INFOGRAPHIC

# Multiple urinary peptides are associated with hypertension: a link to molecular pathophysiology

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**Objectives:** Hypertension is a common condition worldwide; however, its underlying mechanisms remain largely unknown. This study aimed to identify urinary peptides associated with hypertension to further explore the relevant molecular pathophysiology.

**Methods:** Peptidome data from 2876 individuals without end-organ damage were retrieved from the Human Urinary Proteome Database, belonging to general population (discovery) or type 2 diabetic (validation) cohorts. Participants were divided based on systolic blood pressure (SBP) and diastolic BP (DBP) into hypertensive (SBP $\geq$140 mmHg and/or DBP $\geq$90 mmHg) and normotensive (SBP $<$120 mmHg and DBP $<$80 mmHg, without antihypertensive treatment) groups. Differences in peptide abundance between the two groups were confirmed using an external cohort ($n = 420$) of participants without end-organ damage, matched for age, BMI, eGFR, sex, and the presence of diabetes. Furthermore, the association of the peptides with BP as a continuous variable was investigated. The findings were compared with peptide biomarkers of chronic diseases and bioinformatic analyses were conducted to highlight the underlying molecular mechanisms.

**Results:** Between hypertensive and normotensive individuals, 96 (mostly COL1A1 and COL3A1) peptides were found to be significantly different in both the discovery (adjusted) and validation (nominal significance) cohorts, with consistent regulation. Of these, 83 were consistently regulated in the matched cohort. A weak, yet significant, association between their abundance and standardized BP was also observed.

**Conclusion:** Hypertension is associated with an altered urinary peptide profile with evident differential regulation of collagen-derived peptides. Peptides related to vascular calcification and sodium regulation were also affected. Whether these modifications reflect the pathophysiology of hypertension and/or early subclinical organ damage requires further investigation.

**Graphical abstract:** http://links.lww.com/HJH/C445

**Keywords:** blood pressure, CE-MS, hypertension, peptides, urine

**Abbreviations:** BH, Benjamini-Hochberg; BMI, body-mass index; BP, blood pressure; CAD, coronary artery disease; CD99, CD99 antigen; CKD, chronic kidney disease; COL13A1, Collagen alpha-1(XIII) chain; COL15A1, Collagen alpha-1(XV) chain; COL16A1, Collagen alpha-1 (XVI) chain; COL18A1, Collagen alpha-1(XVIII) chain; COL1A1, Collagen alpha-1(I) chain; COL21A1, Collagen alpha-1(XXI) chain; COL22A1, Collagen alpha-1(XXII) chain; COL25A1, Collagen alpha-1(XXV) chain; COL2A1, Collagen alpha-1(II) chain; COL1A2, Collagen alpha-2(I) chain; COL3A1, Collagen alpha-1(III) chain; COL4A3, Collagen alpha-3(IV) chain; DBP, diastolic blood pressure; ECM, extracellular matrix; eGFR, estimated glomerular filtration rate; FGA, Fibrinogen alpha chain; FGB, Fibrinogen beta chain; GSN, Gelsolin; HF, heart failure; IGF2, Insulin-like growth factor II; KRT77, Keratin, type II cytoskeletal 1b; LTBP4, Latent-transforming growth factor beta-binding protein 4; MGP, Matrix Gla protein; MMP, matrix metalloproteinase; ORM1, Alpha-1-acid glycoprotein 1; PIGR, Polymeric immunoglobulin receptor; POTEF, POTE ankyrin domain family member F; SBP, systolic blood pressure; sDBP, standardized DBP; sSBP, standardized SBP; UMOD, Uromodulin

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## INTRODUCTION

The increasing burden of chronic diseases indicates the need for noninvasive, easily accessible, stable, and cost-effective biomarkers. The field of urinary proteomics/peptidomics has developed extensively and consistently over the past two decades, with a focus on kidney and cardiovascular diseases [1–6]. Thousands of proteins present in the human body are ultimately degraded by ubiquitous proteolytic enzymes, resulting in the generation of protein fragments, that is, peptides. By the time these peptides are excreted in urine, enzymatic digestion is completed and thus, they are generally resistant to any additional endogenous proteases. As such, these peptides represent the end-products of endogenous proteolysis and can be assessed as highly stable biomarkers. Simultaneously, given the proximity of the proteome to the phenotype, several of these peptides are reflective of systemic/peripheral (patho) physiology. These properties are essential in clinical biomarker research. Highly significant associations of urine peptides, especially with kidney and cardiovascular disease, have been established in the past [7].

