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Urinary Proteomics and Systems Biology Link Eight Proteins to the Higher Risk of Hypertension and Related Complications in Blacks Versus Whites

De-Wei An[1,2,3] | Dries S. Martens[4] | Gontse G. Mokwatsi[5] | Yu-Ling Yu[2,3] | Babangida S. Chori[4,6] | Agnieszka Latosinska[7] | Godsent Isiguzo[8] | Susanne Eder[9] | Dong-Yan Zhang[1,2,3] | Gert Mayer[9] | Ruan Kruger[5] | Jana Brguljan-Hitij[10] | Christian Delles[11] | Catharina M. C. Mels[5] | Katarzyna Stolarz-Skrzypek[12] | Marek Rajzer[12] | Peter Verhamme[13] | Aletta E. Schutte[5,14] | Tim S. Nawrot[3,4] | Yan Li[1] | Harald Mischak[7] | Augustine N. Odili[6] | Jan A. Staessen[1,2,15]

[1]Department of Cardiovascular Medicine, Shanghai Key Laboratory of Hypertension, Shanghai Institute of Hypertension, State Key Laboratory of Medical Genomics, National Research Center for Translational Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China | [2]Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium | [3]Research Unit Environment and Health, KU Leuven Department of Public Health and Primary Care, University of Leuven, Leuven, Belgium | [4]Center for Environmental Sciences, Hasselt University, Diepenbeek, Belgium | [5]Hypertension in Africa Research Team (HART), SAMRC Extramural Unit for Hypertension and Cardiovascular Disease, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa | [6]Circulatory Health Research Laboratory, College of Health Sciences, University of Abuja, Abuja, Nigeria | [7]Mosaiques Diagnostiques GmbH, Hannover, Germany | [8]Cardiology Unit, Department of Medicine, Alex Ekwueme Federal University Teaching Hospital & Ebonyi State University, Abakaliki, Ebonyi State, Nigeria | [9]Department of Internal Medicine IV, Medical University Innsbruck, Innsbruck, Austria | [10]Division of Hypertension, Department of Internal Medicine, University Medical Center, Ljubljana, Slovenia | [11]School of Cardiovascular and Metabolic Health, University of Glasgow, Glasgow, UK | [12]First Department of Cardiology, Interventional Electrocardiology and Hypertension, Jagiellonian University, Kraków, Poland | [13]Center for Molecular and Vascular Biology, KU Leuven Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium | [14]Faculty of Medicine, UNSW Sydney, Sydney, Australia | [15]Biomedical Sciences Group, Faculty of Medicine, University of Leuven, Leuven, Belgium

Correspondence: Jan A. Staessen (jan.staessen@appremed.org)

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ABSTRACT

Blacks are more prone to salt-sensitive hypertension than Whites. This cross-sectional analysis of a multi-ethnic cohort aimed to search for proteins potentially involved in the susceptibility to salt sensitivity, hypertension, and hypertension-related complications. The study included individuals enrolled in African Prospective Study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT), Flemish Study of the Environment, Genes and Health Outcomes (FLEMENGHO), Prospective Cohort Study in Patients with Type 2 Diabetes Mellitus for Validation of Biomarkers

Abbreviations: African-PREDICT, African Prospective Study on the Early Detection and Identification of Cardiovascular Disease and Hypertension; BMI, body mass index; BP, blood pressure; CE-MS, capillary electrophoresis coupled with mass spectrometry; eGFR, estimated glomerular filtration rate; FLEMENGHO, Flemish Study of the Environment, Genes and Health Outcomes; hs-CRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; IL-8, interleukin-8; KDIGO, Kidney Disease: Improving Global Outcomes; KEGG, Kyoto Encyclopedia of Genes and Genomes; PROVALID, Prospective Cohort Study in Patients with Type 2 Diabetes Mellitus for Validation of Biomarkers; TNF-α, tumor necrosis factor-α; UPP, urinary proteomic profiling; UPRIGHT-HTM, Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial.

De-Wei An and Dries S. Martens contributed equally to this study.

(PROVALID)-Austria, and Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial (UPRIGHT-HTM). Sequenced urinary peptides detectable in 70% of participants allowed the identification of parental proteins and were compared between Blacks and Whites. Of 513 urinary peptides, 300 had significantly different levels among healthy Black (n = 476) and White (n = 483) South Africans sharing the same environment. Analyses contrasting 582 Blacks versus 1731 Whites, and Sub-Saharan Blacks versus European Whites replicated the findings. COL4A1, COL4A2, FGA, PROC, MGP, MYOCD, FYXD2, and UMOD were identified as the most likely candidates underlying the racially different susceptibility to salt sensitivity, hypertension, and related complications. Enriched pathways included hemostasis, platelet activity, collagens, biology of the extracellular matrix, and protein digestion and absorption. Our study suggests that MGP and MYOCD being involved in cardiovascular function, FGA and PROC in coagulation, FYXD2 and UMOD in salt homeostasis, and COL4A1 and COL4A2 as major components of the glomerular basement membrane are among the many proteins potentially incriminated in the higher susceptibility of Blacks compared to Whites to salt sensitivity, hypertension, and its complication. Nevertheless, these eight proteins and their associated pathways deserve further exploration in molecular and human studies as potential targets for intervention to reduce the excess risk of hypertension and cardiovascular complications in Blacks versus Whites.

1 | Introduction

Sub-Saharan countries are transitioning from mortality dominated by communicable, maternal, neonatal, and nutritional causes to noncommunicable diseases [1]. Hypertension is the main driver of this shift in disease burden [2], however, with steadily growing contributions from obesity and Type 2 diabetes [3]. In the United States, adolescent and adult African Americans have the highest hypertension prevalence of all racial groups [4, 5]. American Blacks are more salt sensitive than other racial groups [6], leading to a volume-expanded low-renin type of hypertension. For the same blood pressure (BP) level, Blacks are also more vulnerable to hypertension-related complications [7, 8].

Urine contains over 21,000 endogenous peptides, either generated along the nephron or passing into the tubular fluid through the glomerular sieve [9]. Sequencing these peptides identifies the parental proteins, thereby providing body-wide pathogenic processes [9]. In a multi-ethnic cohort enrolled in Sub-Saharan Africa and Europe, levels of sequenced urinary peptides were compared between races, and divergent results were confronted with the information from public databases with as objective to search for proteins explaining the well-known propensity of salt-sensitive hypertension [4–6] and associated complications in Blacks [7, 8].

