17.0, http://pantherdb.org, access on: March 6, 2023) to perform the analysis of gene ontology (GO) proteins, which included data on molecular function (MF), biological process (BP), cellular component (CC) and protein class (PC). Finally, proteins were included in the Search Tool for the Retrieval of Interacting Genes/Proteins software (STRING, version 12.0, https://string-db.org/, access on August 4, 2023) to identify protein-protein interactions. The interaction network was constructed with a minimum required score of 0.900 (considered "very high").

Criteria used for the definition of biomarker candidate proteins:

The criteria used to define the candidate proteins for biomarkers for CKD were as follows: T-test with p-value ≤ 0.05 corrected by the criterion of FDR, statistically significant differential expression observed by fold-change, production and/or expression in the kidney and/or excretion in the urine, presence only in one of the groups and VIP score value ≥ 2.0.

Results

Characterization of the participants:

Table 1 shows the sociodemographic, behavioral, and biochemical variables of the sample studied. It is noteworthy that in the hemodialysis group, most of the participants were male, had incomplete elementary school and self-declared brown race/color. Regarding the

control group, half of the participants were male, and most had completed higher education and self-declared brown race/color.

As for biochemical tests, the hemodialysis group had a higher average of creatinine, triglycerides and glycemia, while the control group had a higher mean value of total cholesterol.

In addition, the variables that showed a statistically significant difference when comparing the two groups were: age, schooling, tobacco use, level of physical activity, serum creatinine value, GFR value and fasting blood glucose value. Other information can be seen in Table 1.

Table 1. Sociodemographic, behavioral and biochemical characterization of the study participants. Uberlândia, 2023.

Variables		**Control group (n = 10)**	**Hemodialysis group (n = 10)**	**p-value***
Sex	Male, n (%)	5 (41.7)	7 (58.3)	0.361
	Female, n (%)	5 (62.5)	3 (37.5)	
Age in years, mean ± standard deviation		40.3 ± 12.3	60.4 ± 8.2	< 0.001
Schooling	No education / incomplete elementar school, n (%)	0	6 (100)	0.038
	Complete high school / incomplete higher education, n (%)	4 (57.1)	3 (42.8)	
	Complete higher education, n (%)	6 (85.7)	1 (14.3)	
Race/color	White, n (%)	4 (66.7)	2 (33.3)	0.580
	Black, n (%)	1 (33.3)	2 (66.7)	
	Brown, n (%)	5 (45.5)	6 (54.5)	
Marital status	With partner, n (%)	6 (60.0)	4 (40.0)	0.247
	Without a partner, n (%)	4 (40.0)	6 (60.0)	
Nutritional status	Eutrophy, n (%)	5 (45.5)	6 (54.5)	0.337
	Overweight, n (%)	5 (55.5)	4 (44.5)	
Tobacco use	Never smoked, n (%)	10 (66.7)	5 (33.3)	0.010

	Ex smoker, n (%)	0	5 (100)	
Alcohol consumption	Never drinks, n (%)	7 (58.3)	5 (41.7)	0.607
	Less than once a month, n (%)	1 (50.0)	1 (50.0)	
	Once or more a month, n (%)	2 (33.3)	4 (66.7)	
Physical activity	Sedentary or irregularly active, n (%)	0	7 (100)	0.024
	Active or very active, n (%)	10 (76.9)	3 (23.1)	
Creatinine, mean ± standard deviation		0.9 ± 0.1	9.6 ± 1.4	< 0.001
GFR, mean ± standard deviation		94.9 ± 27.1	5.4 ± 1.1	< 0.001
Total cholesterol, mean ± standard deviation		186.3 ± 27.0	172.2 ± 45.1	0.713
Triglycerides, mean ± standard deviation		141.1 ± 40.2	233.1 ± 138.4	0.112
Fasting blood glucose, mean ± standard deviation		83.5 ± 4.9	124.5 ± 45.9	0.011

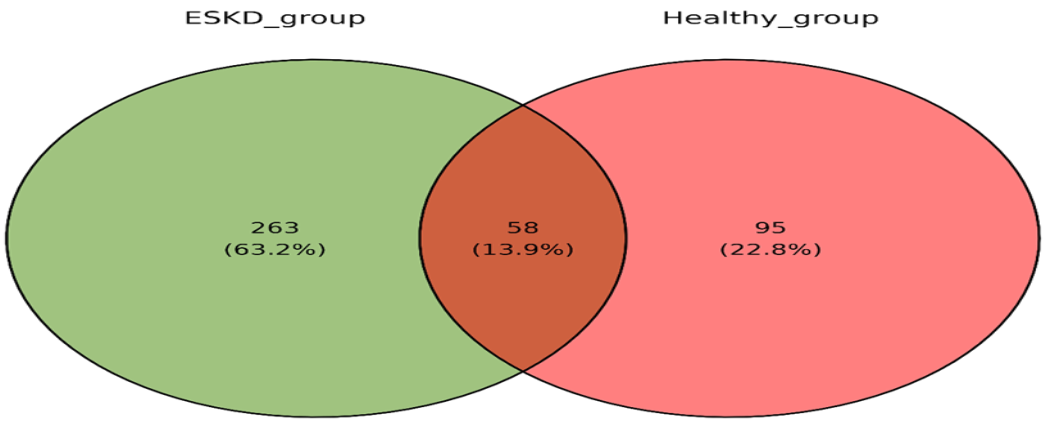
*Significance level $p \leq 0.05$

Urinary proteomic profile:

Regarding the qualitative analysis, 416 proteins were found in the urinary proteomic profile. Of these, 95 proteins were identified only in the control group (22.8%), 263 were detected only in the hemodialysis group (63.2%) and 58 were common in both groups (13.9%).