Hypertension is a complex and mechanistically unexplored condition that is associated with several chronic diseases. This condition appears to play a role not only as a major risk factor, but also as a pathophysiological consequence that further exacerbates chronic diseases. In previous studies focusing on chronic diseases, including heart failure [3], coronary artery disease (CAD) [8], and chronic kidney disease (CKD) [9]), significant differential regulation in the abundance of hundreds of specific urinary peptides was uncovered, likely reflecting disease pathophysiology.

To decipher the molecular mechanisms involved in hypertension, we aimed to identify urinary peptides that differ significantly between patients with hypertension and normotensive individuals. To this end, the urinary peptide data of almost 3000 participants from either population-based or diabetic cohort studies were obtained from the Human Urinary Proteome Database [1] and served as the basis for a series of statistical and bioinformatics analyses to further explore the molecular players associated with this condition.

## MATERIALS AND METHODS

### Study population

Considered were anonymized data from the FLEMENGHO [10] and Generation Scotland: Scottish Family Health Study [3,11] (general population), as well as DIRECT2 [12] and PRIORITY [6,13] (diabetes type 2) studies. On this initial, extracted cohort of $n = 4726$, the following exclusion criteria were applied: established end-organ damage, including eGFR less than 60 ml/min/1.73 $m^2$ (CKD-EPI), macroalbuminuria (total excretion of urinary albumin >300 mg/24 h), left ventricular hypertrophy, heart failure, ischemic heart disease/CAD, transplantation, history of cancer, history of kidney or cardiovascular disease. The general population cohort was investigated for discovery purposes ($n = 749$), whereas the diabetic cohort was used for validation purposes ($n = 2127$). Notably, the aim was not to compare the general population and the diabetic cohort; instead, the latter cohort was used solely to verify that the discovery findings were hypertension-associated. The diabetes cohort was chosen, because no data from an additional independent large cohort of apparently healthy individuals were available. The systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were used to divide each cohort into two groups based on the European guidelines for hypertension: SBP at least 140 mmHg and/or DBP at least 90 mmHg (hypertensive), SBP less than 120 mmHg and DBP less than 80 mmHg, and not undergoing antihypertensive treatment (normotensive). Given that the hypertensive and normotensive groups in both the discovery and validation cohorts differed significantly in their main clinical characteristics (Table 1), an additional external cohort of hypertensive and normotensive individuals ($n = 1352$) was used as a basis for matching the two groups in terms of age, body-mass index (BMI), eGFR, sex, and presence of diabetes to further confirm the results and findings in the absence of these confounding factors. The inclusion and exclusion criteria were similar to those used in the discovery and validation cohorts.

### Urinary peptidomics

Data were extracted from the Human Urinary Proteome Database [1], which contains datasets acquired using capillary electrophoresis coupled with mass spectrometry, as described previously [1,14–16]. Data were evaluated using MosaFinder software and normalized based on the abundance of 29 collagen peptides [17]. Of the 5071 sequenced peptides identified to date, only those present in at least 50% of the entire (discovery+validation) cohort of 2876 individuals (888 peptides) remained for further analyses. Before any statistical analyses, the missing values of that dataset per column were replaced by randomly generated deviates of a uniform distribution in the range between $0.75 \times x$ and $1.25 \times x$ (where x is the minimum column value), similar to the approach described in [18]. For any of these 888 peptides being available in the external, matched cohort, missing values were similarly, independently imputed. A detailed schematic diagram of the study design and workflow is shown in Fig. 1.