Summary

- Which proteins are involved in the higher hypertension risk and salt sensitivity of Blacks compared to Whites is unknown.
- In the current study of urinary proteomics, we found that COL4A1 and COL4A2, FGA and PROC, MGP and MYOCD, and FYXD2 and UMOD are likely candidates underlying the racially different susceptibility to hypertension and salt sensitivity.
- MGP and MYOCD being involved in cardiovascular function, FGA and PROC in coagulation, FYXD2 and UMOD in salt homeostasis, and COL4A1 and COL4A2 in maintaining the integrity of the glomerular basal membrane have high clinical relevance as targets for intervention.

2 | Materials and Methods

2.1 | Study Participants

Anonymized data were extracted from two population studies [10, 11], one cohort of Type 2 diabetic patients [12] and one ongoing clinical trial (Figure 1) [13]. At each recruitment site, the competent local Institutional Review Board approved the study protocol and sharing of anonymized data. All contributing studies complied with the Helsinki Declaration for research involving humans. Participants provided informed written consent. In addition to ethics approval and informed consent, the only selection criterion was the availability of urinary proteomic profiling (UPP) data and basic clinical information, including anthropometrics, hematological and biochemical variables, smoking and drinking habits, medical history, and use of medications. Missing values had a frequency of less than 1% and were imputed using linear regression between correlated variables stratified for cohort, sex, and age bands.

From November 1, 2012, to November 30, 2017, South African Blacks and Whites were enrolled in the African Prospective Study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT) [10]. The inclusion criteria were: adults of either sex, age 20–30 years, free of HIV, BP below 140 mm Hg systolic and 90 mm Hg diastolic, self-reported Black or White race, and confirmed to be healthy and not on chronic medication [10]. The Flemish Study of the Environment, Genes and Health Outcomes (FLEMENGHO) is a longitudinal family based population study [11]. Recruitment started in 1985 and follow-up lasted until December 31, 2019. Participants repeatedly visited the examination center in the catchment area for high-fidelity phenotyping of cardiovascular and renal traits and updating their medical history. The reexamination running from May 30, 2005, to May 31, 2010 included biobanking of a 10-mL aliquot of a 24-h urine sample for UPP [11]. White patients enrolled in the Prospective Cohort Study in Patients with Type 2 Diabetes Mellitus for Validation of Biomarkers (PROVALID)

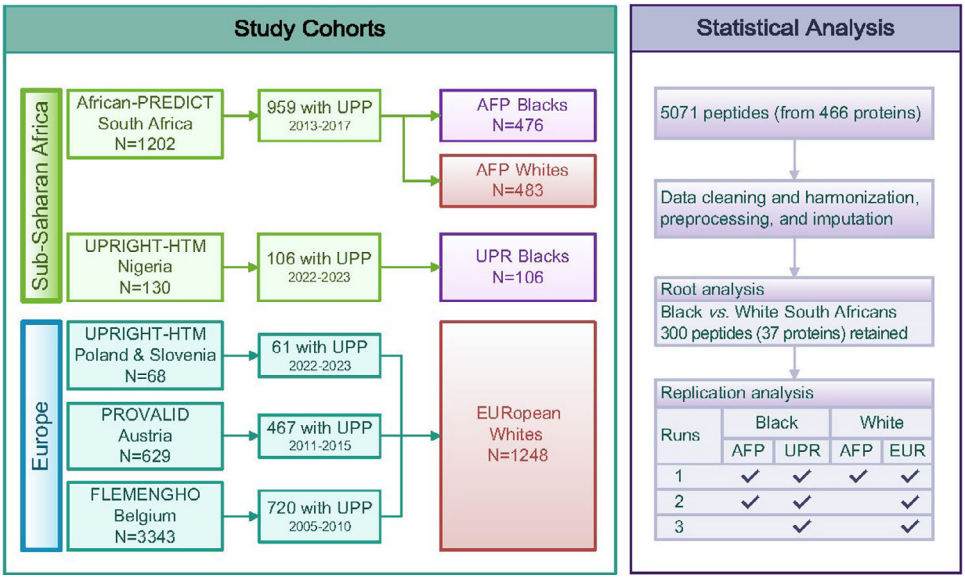

FIGURE 1 | Flow chart illustrating the inclusion of participants and the strategy of the statistical analysis AFP, African-PREDICT; EUR, cohorts of European Whites; UPR, UPRIGHT-HTM.

were recruited in five European countries (Austria, Hungary, Netherlands, Poland, and Scotland). However, based on UPP availability the current analysis only includes Austrians enrolled at general practices in Upper Austria and Tyrol (October 1, 2011–April 1, 2019), henceforth referred to as PROVALID-Austria [12]. The Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial (UPRIGHT-HTM; registration number NCT04299529) is an investigator-initiated clinical trial, in which patients are being recruited in Europe and Sub-Saharan Africa. Eligible patients of either sex, aged 55–75 years, without major disease antecedents, not having symptoms, are required to have five or more risk factors for cardiovascular or chronic kidney disease, preferably including hypertension, Type 2 diabetes, or both [13]. At the time of writing this paper, of 167 randomized patients (June 1, 2021–November 30, 2023), 106 Nigerian Blacks, 52 White Polish, and 9 White Slovenians had their UPP assessed and were included in the present analyses.

2.2 | Study Procedures

In all cohorts, certified laboratories did the hematological and biochemical measurements. Serum creatinine was measured, using Jaffe's method with modifications including isotope-dilution mass spectrometry for calibration [14]. The glomerular filtration rate was estimated from serum creatinine (estimated glomerular filtration rate [eGFR]), using the Chronic Kidney Disease-Epidemiology Collaboration equation [15] and staged according to the Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group Guideline [16]. Given that most pathological processes involve inflammation, high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) were measured in FLEMENGHO and African-PREDICT. In African-PREDICT, serum hs-CRP was determined on the Cobas Integra 400 plus device (Roche, Basel, Switzerland) and the other cytokines, using the MILLIPEX MAP Human High-Sensitivity T Cell Magnetic Bead Panel (EMD Millipore, Merck Missouri, USA) running on a Luminex 200Analyzer (I&L Biosystems, Köningswinter, Germany). In FLEMENGHO, the METS I array was used (Randox Laboratories Ltd., Crumlin, County Antrim, Northern Ireland).

Mosaiques Diagnostics GmbH, Hannover, Germany did the UPP profiling. Before shipment, the African-PREDICT urine samples were blinded to conceal the race of the participants. The methods for sample preparation, capillary electrophoresis coupled with mass spectrometry (CE-MS), peptide sequencing, and the evaluation, calibration, and quality control of the mass spectrometric data have been published and are described in detail in Supporting Information S1 (pp. 2–5) [17]. In the CE-MS step, reference signals of 29 abundant endogenous urinary peptides were run along with the samples as internal standards for calibration of signal intensity. This highly reproducible procedure addresses in a single calibration step both analytical and dilution variances, such as due to variability in renal function [18].