Figure 1 demonstrates the Venn diagram of the proteins found in each group and the proteins common between the groups.

Figure 1. Qualitative characterization of the urinary proteomic profile of the control and hemodialysis groups. Uberlândia, 2023.

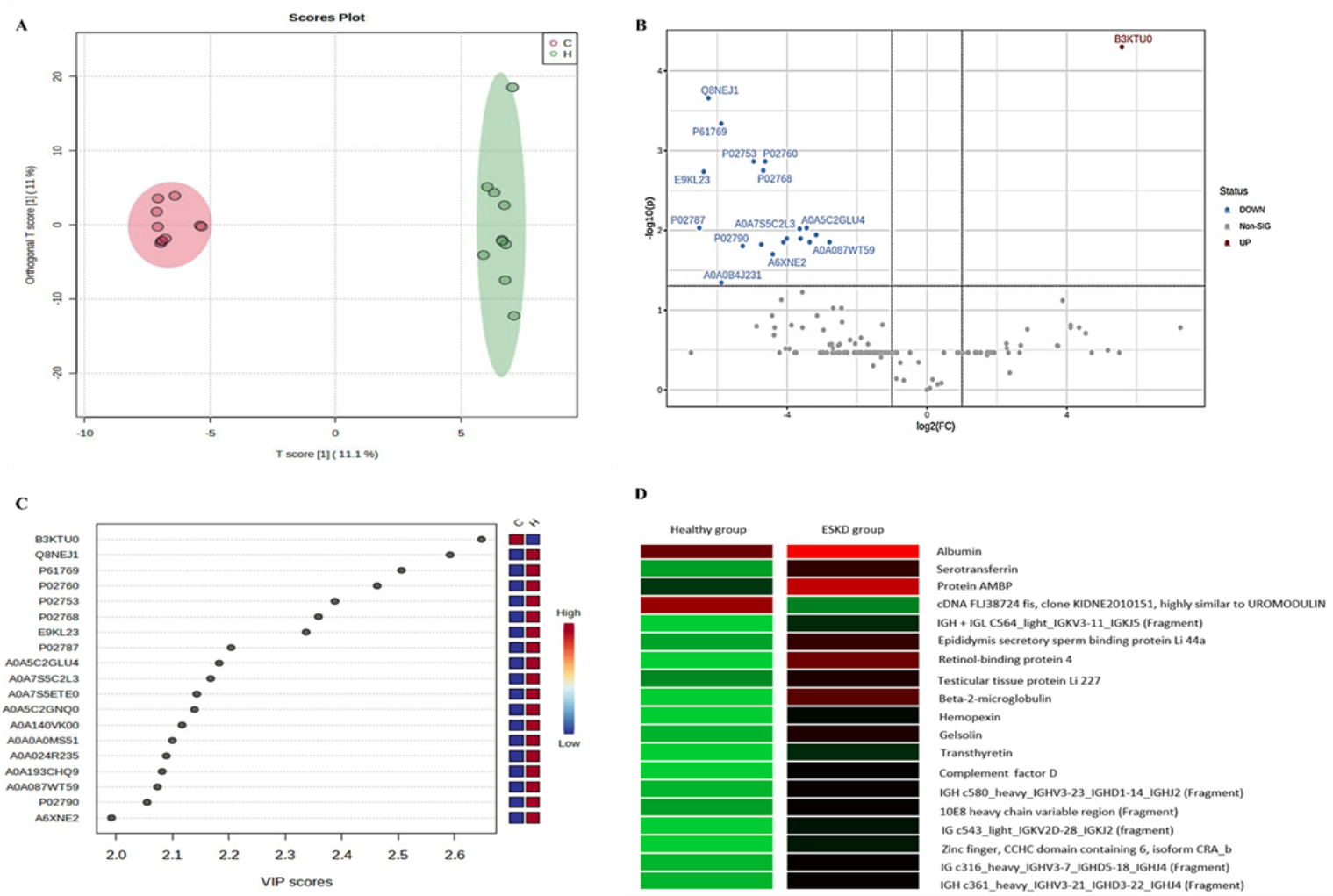


Legend: Venn diagram showing the amount of proteins present in the hemodialysis group (green) and healthy group (red); intersection between the circles: quantity of proteins present in both groups.

When comparing the groups quantitatively, it was seen that, of the 416 proteins initially found, 19 proteins showed a statistically significant difference. Figure 2 shows the OPLS-DA (2A) and Volcano Plot (2B) analysis.

In addition, Figure 2 shows the proteins that obtained a VIP score above 2.0 (2C). It is noted that 18 of the 19 statistically significant proteins were represented in the graph. Only the protein with the access code A0A5C2GDK3 did not reach the minimum score established and therefore, it was not shown in this graph. The analysis of the VIP score is important because high values signal a good contribution of proteins in the separation of groups. Also, the heatmap (2D) of the 19 significant proteins was presented, and most of these presented higher intensity in the hemodialysis group.