### Statistical analysis

#### Differential peptide abundance between the groups

A comparison of peptide abundances between the hypertensive and normotensive groups was performed in the discovery cohort. Next, significantly different peptides (applying Benjamini- Hochberg (BH) correction for multiple testing) were assessed in terms of consistent regulation and nominal significance in the validation cohort. Peptides fulfilling all these three criteria were evaluated based on their consistency in regulation using an external, matched cohort to account for the potential impact of confounders (age, BMI, eGFR, sex, and presence of diabetes), before being considered in subsequent analyses. Linear regression was used to examine the potential relation of hypertension-associated peptides with significant changes in previously identified biomarkers of chronic diseases, including heart failure [3], CAD [8], and CKD [9]. Differential peptide abundance analysis was performed using the Mann–Whitney *U*

**TABLE 1. Clinical characteristics per cohort**

| | Hypertensive (discovery) | Normotensive (discovery) | *P* (discovery) | Hypertensive (validation) | Normotensive (validation) | *P* (validation) | Hypertensive (matched) | Normotensive (matched) | *P* (matched) |
|---|---|---|---|---|---|---|---|---|---|
| *n* | 229 | 177 | | 815 | 106 | | 210 | 210 | |
| Age | 61.1 (13.1) | 39.6 (12.6) | <0.001 | 60.8 (7.7) | 54.4 (8.2) | <0.001 | 33.1 (11.7) | 31.3 (10.3) | 0.093 |
| BMI | 27.7 (5.0) | 24.2 (3.5) | <0.001 | 30.4 (4.8) | 28.5 (6.1) | <0.001 | 25.9 (4.4) | 25.1 (4.0) | 0.048 |
| eGFR | 81.9 (12.0) | 93.9 (16.0) | <0.001 | 91.7 (13.5) | 98.6 (14.3) | <0.001 | 120.4 (29.0) | 124.3 (25.2) | 0.149 |
| SBP | 151.9 (14.8) | 111.1 (5.4) | <0.001 | 147.5 (9.2) | 112.0 (6.4) | <0.001 | 141.2 (11.2) | 111.2 (5.6) | <0.001 |
| DBP | 88.1 (9.5) | 71.1 (5.8) | <0.001 | 83.7 (8.2) | 70.6 (5.7) | <0.001 | 90.6 (8.1) | 72.2 (6.0) | <0.001 |
| sSBP | 148.0 (13.8) | 122.6 (7.0) | <0.001 | 144.8 (9.1) | 118.7 (6.5) | <0.001 | | | |
| sDBP | 86.9 (9.0) | 71.7 (5.9) | <0.001 | 83.5 (8.1) | 72.8 (5.6) | <0.001 | | | |
| Heart rate | 66.5 (10.9) | 63.3 (8.4) | 0.001 | 74.9 (11.5) | 71.0 (8.5) | 0.023 | | | |
| Sex (male) | 144 (62.9) | 61 (34.5) | <0.001 | 473 (58.0) | 46 (43.4) | 0.006 | 159 (75.7) | 140 (66.7) | 0.052 |
| Diabetes | 16 (7.0) | 1 (0.6) | 0.003 | 815 (100.0) | 106 (100.0) | | 53 (25.2) | 57 (27.1) | 0.739 |
| Antihypertensive treatment | 86 (37.6) | 0 (0.0) | <0.001 | 715 (87.7) | 0 (0.0) | <0.001 | 55 (26.2) | 0 (0.0) | <0.001 |

Given is the number (n) of each entire group based on the BP values. A mean (standard deviation) or percentage (number) depending on the variable type is written for the available clinical information. BMI, body-mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; sDBP, standardized DBP; sSBP, standardized SBP.

test. A significance threshold of *P* value less than 0.05 was applied, unless stated otherwise.

## Blood pressure standardization and correlation with peptide abundance

The following clinical parameters were considered as variables for BP standardization: age, BMI, eGFR, sex, antihypertensive treatment, diabetes, and the study cohort. These variables were used as a basis for a stepwise linear regression applying the following approach (also described in [18]): a *P* value threshold of 0.15 was required for a variable to enter ($P < 0.15$) or be removed ($P > 0.15$) from the model. Initially, a model relying only on the variable with the lowest *P* value ($P < 0.15$, as described) was developed. Subsequently, from the variables not included in the model, the variable with the lowest *P* value was added to the previous model. Then, the variable already present in the model (before the addition of that second, newly added variable) was checked again in terms of *P* value and was removed from the model, if it exceeded the designated threshold ($P > 0.15$, as described). This procedure was repeated until all the variables that had not been added to the model, were above the *P* value threshold and thus could not enter the model. On the basis of stepwise linear regression and using the entire (discovery+validation) cohort ($n = 2876$), the variables age, antihypertensive treatment, study cohort, sex, BMI, presence of diabetes, and eGFR remained in the final model and were considered for SBP standardization. Similarly, for DBP, BMI, sex, study cohort, presence of antihypertensive treatment, and age (but not eGFR or presence of diabetes) were considered. For each patient, the BP measures were