2.3 | Statistical Analysis

For database management and statistical analysis, SAS software, version 9.4, was used. Deviation from the normal distribution was assessed by the Shapiro–Wilk statistic. To generate descriptive statistics, means were compared using the large-sample z-test, medians by the Mann–Witney test, and proportions by χ^2 statistic or Fisher exact test if the frequency tables included empty cells. Statistical tests were two sided.

The comparison of the UPP between Blacks and Whites was confined to 513 of 5071 sequenced urinary peptides with a detectable signal in 70% of participants. Levels below the detection threshold were replaced by a value randomly varying from 95% to 105% around the minimum of the distribution of each peptide. The racial comparisons were implemented using general linear models with adjustment for sex, age, body mass

index (BMI), systolic and diastolic BP, eGFR, Type 2 diabetes, and treatment with antihypertensive and lipid-lowering drugs. Because the number of peptides derived from a single protein might increase the likelihood of the protein to be detected, models were weighted by the inverse of the number of peptides derived from the same protein. The significance of the racial differences was corrected for multiple testing by the Bonferroni method [19]. The general linear model assumes normality. The levels of the urinary peptides were therefore rank normalized by sorting measurements from the smallest to the highest value and then applying the inverse cumulative normal function [20]. However, the central tendency and spread of the peptide levels are reported as the median and interquartile range of the nontransformed nonadjusted data.

Analysis of the racial differences was implemented in four predefined steps (Figure 1). First, in a root analysis, the racial UPP differences were assessed in the African-PREDICT cohort, because Black and White South Africans share a similar environment and because the African-PREDICT participants were young and healthy [10], thereby minimizing UPP alterations due to target organ damage. The peptides retaining Bonferroni-corrected significance in the African-PREDICT study were carried through to three replication runs. In the first run, the racial differences were assessed by contrasting all Blacks to all Whites. In the second run, South African and Nigerian Blacks were compared to European Whites, and in the third run, Nigerian Blacks were evaluated against European Whites. Thus, in the second and third replication runs, the White comparator was dissimilar compared to the initial analysis in African-PREDICT and the first replication run.

Two additional analyses were performed. The first had an objective to assess the performance of the age adjustment in the main analyses. Black UPRIGHT-HTM patients were matched in a 1:2 ratio for sex and age (±1 year) with White FLEMENGHO and White PROVALID-Austria participants. In models accounting for BMI, systolic and diastolic BP, and eGFR, the levels of the urinary peptides derived from the eight shortlisted proteins in the main analyses were compared between Blacks and Whites. To assess the role of inflammation, general linear models were run to evaluate the racial differences in the inflammation markers in Black South Africans compared to White South Africans and White Flemish combined. Because different technologies had been employed to assess the markers, their levels were standardized by the recruitment center and expressed in units of standard deviation. The second sensitivity analysis was unadjusted and adjusted for sex, age, BMI, systolic and diastolic BP, and eGFR.

2.4 | Systems Biology

Parental proteins with a role in the regulation of cardiovascular or renal structure or function, potential protein–protein interactions, and tissue expression profiles were identified by consulting the PubMed Human Gene database (http://www.ncbi.nlm.nih.gov/gene), the Uniprot Database (http://www.uniprot.org), and the Global Protein Atlas (http://www.proteinatlas.org). Pathway analysis, using the databases of Reactome (http://reactome.org), the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg), the Gene Ontology Database (http://www.geneontology.org), and the Disease Ontology Database (http://www.disease-ontology.org), was the final step in the analysis. Overrepresentation analysis was conducted using the Reactome Pathway Analysis [21], Clusterprofiler [22], and DOSE [23] packages in R (version 4.3.3). Enriched pathways in the Reactome and the KEGG analyses included at least two proteins identified from the retained urinary peptides, had a significant q value with false discovery rate correction, and were visualized using R software to create DotPlots [21]. Enriched pathways in the Gene Ontology and Disease Ontology Databases included three or more proteins.

3 | Results

3.1 | Characteristics of Participants

As shown in Table 1, the 2313 study participants included 582 (25.2%) Blacks recruited in Nigeria (UPRIGHT-HTM) and South Africa (African-PREDICT) and 1731 (74.8%) Whites enrolled in Austria (PROVALID-Austria), Belgium (FLEMENGHO), Poland and Slovenia (UPRIGHT-HTM), and South Africa (African-PREDICT) with a balanced representation of women (49.7%) and men (50.3%). Median age was 40.9 years in all participants and 26.0 years in Blacks and 49.7 years in Whites. Given the age difference, systolic and diastolic BP and BMI were significantly lower ($p < 0.001$) in Blacks than Whites, whereas eGFR was higher in Blacks. A detailed description of each cohort, broken down by race for African-PREDICT and UPRIGHT-HTM and broken down by sex for all cohorts, is available in Supporting Information S1 (Tables S1–S7). With the exception of the small and racially diverse UPRIGHT-HTM cohort (Table S7), heart rate and high-density lipoprotein cholesterol were higher in women than men, whereas the opposite trend was observed for body height, body weight, waist circumference, systolic and diastolic BP, hemoglobin, hematocrit, and serum creatinine. Among women, 186 of 475 (39.2%) used hormonal contraception in African-PREDICT (Table S2) and 82 of 362 (22.6%) in FLEMENGHO (Table S4). The other cohorts did not include women of reproductive age.

3.2 | Urinary Proteomic Results

Analysis of the African-PREDICT data with cumulative adjustments applied for sex, age, BMI, systolic and diastolic BP, and eGFR revealed that 300 of the 513 sequenced urinary peptides with a detectable signal in at least 70% of participants had Bonferroni-corrected different levels in Black compared to White South Africans. These peptides were the standard set carried through to further analyses first contrasting all Blacks to all Whites ($n = 582$ vs. 1731), next all Blacks to White Europeans ($n = 582$ vs. 1248), and finally Nigerian Blacks to European Whites ($n = 106$ vs. 1248). As appropriate, analyses were additionally adjusted for the use of antihypertensive and lipid-lowering drugs.

Of the urinary peptides keeping Bonferroni-corrected significant racial differences, many were collagen fragments and fewer originated from noncollagenous proteins. Of the 33 peptides derived from noncollagenous proteins with replication in 1–3 runs in addition to African-PREDICT, all but one UMOD

TABLE 1 | Characteristics of Black and White participants.