Figure 2. Quantitative characterization of the urinary proteomic profile of the control and hemodialysis groups. Uberlândia, 2023.



Legend: Graph 2A – proteins found in the control group (red) and hemodialysis group (green). Graph 2B – each point represents a protein; blue dots: proteins least expressed in the control group; red dot: protein most expressed in the control group; gray dots: proteins that did not show a statistically significant difference. Graph 2C – vertical axis: codes for the 19 proteins with a VIP score above 2.0; horizontal axis: VIP score values between 2.0 and 2.6. Graph 2D – green bars: absence of intensity; red bars – maximum intensity; lines: proteins; columns: healthy (control) or hemodialysis group.

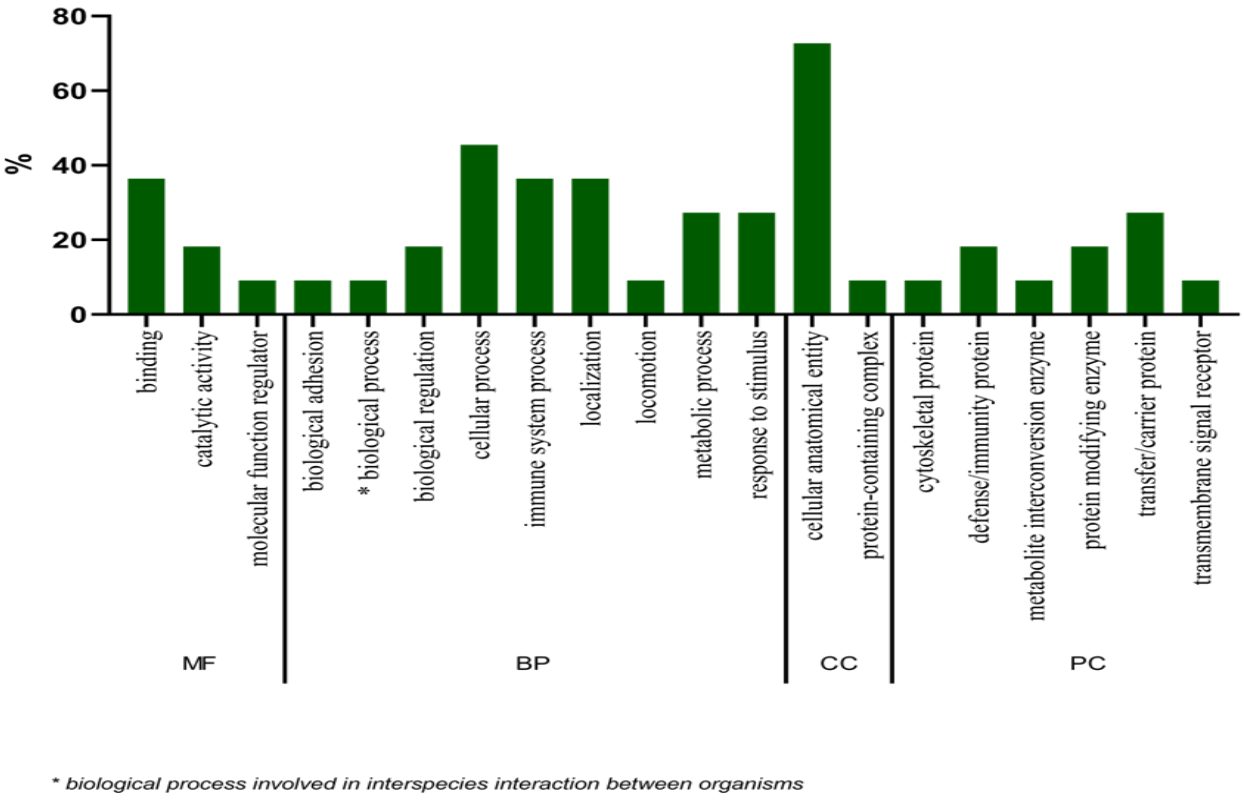
Characterization of significant proteins:

Supplementary Table 1 (S1) shows the significant proteins, besides the accessions collected in the UniProt database and in which group(s) (control and/or ESKD group) the proteins were found.

Gene ontology analysis of proteins:

In Figure 3, the GO analysis of proteins with statistically significant differences between the two groups was presented. It is noted that in relation to molecular function and biological process, proteins had a greater association with binding and cellular process functions, respectively. Regarding the cellular component, there is a greater participation of proteins in the cellular anatomical entity. Finally, regarding the class of proteins, it is noteworthy that these were mainly related to the class of transfer/carrier protein.

Figure 3. GO analysis of the 19 statistically significant proteins ($p \leq 0.05$). Uberlândia, 2023.