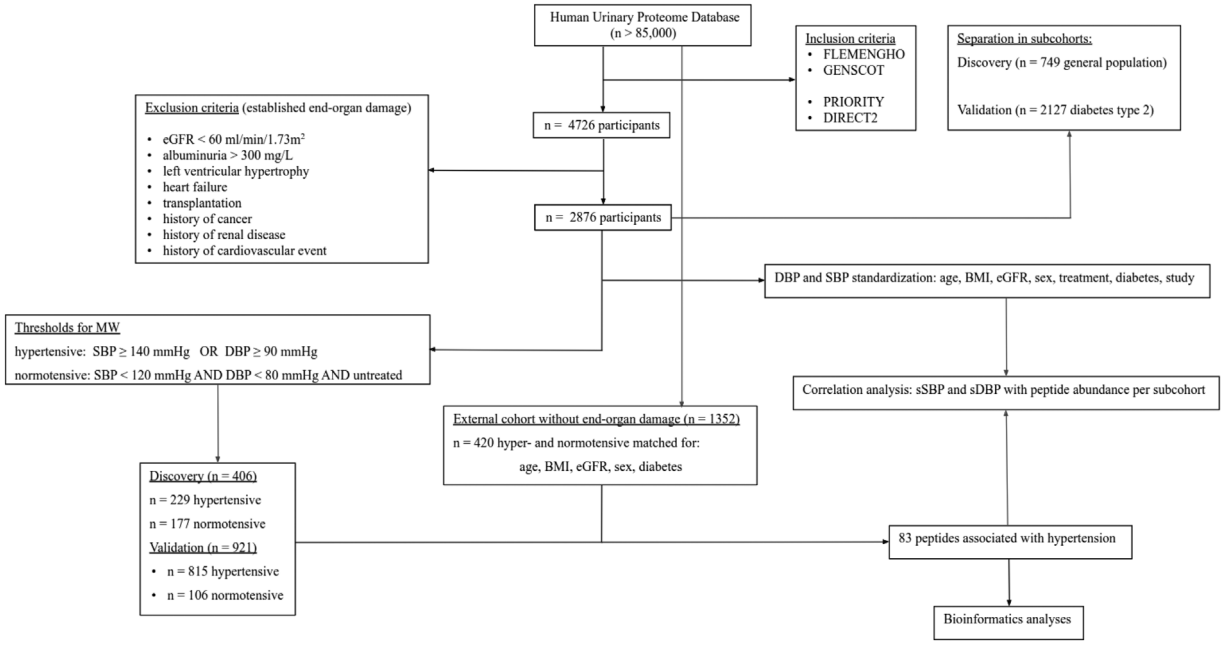


**FIGURE 1** Study design and workflow. BH, Benjamini-Hochberg; BMI, body-mass index; BP, blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; sDBP, standardized DBP; sSBP, standardized SBP.

standardized using each time the respective beta coefficients of the aforementioned clinical variables, based on the formula:

$$\text{Standardized BP measure} = \text{BP measure} - \sum(\text{variable beta coefficient} * [\text{variable} - \text{mean (variable)}]).$$

Finally, using the entire discovery cohort (general population, $n = 749$), a correlation analysis between these standardized BP measures (sSBP and sDBP) and the significant, consistently regulated peptides was performed to reveal any relevant associations.

## Bioinformatics analysis

Bioinformatics analyses were employed to place the urinary proteomic findings in a biological context. Protein-protein interactions were investigated using the STRING database, based on default settings. The output was subjected to k-means clustering, based on a specified number of clusters ($n = 3$).