Characteristic	All individuals	By race		
		Blacks	Whites	*p* value
No. in group	2313	582	1731	
Sex, no.(%)				
Women	1150 (49.7)	290 (49.8)	860 (49.7)	0.95
Hormonal contraception, no. (% of women)	268 (11.6)	88 (15.1)	180 (10.4)	0.002
Men	1163 (50.3)	292 (50.2)	871 (50.3)	0.95
Region of enrollment, no. (%)				
Tyrol, Austria	467 (20.2)	—	467 (27.0)	—
Flanders, Belgium	720 (31.1)	—	720 (41.6)	—
Eastern Europe	61 (2.6)	—	61 (3.5)	
Abuja, Nigeria	106 (4.6)	106 (18.2)	—	—
Potchefstroom, South Africa	959 (41.5)	476 (81.8)	483 (27.9)	—
Clinical measurements				
Age, median (IQR) (year)	40.9 (25.2-61.2)	26.0 (22.7-30.0)	49.7 (27.2-64.2)	<0.001
Age, mean (SD) (year)	43.5 (19.4)	31.6 (14.6)	47.5 (19.2)	<0.001
Body height, mean (SD) (cm)	168.7 (9.6)	164.6 (8.5)	170.1 (9.6)	<0.001
Body weight, mean (SD) (kg)	77.9 (18.3)	69.7 (16.5)	80.7 (18.0)	<0.001
Body mass index, mean (SD) (kg/m^2)	27.3 (5.9)	25.8 (6.3)	27.8 (5.6)	<0.001
Waist circumference, mean (SD) (cm)	89.2 (15.0)	82.7 (13.4)	91.4 (14.9)	<0.001
Blood pressure, mean (SD) (mm Hg)				
Systolic	127.5 (16.7)	123.7 (15.0)	128.8 (17.1)	<0.001
Diastolic	79.6 (9.5)	80.6 (9.7)	79.3 (9.3)	0.004
Heart rate, mean (SD) (bpm)	64.2 (9.8)	64.9 (11.7)	63.9 (9.1)	0.060
Risk factors No.(%)				
Body mass index ≥30 kg/m^2	658 (28.5)	129 (22.2)	529 (30.6)	<0.001
Total serum cholesterol ≥190 mg/dL	737 (31.9)	30 (5.2)	707 (40.8)	<0.001
Current use of smoking materials	387 (16.7)	121 (20.8)	266 (15.4)	<0.001
Habitual consumption of alcoholic beverages	1257 (54.4)	271 (46.6)	986 (57.0)	<0.001
Hypertension	937 (40.5)	104 (17.9)	833 (48.1)	<0.001
Diabetes	523 (22.6)	18 (3.1)	505 (29.2)	<0.001
Previous cardiovascular disease	85 (3.7)	0 (0.0)	85 (4.9)	<0.001
Hematological and biochemical variables				
Hemoglobin, mean (SD) (mg/dL)	14.1 (1.6)	14.2 (2.0)	14.0 (1.5)	0.044
Hematocrit, mean (SD) (%)	41.9 (4.2)	42.2 (5.5)	41.8 (3.7)	0.128
Blood glucose, mean (SD) (mg/dL)	105.7 (47.0)	93.8 (19.2)	109.8 (52.6)	<0.001
Total serum cholesterol, mean (SD) (mg/dL)	171.2 (52.5)	144.1 (50.1)	181.0 (50.0)	<0.001
HDL serum cholesterol, mean (SD) (mg/dL)	48.4 (15.7)	44.3 (15.5)	49.8 (15.5)	<0.001
Total/HDL serum cholesterol ratio, mean (SD)	3.74 (1.25)	3.45 (1.32)	3.84 (1.21)	<0.001
Serum creatinine, mean (SD) (mg/dL)	0.87 (0.25)	0.76 (0.23)	0.91 (0.25)	<0.001
eGFR, mean (SD) (mL/min/1.73 m^2)	99.1 (33.8)	131.0 (32.3)	88.4 (26.8)	<0.001

(Continues)

TABLE 1 | (Continued)

Characteristic	All individuals	By race		
		Blacks	Whites	*p* value
KDIGO stage of eGFR				
≥90 mL/min/1.73 m^2	1193 (51.6)	499 (85.7)	694 (40.1)	<0.001
60–89 mL/min/1.73 m^2	883 (38.2)	60 (10.3)	823 (47.5)	<0.001
45–59 mL/min/1.73 m^2	172 (7.4)	19 (3.3)	153 (8.8)	<0.001
<45 mL/min/1.73 m^2	65 (2.8)	4 (0.7)	61 (3.5)	<0.001
Use of prescription drugs, no. (%)				
Antihypertensive drugs	809 (35.0)	100 (17.2)	709 (41.0)	<0.001
Antidiabetic agents	496 (21.4)	16 (2.8)	480 (27.7)	<0.001
Lipid-lowering drugs	522 (22.6)	37 (6.4)	485 (28.0)	<0.001

Note: Eastern Europe refers to 52 Polish and 9 Slovenians enrolled in UPRIGHT-HTM. Hypertension is a blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic or use of antihypertensive medications. If systolic and diastolic are in different categories, the highest category is applied for classification. Diabetes is the use of antidiabetic drugs, fasting blood glucose of ≥126 mg/dL, random blood glucose of ≥200 mg/dL, a self-reported diagnosis, or diabetes documented in practice or hospital records. eGFR is estimated from serum creatinine, using the Chronic Kidney Disease-Epidemiology Collaboration formula, and staged according to the Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group classification. Habitual alcohol consumption is using alcoholic beverages daily or weekly. An ellipsis indicates not applicable or that the *p* value was not computed. SI conversion factors: to convert hemoglobin to mmol/L multiply by 0.0259; glucose from mg/dL to mmol/L, multiply by 0.0555; cholesterol from mg/dL to mmol/L, multiply by 0.0259; creatinine from mg/dL to μmol/L, multiply by 88.4; eGFR from mL/min/1.73 m^2 to mL/s/1.73 m^2, multiply 0.0167.

Abbreviations: eGFR, estimated glomerular filtration rate; IQR, interquartile range; SD, standard deviation; UPRIGHT-HTM, Urinary Proteomics Combined with Home Blood Pressure Telemonitoring for Health Care Reform Trial.

fragment had lower urinary levels in Blacks than Whites (Table 2). Details including amino-acid sequence and numerical data are listed in Table S8. The collagen-derived urinary peptides keeping significance and consistent directionality in African-PREDICT and in at least two replication runs are tabulated in Table 2. Of these 158 peptides, four peptides with short chain (number of amino acids ranging from 8 to 11) derived from COL1A1 and one peptide (35 amino acids) originating from COL13A1 had higher levels in Blacks than Whites. The other collagen-derived peptides with duplicate or triplicate replication with consistent directionality on top of African-PREDICT had lower urinary levels in Blacks than Whites and predominantly originated from COL1A1, COL1A2, COL2A1, and COL3A1 (Table 2). Notably, of the three peptides originating from COL4A1 or COL4A2, all were replicated in three runs (Table 2). Table S9 lists the results of the sex- and age-matched analysis comparing the levels of urinary peptides in Black UPRIGHT-HTM patients ($n = 106$) and White FLEMENGHO ($n = 106$) and PROVALID-Austria ($n = 106$) participants combined. One peptide for each shortlisted protein with a substantial racial difference in the overall analysis of all participants was chosen for analysis. Consistent with the main analysis, the racial differences in the urinary levels were significantly different for these eight proteins ($p \leq 0.023$).