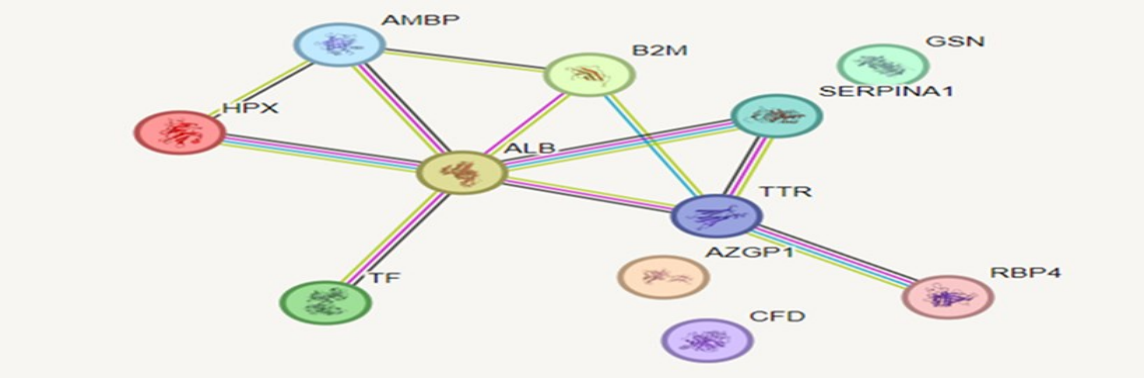


Legend: MF = molecular function, BP = biological process, CC = cellular component, PC = class of proteins.

Protein interaction analysis:

In Figure 4, the functional interactions found among the proteins were presented using the STRING software. It is important to highlight that the nodes are the proteins (n = 11), and the edges are the interactions between them (n = 11). Of the 19 proteins with significant difference between the groups, eight presented interactions according to the analysis performed.

Figure 4. Functional interactions among proteins. Uberlândia, 2023.



Legend: B2M = beta-2-microglobulin, AMBP = alpha-1-microglobulin, ALB = albumin, HPX = hemopexin, SERPINA1 = alpha-1-antitrypsin, TTR = transthyretin, RBP4 = retinol-binding protein 4, GSN = gelsolin, AZGP1 = zinc-alpha-2-glycoprotein, TF = transferrin, CFD = complement factor D.

Biomarker candidate proteins:

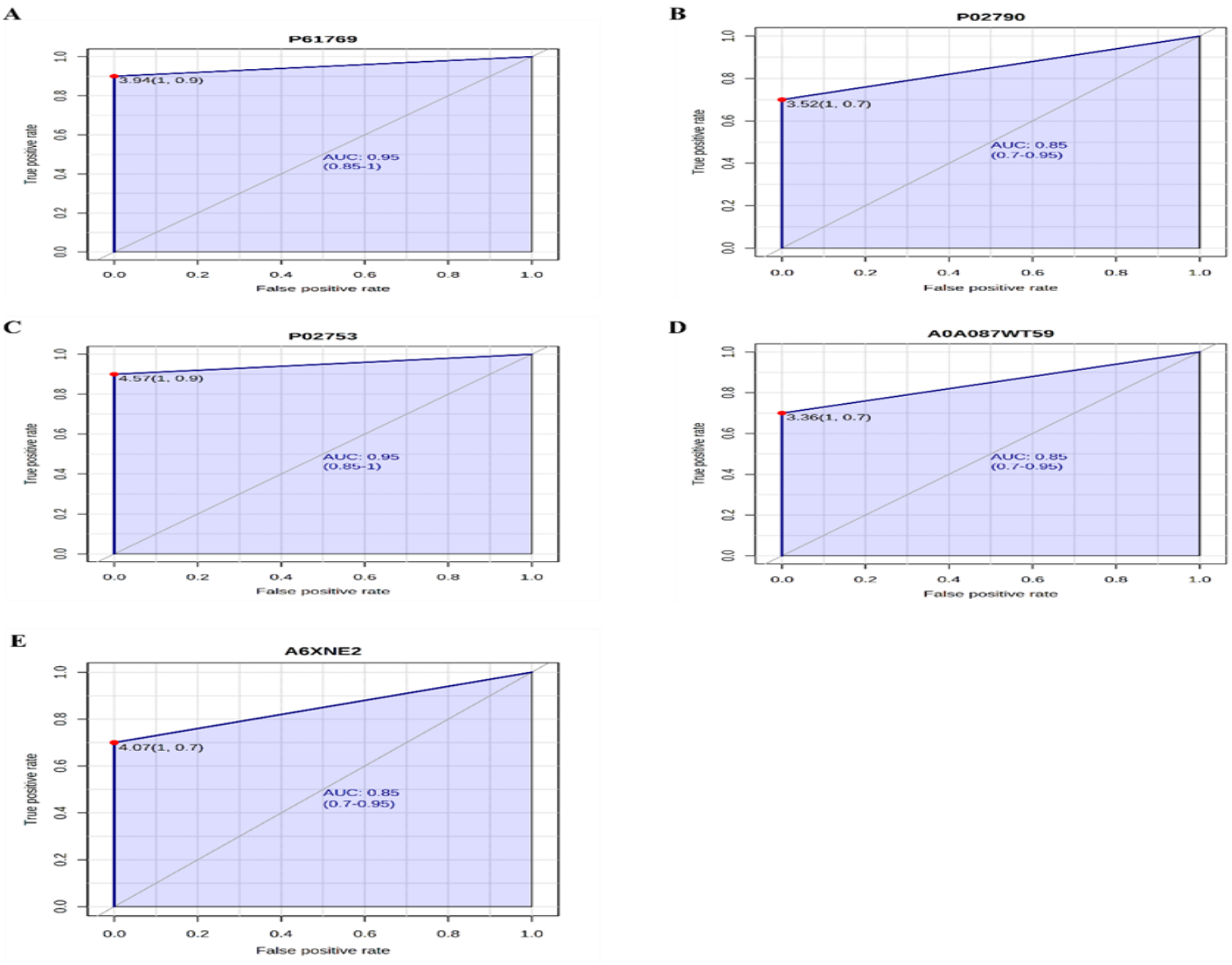
By using the criteria for defining candidate proteins that were previously described, we found that the proteins that met all established criteria were: hemopexin, beta-2-microglobulin, retinol-binding protein 4, transthyretin and factor D. Therefore, in addition to being statistically different between the two groups, these proteins were only found in the hemodialysis group. Moreover, it was seen that there is a small elimination of beta-2-microglobulin in the urine in healthy adults, however, in cases of dysfunction of the renal proximal tubules, urinary excretion of this protein may increase [23]. Also, hemopexin can be synthesized by the kidneys [24] and retinol-binding protein 4 can be produced in the kidneys [25] and a small part is excreted by urine [26]. Furthermore, in cases of transthyretin mutation, it can be deposited in the kidneys and cause a condition called amyloidosis [27]. Finally, it is known that the kidneys play a role in regulating the concentration of factor D through glomerular filtration [28].