## Software

Data processing and analyses were based on R programming [19] (version 4.3.2) running on Ubuntu 22.04 computer software. The collection of R packages in the 'tidyverse' [20] package as well as the 'broom' [21] and 'janitor' [22] packages were extensively used. Random deviations were generated based on the runif function of the stats package after applying the function set.seed (2020). Matching between hypertensive and normotensive participants at 1 : 1 ratio was based on the matchit function of the 'MatchIt' package [23]. The Mann–Whitney *U* test for peptide differential abundance comparison was performed based on the col_wilcoxon_twosample (exact = FALSE) of the 'matrixTests' package [24]. *P* value less than 0.05 was the threshold for both the adjusted (for multiple testing) and nominal significance. For the former, the BH method was performed via the function p.adjust (method = 'BH') of the 'stats' package. Stepwise linear regression was based on the function ols_step_both_p (pent = 0.15, prem = 0.15) of the 'olsrr' package [25] using the output of the lm function of the 'stats' package with the clinical variables already described. The association of the peptides with the BP as a continuous variable was based on the rcorr(type = 'spearman') function of the 'Hmisc' package [26]. Protein-protein interaction analysis and subsequent clustering were performed using STRING (https://version-12-0.string-db.org/) [27]. Linear regressions that considered discovery and chronic disease fold-changes were performed using MedCalc (version 12.1.0.0; MedCalc Software, Mariakerke, Belgium).

# RESULTS

The discovery cohort included 749 individuals from the general population, whereas the validation cohort included 2127 patients with type 2 diabetes. On the basis of the applied definition of cases (hypertensive) and controls (normotensive), excluding individuals in which normotension or hypertension could not be established with sufficient confidence, the discovery cohort consisted of 229 hypertensive and 177 normotensive individuals, whereas the validation cohort consisted of 815 hypertensive and 106 normotensive individuals, reflecting the increased risk of hypertension in diabetic patients. As several relevant demographic and clinical characteristics in the discovery cohort showed significantly different distributions between normotensive and hypertensive individuals, an additional, external cohort, matched for age, BMI, eGFR, sex, and presence of diabetes, was included in the study. The matched cohort consisted of 420 hypertensive and normotensive participants. The clinical and demographic characteristics of the discovery and validation cohorts are presented in Table 1. The study design and workflow are shown in Fig. 1.

## Urinary peptide associations with blood pressure

Using the discovery cohort, a comparison of peptide levels between the hypertensive and normotensive groups was initially performed, revealing 308 peptides that were significantly different (*P* values adjusted for multiple testing). Of these, 205 showed the same abundance trend in both the discovery and the diabetic validation cohort, with 96 reaching nominal significance in the validation cohort. As a result of the significant difference regarding clinical confounders between the hypertensive and normotensive individuals in the study, as an additional step of evaluation, an analysis of these 96 peptides was performed between the two groups, using an external, matched cohort. The analysis confirmed consistent regulation of 83 of the 96 peptides, and only those were considered for subsequent analyses. These peptides were derived from 24 proteins, 12 of which were collagens (the parental proteins of 68 peptides): Collagen alpha-1(I) (COL1A1) (34 peptides), Collagen alpha-1(III) (COL3A1) (13), Collagen alpha-1(II) (COL2A1) and Collagen alpha-2(I) (COL1A2) (four each), Collagen alpha-3(IV) (COL4A3) (three), Collagen alpha-1(XVI) (COL16A1), Collagen alpha-1(XVIII) (COL18A1), and Collagen alpha-1 (XXII) (COL22A1) (two each), and Collagen alpha-1(XIII) (COL13A1), Collagen alpha-1(XV) (COL15A1), Collagen alpha-1(XXI) (COL21A1), and Collagen alpha-1(XXV) (COL25A1) (one each). For non-collagen proteins, three peptides were derived from Fibrinogen alpha (FGA), two from Matrix Gla protein (MGP), and one from Alpha-1-acid glycoprotein 1 (ORM1), CD99 antigen (CD99), Fibrinogen beta (FGB), Gelsolin (GSN), Insulin-like growth factor II (IGF2), Keratin, type II cytoskeletal 1b (KRT77), Latent-transforming growth factor beta-binding protein 4 (LTBP4), POTEF (POTE ankyrin domain family member F), Polymeric immunoglobulin receptor (PIGR), and Uromodulin (UMOD). The 20 most BH-significant peptides (based on the discovery cohort), along with their regulation trends, are listed in Table 2. To further confirm the relevance of these peptides in the context of BP, their continuous correlation with sSBP and sDBP was investigated using Spearman's rank method (using the entire discovery cohort of $n = 749$). The range for the Spearman's rank correlation