3.3 | Inflammation Markers

Circulating hs-CRP, IL-6, IL-8, and TNF-α were available in 476 Black South Africans versus 484 White South Africans and 618 White Flemish (Table S10). Their levels were expressed in dimensionless units of SD to account for the different techniques used South Africans and Flemish to measure their levels. With adjustments applied for sex, age, BMI, systolic and diastolic BP, and eGFR, the only Black versus White difference was detected in hs-CRP (0.07 vs. −0.03; $p = 0.0021$). hs-CRP, IL-6, IL-8, and TNF-α levels were similar in Blacks and Whites ($p \geq 0.52$).

3.4 | Information From Public Databases

Based on the information extracted from public databases (Figure 2), the proteins retained in Table 2 were ranked based on the published evidence demonstrating their implication in cardiovascular or renal structure or function. Of the noncollagenous proteins KRT2, MUC2, S100A7, and SAA2 were classified as irrelevant and LMAN2, PIGR, XYLT2, HBD, and IGKV4-1 as unlikely candidates. Of the remaining noncollagenous proteins, those with likely direct or indirect involvement in cardiovascular or renal structure or function as based on their function or expression profiles (Figure S1 in Supporting Information S1) included CD99, FAT1, FGA, FXYD2, MGP, MYOCD, PROC, and UMOD. FGA, FXYD2, and PROC are FDA-approved drug targets.

Collagens, in particular, fibrillar collagens with high abundance throughout the human body (COL1A1, COL1A2, COL2A1, COL3A1, COL6A1, COL7A1, and COL9A2) are important structural components involved in the scaffolding of most organs, supporting the musculoskeletal system, making up the extracellular matrix (ECM) and the connective tissue underlying epithelia. Because of their body-wide roles, collagens were not further considered with the exception of COL4A1 and COL4A2, which are ubiquitous constituents of basal membranes and which are the principal component of the glomerular basement membrane.

TABLE 2 | Racial differences in urinary peptide levels derived from noncollagenous proteins or collagens.

Name (UPA)	Symbol	No. of peptides	ΔBW (no. of peptides)			No. of replications		
			B–W	B < W	B > W	In 1/3 runs	In 2/3 runs	In 3/3 runs
Peptides derived from non-collagenous proteins								
CD99 antigen (P14209)	CD99	6	0	6	0	0	4	2
Protocadherin fat 1 (Q14517)	FAT1	1	0	1	0	0	1	0
Fibrinogen α chain (P02671)	FGA	6	0	6	0	2	2	1
Na/K-transporting ATPase subunit γ (P54710)	FXYD2	2	0	2	0	1	0	1
Hemoglobin subunit δ (P02042)	HBD	1	0	1	0	0	0	1
Immunoglobulin κ (P06312)	IGKV4-1	1	0	1	0	0	1	0
Keratin, Type II cytoskeletal 2 epidermal (P35908)	KRT2	1	0	1	0	0	0	1
Vesicular integral-membrane protein VIP36 (Q12907)	LMAN2	1	0	1	0	0	0	1
Matrix Gla protein (P08493)	MGP	1	0	1	0	0	1	0
Mucin-2 (Q02817)	MUC2	1	0	1	0	0	0	1
Myocardin (Q8IZQ8)	MYOCD	1	0	1	0	0	1	0
Protocadherin-7 (O60245)	PCDH7	1	0	1	0	0	1	0
Polymeric immunoglobulin receptor (P01833)	PIGR	3	0	3	0	0	2	1
Vitamin K-dependent protein C (P04070)	PROC	1	0	1	0	1	0	0
Protein S100-A7 (P31151)	S100A7	1	0	1	0	0	0	0
Serum amyloid A-2 protein (P0DJI9)	SAA2	1	0	1	0	0	0	1
Uromodulin (P07911)	UMOD	4	0	3	1	3	0	1
Xylosyltransferase 1 (Q86Y38)	XYLT1	1	0	1	0	0	1	0
Collagen-derived peptides								
Collagen alpha-1(I) chain (P02452)	COL1A1	79	7	67	5	0	39	28
Collagen alpha-2(I) chain (P08123)	COL1A2	11	0	11	0	0	5	6
Collagen alpha-1(II) chain (P02458)	COL2A1	10	1	9	0	1	5	3
Collagen alpha-1(III) chain (P02461)	COL3A1	41	1	38	2	1	27	13
Collagen alpha-1(IV) chain (P02462)	COL4A1	1	0	1	0	0	0	1
Collagen alpha-2(IV) chain (P08572)	COL4A2	2	0	2	0	0	0	2
Collagen alpha-2(V) chain (P05997)	COL5A2	3	0	3	0	0	1	2
Collagen alpha-3(V) chain (P25940)	COL5A3	1	0	1	0	0	0	1
Collagen alpha-1(VI) chain (P12109)	COL6A1	2	0	2	0	0	2	0
Collagen alpha-2(VI) chain (Q14055)	COL6A2	2	0	2	0	0	0	2
Collagen alpha-1(VII) chain (Q02388)	COL7A1	2	0	2	0	0	1	1
Collagen alpha-2(IX) chain (Q14055)	COL9A2	1	0	1	0	0	0	1
Collagen alpha-1(XIII) chain (Q5TAT6)	COL13A1	3	1	1	1	0	0	0
Collagen alpha-1(XV) chain (P39059)	COL15A1	1	1	0	0	0	0	0
Collagen alpha-1(XVI) chain (Q07092)	COL16A1	2	0	2	0	0	1	1
Collagen alpha-1(XVIII) chain (P39060)	COL18A1	1	0	1	0	0	0	1
Collagen alpha-1(XIX) chain (Q14993)	COL19A1	1	1	0	0	0	0	0
Collagen alpha-1(XXII) chain (Q8NFW1)	COL22A1	2	1	1	0	0	2	0
Collagen alpha-1(XXV) chain (Q9BXS0)	COL25A1	1	0	1	0	0	0	1

The findings are based on a comparison of South African Blacks and Whites and three replication runs: (i) comparison of Black South Africans and Nigerians with White South Africans, Flemish, and Austrians; (ii) comparison of Black South Africans and Nigerians with White Europeans (Flemish and Austrians); and (iii) comparison of Black Nigerians with White Europeans (Flemish and Austrians). UPA is the Uniprot accession number (https://www.uniprot.org). ΔBW indicates a gradient in the racial differences: B–W: inconsistent racial gradient; B < W, consistently lower levels in Blacks than Whites and B > W, consistently higher levels in Blacks than Whites. No. of replications refers to the number of confirmatory runs, in which urinary peptides had racially different levels with consistent directionality and Bonferroni-corrected significance in all analysis steps. Detailed results for peptides derived from non-collagenous peptides are available in Table S9 in Supporting Information S1 and for collagen-derived peptides in Supporting Information S2.