Accuracy analysis of biomarker candidate proteins:

Figure 5 shows the ROC curve of the proteins beta-2-microglobulin (2A) hemopexin (2B), retinol-binding protein 4 (2C), transthyretin (2D) and factor D (2E). The proteins beta-2-

microglobulin and retinol-binding protein 4 showed the same AUC value, which resulted in 0.95 [with confidence interval (CI) between 0.85 to 1]. Hemopexin, transthyretin and factor D proteins presented AUC of 0.85 (CI between 0.7 and 0.95). Thus, it can be affirmed that these proteins presented high accuracy for the differentiation of the groups because, according to the interpretation of the AUC, the result of the analysis of the beta-2-microglobulin and retinol binding protein 4 was considered excellent and the result of analysis of hemopexin, transthyretin and factor D was good.

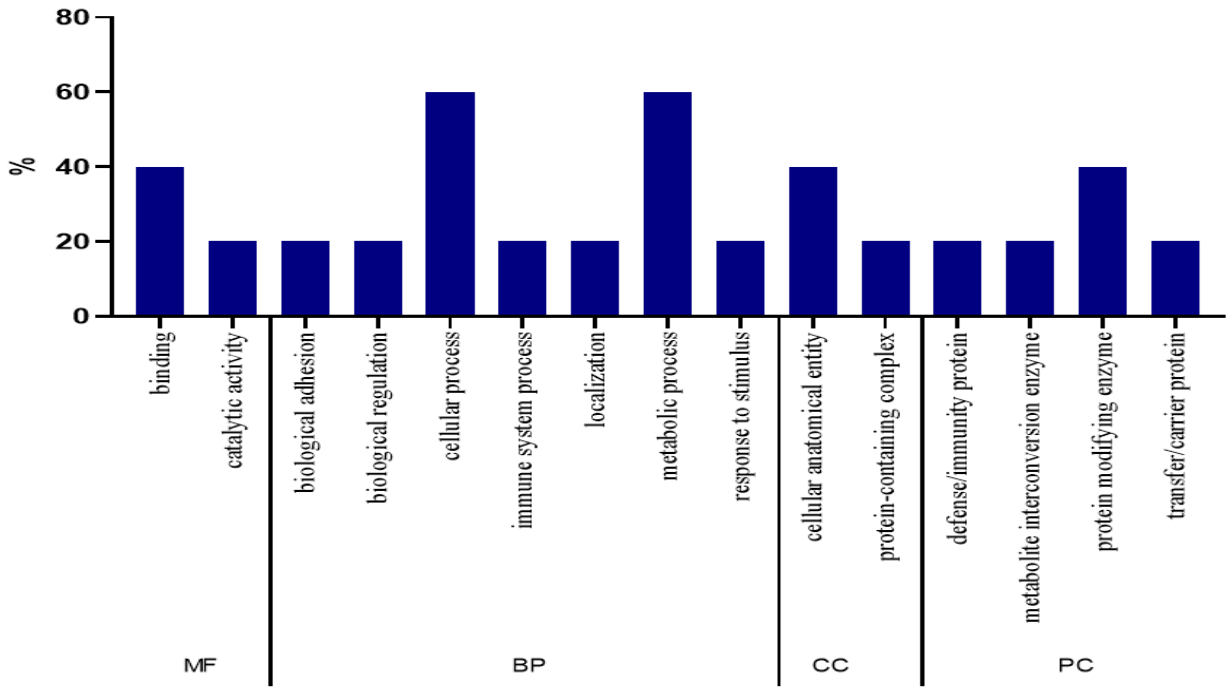
Figure 5. ROC curve referring to the accuracy analysis of candidate biomarker proteins for CKD. Uberlândia, 2023.



Gene ontology analysis of biomarker candidate proteins for CKD:

In Figure 6, the GO analysis of the five candidate biomarker proteins was presented. It is noteworthy that in relation to molecular function, proteins had a greater association with binding. Regarding the biological process, these were mainly related to cellular and metabolic processes. As for the cellular component, there is a greater participation of proteins in the cellular anatomical entity. Regarding the class of proteins, these were especially related to the protein modifying enzyme class.