**TABLE 2. Results of the peptide differential abundance and Spearman's rank correlation analyses as well as regulation of previously established biomarkers in chronic diseases**

| Protein | Sequence | Adj. *P* Discovery | FC Discovery | *P* Validation | FC Validation | FC Matched | FC HF | FC CAD | FC CKD | Cor *P* sSBP Discovery | Cor rho sSBP Discovery | Cor *P* sDBP Discovery | Cor Rho sDBP Discovery |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MGP | cDDYRLcE | 9.96E-19 | 2.61E+00 | 2.14E-08 | 1.57E+00 | 1.30E+00 | 1.93E+00 | | | 1.18E-02 | 9.19E-02 | 5.40E-05 | 1.47E-01 |
| MGP | cDDYRLc | 2.83E-13 | 2.89E+00 | 1.18E-05 | 1.65E+00 | 1.33E+00 | 2.12E+00 | | | 1.42E-02 | 8.96E-02 | 1.09E-03 | 1.19E-01 |
| COL25A1 | PGpPGPHGPPGpmGPHGLpGP | 2.83E-13 | 2.07E+00 | 7.86E-03 | 1.27E+00 | 1.11E+00 | 7.24E-01 | | | 7.05E-01 | 1.39E-02 | 5.39E-04 | 1.26E-01 |
| COL18A1 | DDILASPPRLPEPQPYPGAPHHS | 4.69E-13 | 3.11E+00 | 3.99E-04 | 1.56E+00 | 1.69E+00 | 1.57E+00 | | | 2.68E-01 | 4.05E-02 | 8.01E-03 | 9.68E-02 |
| COL1A1 | ApGDRGEpGPpGPAG | 8.37E-10 | 7.96E-01 | 1.59E-02 | 9.25E-01 | 9.74E-01 | 7.72E-01 | 7.50E-01 | 7.11E-01 | 5.27E-01 | -2.32E-02 | 1.35E-01 | -5.47E-02 |
| CD99 | DDPRPPNPPKPMPNPNPNHPSSSGS | 8.37E-10 | 3.97E+00 | 3.86E-03 | 1.53E+00 | 1.77E+00 | | | | 2.74E-01 | 4.00E-02 | 4.55E-04 | 1.28E-01 |
| COL1A1 | GSpGSpGPDGKTGPpGPAG | 2.11E-09 | 7.88E-01 | 1.44E-03 | 8.79E-01 | 9.24E-01 | 8.80E-01 | | 9.15E-01 | 6.90E-02 | -6.65E-02 | 4.77E-02 | -7.24E-02 |
| COL1A1 | LDGAKGDAGPAGPKGEpGSpGENGApG | 2.45E-09 | 6.86E-01 | 4.49E-02 | 9.01E-01 | 9.53E-01 | | | | 6.81E-01 | -1.51E-02 | 2.57E-02 | -8.15E-02 |
| COL2A1 | DGpSGAEGpPGp | 3.16E-09 | 1.61E+00 | 1.22E-06 | 1.35E+00 | 1.16E+00 | 1.16E+00 | | | 2.39E-02 | 8.25E-02 | 2.98E-05 | 1.52E-01 |
| COL1A1 | EGSpGRDGSpGAKGDRG | 5.21E-09 | 5.55E-01 | 6.50E-06 | 6.14E-01 | 8.73E-01 | 5.95E-01 | | 3.45E-01 | 7.18E-01 | -1.32E-02 | 1.99E-02 | -8.51E-02 |
| COL3A1 | DGESGRpGRpG | 2.45E-08 | 5.16E-01 | 5.61E-05 | 6.59E-01 | 8.53E-01 | 3.96E-01 | | | 6.87E-03 | -9.87E-02 | 9.52E-04 | -1.20E-01 |
| COL4A3 | GSPGPpGTpGEpGMQGE | 2.04E-06 | 3.25E+00 | 2.52E-02 | 1.49E+00 | 1.02E+00 | | | | 6.52E-01 | -1.65E-02 | 4.67E-01 | 2.66E-02 |
| COL1A2 | RTGEVGAVGPpGFAGEKGPSGEAGTAGpPGTpGPQG | 3.38E-06 | 7.58E-01 | 4.95E-03 | 8.63E-01 | 8.30E-01 | 8.71E-01 | | 7.06E-01 | 8.11E-02 | -6.38E-02 | 1.09E-02 | -9.30E-02 |
| COL1A1 | ADGQpGAKGEpGDAGAKGDAGPpGPAGP | 8.30E-06 | 7.03E-01 | 4.43E-02 | 8.19E-01 | 7.58E-01 | | | 7.01E-01 | 1.14E-01 | -5.78E-02 | 1.36E-02 | -9.02E-02 |
| COL1A1 | EGSpGRDGSpGAKGDRGET | 8.50E-06 | 7.14E-01 | 2.19E-08 | 6.20E-01 | 9.84E-01 | 4.93E-01 | | 3.49E-01 | 3.85E-01 | -3.18E-02 | 6.39E-02 | -6.77E-02 |
| COL3A1 | DGVPGKDGPRGPT | 1.03E-05 | 6.29E-01 | 1.80E-04 | 7.06E-01 | 8.84E-01 | 3.21E-01 | | | 5.97E-01 | 1.94E-02 | 4.77E-01 | -2.60E-02 |
| COL1A1 | DGQPGAKGEpGDAG | 1.14E-05 | 6.13E-01 | 1.03E-05 | 5.21E-01 | 8.22E-01 | 5.57E-01 | | | 6.58E-01 | 1.62E-02 | 1.11E-01 | -5.83E-02 |
| COL4A3 | GppGDpGSPGSP | 2.02E-05 | 1.36E+00 | 1.50E-05 | 1.34E+00 | 1.25E+00 | 1.08E+00 | | | 2.71E-01 | 4.03E-02 | 8.27E-02 | 6.34E-02 |
| COL1A1 | ADGQpGAKGEpGDAGAKGDAGPPGPAGP | 2.13E-05 | 7.59E-01 | 4.51E-05 | 7.61E-01 | 8.01E-01 | | | 6.98E-01 | 9.39E-01 | -2.81E-03 | 3.62E-01 | -3.34E-02 |
| COL1A1 | pGpAGEKGSpGADGPAGAP | 2.58E-05 | 1.21E+00 | 6.67E-05 | 1.25E+00 | 1.34E+00 | | | | 4.59E-01 | 2.71E-02 | 1.31E-02 | 9.07E-02 |