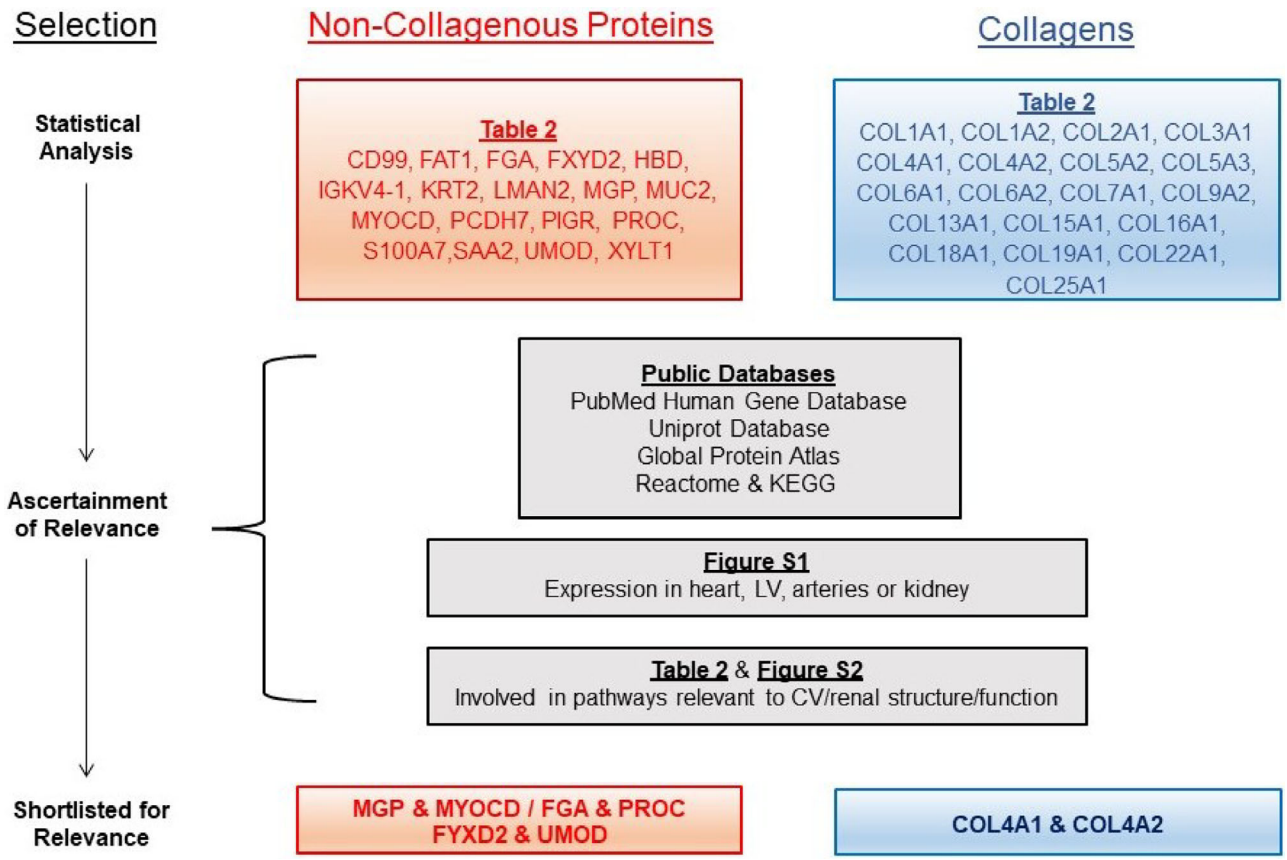

FIGURE 2 | Selection strategy of proteins with relevance to cardiovascular or renal structure or function Names of the collagens and noncollagenous symbols represented by symbols are listed in Table 2. CV, cardiovascular; LV, left ventricle.

3.5 | Pathway Analysis

Consulting the Reactome and KEGG databases and using as input COL4A1, COL4A2, CD99, FAT1, FGA, FXYD2, MGP, MYOCD, PROC, and UMOD identified 27 (Reactome) and 10 (KEGG) enriched pathways (q value $\leq$0.039; Figure S2 in Supporting Information S1). The most relevant pathways identified by Reactome included processes related to hemostasis, platelet activity, collagens, ECM biology, and signal transduction. In line with the Reactome analysis, the most relevant KEGG pathways also involved the ECM and protein digestion and absorption. The GO terms for processes and diseases are listed in Table 3; the Disease Ontology terms comprised cerebral small-vessel disease, myocardial infarction, and atherosclerotic cardiovascular disease.

4 | Discussion

In a search for proteins possibly explaining the high susceptibility of Blacks to salt sensitivity, hypertension, and hypertension-related complications [4–8], the current analysis followed a design with a high a-priory likelihood to detect relevant proteins. In a root analysis of the UPP data, the racial UPP differences were first assessed in the African-PREDICT cohort, because Black and White South Africans share a similar environment and because the African-PREDICT participants were young and healthy [10], thereby minimizing UPP alterations due to target organ damage. Three additional runs, two of which excluded South African Whites as comparator, confirmed the racial differences in the UPP. In short, this study identified racially different levels of urinary peptides derived from COL4A1, COL4A2, CD99, FAT1, FGA, FXYD2, MGP, MYOCD, PROC, and UMOD. As summarized in Figure 2, the information extracted from relevant gene and protein databases, tissue expression profiles (Figure S1), and the pathway analyses (Figure S2) support a potential role of the shortlisted proteins. Enriched pathways included hemostasis, platelet activity, collagens and ECM biology, and protein digestion. The Disease Ontology terms point to small-vessel disease and coronary and macrovascular atherosclerotic disease.