Figure 6. GO analysis of biomarker candidate proteins. Uberlândia, 2023.



Legend: MF = molecular function, BP = biological process, CC = cellular component, PC = class of proteins.

Discussion

In the present study, 416 proteins were detected in the urinary proteomic profile of the control and hemodialysis groups. Of these, 19 proteins were considered statistically significant when using the p-value≤0.05 and the FDR correction. Also, five proteins were identified as potential candidates for biomarkers for CKD.

As for sociodemographic characteristics, the hemodialysis group had a higher mean age, which is in line with recent research showing that advanced age is associated with the onset and rapid progression of the disease [29]. It was also possible to observe that most participants in the hemodialysis group had no education or only incomplete elementary school. According to the National Health Survey (PNS) conducted by the Brazilian Institute of Geography and Statistics (IBGE) with a representative sample of the Brazilian population, individuals without education or with incomplete elementary school had a higher number of medical diagnoses of CKD when compared to people with higher education level [30].

Regarding tobacco use, half of the participants in the hemodialysis group were former smokers. A systematic review showed that tobacco use was associated with substantial risk of developing CKD, which can be explained by changes caused by the nephrotoxic effects of smoking, such as: endothelial cell dysfunction, pro-inflammation, oxidative stress, among others [31]. Regarding the level of physical activity of the participants, it was observed that the hemodialysis group was considered sedentary or insufficiently active. This data corroborates a study that shows that people with CKD usually have a lower level of physical activity compared to the general population and this can cause reduction of neuromuscular and cardiorespiratory activity and worsening of quality of life. Also, it is known that the increased level of physical activity may contribute to the deceleration of renal dysfunction [32].

Regarding the biochemical tests, as expected, the values of serum creatinine, GFR and fasting blood glucose of the participants in the hemodialysis group were higher. Increased creatinine in the blood and the consequent reduction of GFR are associated with an increased risk of progression of CKD [33]. Regarding the mean fasting blood glucose, it is emphasized that persistent hyperglycemia caused by type 2 DM promotes microvascular injury and complications, such as diabetic nephropathy [34].

549 Concerning the proteomic analysis, the present study identified proteins that were able
550 to characterize and differentiate the urinary proteomic profiles of the two groups. Through
551 ontological analysis of significant proteins, it was found that there was a higher percentage of
552 proteins that participated in biological processes that may be related to inflammation, such as
553 cellular process and immune system process. Furthermore, when analyzing the ontology of
554 candidate proteins for biomarkers, it was noted that they were associated with biological
555 processes, such as: adhesion and biological regulation, cellular and metabolic processes,
556 immune system processes, response to stimulus and localization. In agreement, some studies
557 have related these processes to low-grade systemic inflammation [35] and oxidative stress
558 (caused by changes in metabolic processes) [36], which are present in patients with CKD and
559 can be characterized by high levels of circulating inflammatory proteins and the presence of
560 oxidative stress biomarkers, respectively [37]. Moreover, the accumulation of uremic toxins
561 that occurs in CKD may contribute to the adhesion and migration of leukocytes [37, 38], which
562 increase the risk of infections and CVD in patients with CKD [38].

563 Also, it was observed that the proteins that met all the established criteria to be
564 considered biomarker candidates were: hemopexin, beta-2-microglobulin, retinol-binding
565 protein 4, transthyretin and factor D. Through the ROC curves, it was possible to verify that
566 these proteins showed high accuracy and thus potential candidates for biomarkers for CKD.

567 Hemopexin is an acute phase glycoprotein, that is, its plasma concentration may
568 increase in the occurrence of inflammatory events [24]. The main function of this protein is to
569 eliminate the free heme present in the systemic circulation [39]. Some studies have indicated
570 that hemopexin is able to induce the rearrangement of the nephrine-dependent cytoskeleton in
571 the podocytes and affect the permeability of the glomerular filtration barrier by decreasing the
572 glycocalyx, which can lead to proteinuria [39, 40]. Thus, because proteinuria is an important

573 marker of renal damage, it is suggested that increased hemopexin may be indirectly related to
574 the occurrence of this condition and, consequently, to the development of CKD.

575 Beta-2-microglobulin (β2m) is a polypeptide that is bound to the main
576 histocompatibility complex (MHC) class I protein on the surface of nucleated cells. This
577 molecule performs the function of antigen presentation, which contributes to the proper
578 functioning of the immune system [41]. Moreover, β2m is considered a marker of uremic toxins
579 and is filtered exclusively by the renal glomerulus [42]. Some studies have demonstrated the
580 association of β2m with general mortality, cardiovascular events, and disease progression in
581 patients with CKD [42, 43]. Also, important levels of β2m are related to a higher risk of
582 developing dialysis-related amyloidosis [44]. Finally, it should be noted that increased levels of
583 β2m in urine may occur in cases of dysfunction of the reabsorption of the proximal renal
584 tubules, which can cause proteinuria [41].

585 Retinol-binding protein 4 (RBP4) is a serum polypeptide formed by 201 amino acids.
586 The main sites of synthesis are the liver and adipose tissue, but it can also be produced in smaller
587 amounts in the lungs, testes, kidneys, brain, and retina [25]. The key role of this protein is the
588 transport of retinol from the liver to the target tissue [25]. In the blood circulation, the retinol-
589 RBP4 complex binds to the transthyretin because this binding stabilizes the complex, decreases
590 the elimination of RBP4 in renal filtration and favors the recycling of RBP4 after the absorption
591 of retinol in cells [25]. The retinol free fraction of RBP4 is submitted to glomerular filtration
592 and degraded by the proximal tubules of the kidneys [26]. However, a small portion of RBP4
593 is excreted in urine. Thus, when there is increased elimination of this protein in the urine, there
594 is indicative of tubular injury [26]. In agreement, studies indicate that RBP4 can be considered
595 a biomarker of glomerular diseases - such as diabetic nephropathy [45, 46] - and proximal
596 tubulopathies, such as Falconi syndrome [46]. Also, some studies have shown that in patients
597 with CKD, the levels of this protein in the urine were significantly increased [26, 47].

598 Transthyretin (TTR) is a plasma protein composed of 4 identical subunits containing
599 127 amino acids. This protein is synthesized in the liver, choroid plexus, and pigmentary
600 epithelia of the retina and ciliary of the eye and is responsible for the transport of thyroxine and
601 retinol-RBP4 complex [48]. In addition, when mutation occurs in the TTR gene through the
602 destabilization of the subunits, the protein monomers aggregate in amyloid fibrils and can be
603 deposited in various tissues, such as: heart, nerves, kidneys, and gastrointestinal tract [27]. In
604 the kidneys, TTR deposition may cause dysfunction of the lower urinary tract [49], nephrotic
605 syndrome and/or progressive renal failure [27]. Thus, in individuals with symptoms and/or
606 family history of amyloidosis, it is necessary to investigate the mutation through the molecular
607 test [50]. If the mutation is proven, clinical interventions are essential to prevent the
608 development of complications such as nephrotic syndrome and renal failure. Therefore, TTR
609 can be considered a biomarker for the identification of CKD only in individuals with this
610 mutation.

611 Factor D is a Serino protease that participates in the alternative pathway of the
612 Complement System. This protein is produced and secreted into the blood circulation by
613 adipocytes. Under healthy conditions, factor D - as well as other low molecular weight proteins
614 - is filtered through the renal glomerulus and completely reabsorbed inside the tubules, where
615 it is catabolized [28]. However, in patients with end-stage kidney disease, plasma levels of this
616 protein increased about 10 times due to deficient glomerular filtration [28]. In addition,
617 dysregulation of the alternative pathway may cause some inflammatory glomerular diseases,
618 which leads to glomerular lesion and consequently hematuria and proteinuria, which contribute
619 to the development of CKD [28].

620 As strengths of this study, we highlight the choice of liquid chromatography method
621 coupled to mass spectrometry, quality of protein separation in the matrix by means of liquid
622 chromatography and the chemical identification capability of mass spectrometry equipment.

Another point to be highlighted is the fact that, so far, there are few similar studies published in the literature, which makes this study bring added information about this topic. In addition, the protocols used for sample preparation and analysis were validated in several studies in the scientific literature.

However, this study also has limitations: the small sample size and the absence of pools can be explained by the proteomic analysis being a technique of high cost; the groups showed significant differences for tobacco consumption and physical activity, which may influence the interpretation of proteomic analysis data, as these individual factors may affect the comparison of groups; the control group was formed by participants with lower mean age - a fact that may also have influenced the comparison between the groups - however, this reflects the general population since, the advanced age is associated with higher chances of developing DM, AH, CVD and CKD, which were exclusion criteria for the control group in the present study; data were collected at a single time, that is, there was no follow-up of the participants over time to verify whether the changes observed between the groups remained during the time; finally, due to the sociodemographic and lifestyle information being self-reported, may have occurred memory bias of the participants when answering some question.

Finally, the proteins found were able to characterize and differentiate the urinary proteomic profiles of the two groups analyzed. In addition, the proteins hemopexin, beta-2-microglobulin, retinol-binding protein 4, transthyretin and factor D can be considered candidate biomarkers for CKD because the mechanisms of action of these proteins are involved with the pathophysiology of the disease. It is suggested that longitudinal studies be performed on a representative sample of the population so that it is possible to validate whether the five proteins found can be used in conjunction with the currently available methods for the diagnosis and monitoring of CKD progression.

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836 **Appendix**

837 **Table S1.** Characterization of the 19 proteins with statistically significant differences.