The human *COL4A1* and *COL4A2* genes locate to chromosome 13, *COL4A3* and *COL4A4* to chromosome 2, and *COL4A5* and *COL4A6* to the X chromosome [24]. COL4A1 and COL4A2 are present in almost all basement membranes, while the distribution of COL4A3 through COL4A6 is more restricted spatially and temporally [25]. Type IV collagen is the major structural component of the glomerular basement membrane, forming a chicken-wire meshwork together with laminins, proteoglycans, and nidogen [26]. *COL4A1* and *COL4A2* mutations cause multisystem disorders with high penetrance [24] that is further modulated by other polygenic risk factors [27]. Renal function is severely impaired in Alport syndrome and thin basement membrane disease. Alterations in the collagen IV coding genes also entail systemic small-vessel disease (Figure S2), as confirmed by skin and kidney biopsies [24], causing sporadic intracerebral

TABLE 3 | Gene Ontology (GO) search.

Search target (GO term identifier)	GO term description	p_{FDR}	Proteins included
Biological process			
GO:0009888	Tissue development	0.0069	FAT1/MGP/MYOCD/PROC/UMOD/COL4A1/COL4A2
GO:0098609	Cell–cell adhesion	0.011	CD99/FAT1/FGA/PCDH7/UMOD
GO:0098742	Cell–cell adhesion via plasma–membrane adhesion molecules	0.027	FAT1/PCDH7/UMOD
GO:0007155	Cell adhesion	0.028	CD99/FAT1/FGA/PCDH7/UMOD
GO:0072001	Renal system development	0.028	MYOCD/UMOD/COL4A1
GO:0050878	Regulation of body fluid levels	0.032	FGA/PROC/UMOD
GO:0035295	Tube development	0.040	MYOCD/UMOD/COL4A1/COL4A2
GO:0051241	Negative regulation of multicellular organismal process	0.040	FGA/MYOCD/PROC/COL4A2
GO:0060429	Epithelium development	0.041	FAT1/PROC/UMOD/COL4A1
GO:0007275	Multicellular organism development	0.043	FAT1/MGP/MYOCD/PROC/UMOD/COL4A1/COL4A2
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	0.043	MYOCD/COL4A1/COL4A2
GO:0048514	Blood vessel morphogenesis	0.043	MYOCD/COL4A1/COL4A2
Disease			
DOID:0112313	Brains small vessel disease	<0.0001	FGA/COL4A2/COL4A1
DOID:5844	Myocardial infarction	0.0096	MGP/FGA/PROC/COL4A2
DOID:1936	Atherosclerosis	0.024	MGP/PROC/COL4A2
DOID:2348	Atherosclerotic cardiovascular disease	0.024	MGP/PROC/COL4A2
DOID:2349	Arteriosclerosis	0.028	MGP/PROC/COL4A2

Findings are based on the Gene Ontology Database (http://www.geneontology.org) and the Disease Ontology Database (http://www.disease-ontology.org). p_{FDR} is the statistical significance corrected for the false discovery rate.

hemorrhage and stroke in adults. Intraframe *COL4A2* deletions lead to fetal intracerebral hemorrhage and hemiplegic cerebral palsy with related phenotypes in adults depending on penetrance [28]. Other features associated with COL4A1 and COL4A2 involve the cardiovascular system with valvular cardiac disease and supraventricular arrhythmias [24].

MYOCD is the most important coactivator of serum response factor (SRF). This protein plays a critical role in the development of cardiac myocytes and cardiovascular smooth muscle cells [29]. The binding of MYOCD to SRF transcriptionally activates a variety of downstream muscle-specific genes, such as *Sm22a*, *Acta2*, and *Myh11*. MYOCD expression results in a contractile and differentiated smooth muscle phenotype, whereas deficient MYOCD expression leads to a dedifferentiated phenotype, a hallmark in atherosclerosis [29]. Clinical studies showed increased *MYOCD*-derived mRNA levels in circulating cells or cardiac tissues from patients with idiopathic or hypertrophic cardiomyopathy or essential hypertension [30]. In experimental studies [31], *MYOCD* knockdown mice inhibited expression of the ATP binding cassette transporter A1 (ABCA1), a key membrane-associated lipid transporter, which maintains intracellular lipid homeostasis in human aortic smooth muscle cells, leading to reduced cholesterol efflux and increased intracellular cholesterol content.

Vascular smooth muscle cells and the endothelium synthesize a small secretory protein (11 kDa), which is named matrix Gla protein (MGP), because it contains five γ-carboxyglutamate (Gla) amino-acid residues. Activation of MGP requires two post-translational modifications: serine phosphorylation and vitamin K-dependent γ-glutamate carboxylation [32]. Active MGP, once released into the extracellular space, acts as a potent local inhibitor of calcification. However, growing evidence implicates activated MGP in maintaining microvascular integrity and preserving the structure and function of vital organs, including the retina, kidney, heart, and large arteries [32]. In line with this interpretation, in African-PREDICT [33], Blacks had lower circulating MGP levels than Whites, which might result in less protection of vital organs affected by hypertension. Additionally, African-PREDICT Blacks compared with Whites had smaller retinal arteriolar and venular diameters [34].

Of the sodium load filtered at the glomerulus, about 60%–70% is reabsorbed by the proximal renal tubules, while about 90% of the sodium escaping the proximal nephron is reabsorbed in the

thick ascending limb (TAL) and the more distal nephron [35, 36]. In South African Blacks compared to Belgian Whites, more of the filtered sodium load is reabsorbed in the proximal nephron [37]. Furthermore, segmental sodium reabsorption along the nephron is highly heritable [37]. These observations corroborate that renal sodium handling is racially different. In line with these observations [35, 37], the present analyses identified different urinary levels of one peptide derived from FXYD2 and four originating from UMOD (Table 2). Both FXYD2 and UMOD are implicated in renal sodium homeostasis and salt sensitivity.

In humans, there are seven tissue-specific FXYD proteins, of which FXYD2 (the γ-subunit) is expressed as two splice variants, the γa and γb subunits in the kidney and pancreas (Figure S1) [38]. In the kidney, both γ-subunit variants are primarily expressed in the TAL and induce a similar reduction of the affinity of Na+/K+-ATPase for sodium. FXYD4 is exclusively expressed in the distal nephron and enhances the affinity of Na+/K+-ATPase for sodium. FXYD2 and FXYD4 fine-tune sodium affinity along the nephron: low in the proximal tubule, connecting tubule, and medullary ascending limb (FXYD2); intermediate in the cortical ascending limb (no FXYD detected); and high in the collecting duct (FXYD4) [39]. Given that in Blacks compared to Whites, sodium reabsorption is higher in the proximal nephron where FXYD2 is expressed, making the involvement of FXYD2 in the greater salt sensitivity of Blacks a likely hypothesis.

UMOD, a mucin-like glycoprotein also known as Tamm-Horsfall protein, is a major constituent of urine and is exclusively produced in the TAL [40]. Salt loading might decrease its secretion [41], perhaps explaining why urinary UMOD-derived peptide fragments may be up- or downregulated depending on salt intake. Genome-wide association studies [42] and longitudinal cohort studies [43] identified common variants in the promoter of the *UMOD* gene, which in humans causes hypertension and susceptibility to chronic kidney disease. UMOD regulates the activity of the sodium–potassium–chloride transporter (NKCC2) and the renal outer medullary potassium channel (ROMK), the two main ion transporters involved in NaCl reabsorption and potassium excretion by the TAL. Overexpression of UMOD in transgenic mice stimulates sodium uptake in the TAL by potentiating the effects of TNF-α on NKCC2 expression and causes salt-sensitive hypertension [44]. Furosemide, the specific NKCC2 inhibitor, reverses these effects [42].

In a translational experiment [45], 6 salt-sensitive and 10 salt-resistant never-treated hypertensive patients were studied. After a 2-h calibration period, during which the patient rested in the supine position, 2 L of a 0.9% NaCl solution was infused over 2 h. Blood and urine samples were collected at the start and completion of the infusion and at the end of a 2-h recovery period for measurement of the circulating components of the renin–angiotensin system (RAS) and urinary UPP. The urinary sodium excretion was similar in salt-sensitive and salt-resistant patients. However, salt-sensitive patients regulated RAS differently from salt-resistant patients [45]. The UPP analysis revealed a differential upregulation of glutamyl aminopeptidase (ENPEP), plasminogen activator (PLAU), epidermal growth factor (EGF), and Xaa-Pro aminopeptidase 2 precursor (XPNPEP2) as key molecules of salt sensitivity through modulation of the epithelial sodium channel (ENaC) along the distal tubule [45]. These findings are in keeping with the key role of the regulation of sodium transport along the nephron in determining the racial divergence is salt sensitivity, as captured in the current study by FYXD2 and UMOD.

4.1 | Study Limitations

This report includes Blacks born and living in Sub-Saharan Africa and therefore adds to the reports focusing on African Americans [4–6]. To minimize environmental biases and the influence of subclinical target organ damage on the UPP, the initial set of racially different peptides was derived in young and healthy African-PREDICT participants. Subsequent runs excluding White South Africans from the racial comparator were confirmatory. Nevertheless, this study has limitations. First, Blacks typically had lower urinary peptide levels. Inaccurate urine sampling does not explain this observation, because urine collection was supervised in African-PREDICT [10]. South African Blacks compared to South African Whites had lower body height and weight (Table S2) and therefore a lower pool of parental proteins. To account for analytical and dilution variances, the CE-MS procedure incorporated reference signals of 29 endogenous urinary peptides as internal standards for the calibration of signal intensity [18]. Centering the UPP data from each cohort separately before combining datasets [46] additionally addressed batch effects and produced confirmatory results. Second, although analyses were multivariable adjusted, the impact of unmeasured confounders cannot be excluded. A sensitivity analysis suggested that inflammation, which is dependent on sex, age, and risk factors, is unlikely to have played a role in the racial differences in the UPP markers. The adequacy of the age adjustment in the general linear models was confirmed in a sex- and age-matched analysis comparing one peptide derived from the shortlisted proteins between Nigerian Blacks and European Whites. Third, there is a vast body of evidence from experimental, clinical, and epidemiological studies confirming that Blacks are more salt sensitive than Whites and have an increased risk of hypertension and hypertension-related complications [5, 6]. These concepts prevail as the current state of scientifically generated insights but were not further explored in the present study participants, given the confounding by regional differences in the distribution of risk factors, such as age, access to medical care, lifestyle, and dietary habits. Finally, the levels of the urinary peptides do not provide any information on the expression of the encoding genes. Additionally, proteases active along the nephron and distal urinary tract might affect the urinary peptide fragments detected. However, in a placebo-controlled study of a dipeptidyl peptidase 4 inhibitor, the UPP included pairs of peptide chains, including the substrate for the protease activity (e.g., PPGPPGKNGDDGEAGKPG) and the resulting breakdown product (e.g., GPPGKNGDDGEAGKPG) [47]. In the current study, none of the peptides retained in the final analysis contained such peptide pairs.

5 | Conclusions

MGP and MYOCD are involved in cardiovascular structure and function, FGA and PROC in cloth formation, FXYD2 and UMOD in salt homeostasis, and COL4A1 and COL4A2 in maintaining

the integrity of the glomerular basal membrane. These proteins have high clinical relevance in differentiating health from disease and play a role in the higher susceptibility to hypertension and salt sensitivity of Blacks compared to Whites. FGA, FXYD2, and PROC are FDA-approved drug targets. The current study adds additional proteins, variation in the encoding genes, and downstream signaling pathways to the pathogenic mechanism requiring further exploration in clinical and experimental studies to address the excess hypertension risk and salt sensitivity in Blacks.

Author Contributions

Jan A. Staessen: had full access to the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. **Jan A. Staessen:** concept and design. **Gontse G. Mokwatsi, Babangida S. Chori, Godsent Isiguzo, Susanne Eder, Gert Mayer, Ruan Kruger, Jana Brguljan-Hitij, Catharina M.C. Mels, Katarzyna Stolarz-Skrzypek, Marek Rajzer, Augustine N. Odili, and Aletta E. Schutte:** acquisition of the data. **Agnieszka Latosinska and Harald Mischak:** acquisition of the urinary proteomic data. **De-Wei An, Dries S. Martens, and Jan A. Staessen:** statistical analysis. **De-Wei An, Dries S. Martens, Jan A. Staessen:** drafting the manuscript. **Peter Verhamme, Yan Li, Harald Mischak, Aletta E. Schutte, Tim S. Nawrot, Augustine N. Odili, and Jan A. Staessen:** supervision. **All authors:** critical review of the manuscript for important content.

Conflicts of Interest

Agnieszka Latosinska is an employee of Mosaiques Diagnostics GmbH, Hannover, Germany. Harald Mischak is the co-founder and co-owner of Mosaiques Diagnostics GmbH. The other authors declare no conflict of interest.

Role of the Funder

The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Data Availability Statement

All relevant data are within the paper. Informed consent given by study participants did not include data sharing with third parties. However, anonymized data can be made available to investigators for targeted noncommercial research based on a motivated request to be submitted to Jan A. Staessen and pending ethical clearance by the Ethics Review Board of each institution involved in the study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.