838 Uberlândia, 2023.

Protein name	**Alternative names**	**UniProt access**	**Group(s) where the protein was found**
Albumin	cDNA FLJ78413, highly similar to Homo sapiens albumin, mRNA; cDNA FLJ95666, highly similar to Homo sapiens albumin (ALB), mRNA; Serum albumin; cDNA FLJ54371, highly similar to serum albumin; cDNA FLJ50830, highly similar to serum albumin; Isoform 2 of Albumin; Isoform 3 of Albumin	P02768	Control and Hemodialysis
Serotransferrin	cDNA FLJ54111, highly similar to Serotransferrin; Transferrin, isoform CRA_c; cDNA FLJ56687, highly similar to Serotransferrin; TF protein (fragment)	P02787	Control and Hemodialysis

Protein AMBP	Inter-alpha-trypsin inhibitor (fragment)	P02760	Control and Hemodialysis
cDNA FLJ38724 fis, clone KIDNE2010151, highly similar to UROMODULIN	Uromodulin; Isoform 2 of Uromodulin; Isoform 3 of Uromodulin; Isoform 4 of Uromodulin; Isoform 5 of Uromodulin; cDNA FLJ45746 fis, clone KIDNE2018727, highly similar to UROMODULIN	P07911 B3KTU0	Control and Hemodialysis
IGH + IGL c564_light_IGKV3-11_IGKJ5 (Fragment)	IG c185_light_IGKV3-11_IGKJ2 (Fragment) IGL c526_light_IGKV3-20_IGKJ1 (Fragment); IGL c1611_light_IGKV3-11_IGKJ5 (Fragment); IGL c2008_light_IGKV3-11_IGKJ4 (Fragment); IGL c3277_light_ IGKV3-11_IGKJ4 (Fragment); IGH + IGL c620_light_ IGKV3-11_IGKJ5 (Fragment)	A0A5C2GDK3	Hemodialysis
Epididymis secretory sperm binding protein Li 44[a]	Alpha-1-antitrypsin; Epididymis secretory sperm binding protein; Isoform 2 of alpha-1-antitrypsin; Alpha-1-antitrypsin (fragment); PRO2275; Isoform 3 of alpha-1-antitrypsin; Alpha-1-antitrypsin Valcamonica variant (fragment); Alpha-1-antitrypsin null variant (fragment); Serpina 1; Alpha-1-antitrypsin MBrescia variant (fragment); Alpha-1-antitrypsin short transcript variant 1C4; Alpha-1-antitrypsin null genova variant (fragment); Alpha-1-antitrypsin null (Brescia) variant (fragment)	E9KL23	Control and Hemodialysis

Retinol-binding protein 4	Retinol-binding protein	P02753	Hemodialysis
Testicular tissue protein Li 227	Zinc-alpha-2-glycoprotein; AZGP1 protein (fragment)	P25311 A0A140VK00	Control and Hemodialysis
Beta-2-microglobulin	Beta-2-microglobulin (fragment)	P61769	Hemodialysis
Hemopexin	Epididymis secretory sperm binding protein; cDNA FLJ56652, highly similar to hemopexin	P02790	Hemodialysis
Gelsolin	Isoform 2 of gelsolin; Isoform 3 of gelsolin; Isoform 4 of gelsolin	P06396 A0A0A0MS51	Control and Hemodialysis
Transthyretin	-	P02766 A0A087WT59	Hemodialysis
Complement factor D	-	P00746 A6XNE2	Hemodialysis
IGH c580_heavy_IGHV3-23_IGHD1-14_IGHJ2 (Fragment)	IGH c2982_heavy_IGHV3-23_IGHD2-8_IGHJ2 (Fragment)	A0A7S5C2L3	Control and Hemodialysis
10E8 heavy chain variable region (Fragment)	IGH + IGL c351_heavy_IGHV3-15_IGHD5-18_IGHK4 (fragment); IGH + IGL C38_heavy_IGHV3-15_IGHD1-14_IGHJ4 (fragment); IG c730_heavy_IGHV3-15_IGHD7-27_IGHJ6 (fragment); IG c255_heavy_IGHV3-15_IGHD3-10_IGHJ4 (fragment); IG c684_heavy_IGHV3-15_IGHD3-9_IGHJ3 (fragment);	A0A193CHQ9	Control and Hemodialysis

	IG c260_heavy_IGHV3-15_IGHD3-9_IGHJ3 (fragment); IG c1073_heavy_ IGHV3-15_IGHD3-22_IGHJ5 (fragment); IG c155_heavy_IGHV3-15_IGHF3-22_IGHJ4 (fragment); IGH + IGL c27_heavy_IGHV3-15_IGHD4-17_IGHJ4 (fragment); Immunoglobulin heavy chain variable region (fragment)		
IG c543_light_IGKV2D-28_IGKJ2 (Fragment)	Immunoglobulin kappa variable 2-28; Immunoglobulin kappa variable 2-40; HRV Fab N8-VL (fragment); Cold agglutinin FS-1 L-chain (fragment); ACX82 (fragment)	A0A5C2GNQ0	Hemodialysis
Zinc finger, CCHC domain containing 6, isoform CRA_b	Terminal uridylyltransferase 7; Isoform 4 of terminal uridylyltransferase 7; Isoform 6 of terminal uridylyltransferase 7	A0A024R235	Hemodialysis
IG c316_heavy_IGHV3-7_IGHD5-18_IGHJ4 (Fragment)	IG c573_heavy_IGHV3-7_IGHD3-3_IGHJ4 (fragment)	A0A5C2GLU4	Control and Hemodialysis
IGH c361_heavy_IGHV3-21_IGHD3-22_IGHJ4 (Fragment)	IGH + IGL c36_heavy_IGHV3-15_IGHD3-3_IGHJ6 (fragment); IG c775_heavy_IGHV3-15_IGHD3-10_IGHJ4 (fragment)	A0A7S5ETE0	Control and Hemodialysis

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