The analysis was performed separately for the discovery, validation, and matched cohorts between hypertensive and normotensive groups. Listed are the 20 (out of the 83) most significant peptides identified as significantly different in both the discovery (*P* value adjusted for multiple testing) and the validation cohorts (nominal significance) and demonstrating consistent regulation across both of these cohorts as well as the external matched cohort. Findings are sorted based on increasing adjusted *P* values in the discovery cohort. Moreover, the fold change comparison with previously established biomarkers in chronic diseases revealed no substantial subclinical organ damage. CAD, coronary artery disease; CKD, chronic kidney disease; Cor, correlation; DBP, diastolic blood pressure; FC, fold change; HF, heart failure; SBP, systolic blood pressure; sDBP, standardized DBP; sSBP, standardized SBP.

coefficients for the sSBP and sDBP was -0.10 to +0.09 and -0.12 to +0.15, respectively. All results and findings are listed in Supplementary Table 1, http://links.lww.com/HJH/C444.

## Comparison with urinary peptides associated with kidney and cardiovascular disease

Although a cohort inclusion criterion was the absence of clinical signs of end-organ damage, this was not sufficient to exclude the presence of subclinical organ damage. Therefore, additional steps were performed to investigate the relation of the defined 83 hypertension-associated peptides with previously established peptidomic biomarkers for chronic diseases, such as heart failure [3], CAD [8], and CKD [9]. The results for all the 83 peptides are listed in Supplementary Table 1, http://links.lww.com/HJH/C444. Of the 273 chronic kidney disease biomarkers, 25 (9.2%) peptides were common with the 83 hypertension-associated peptides, demonstrating significantly associated fold changes ($R^2 = 0.67$, $P < 0.0001$). Among the 577 urinary peptides that were described to be associated with heart failure, 44 (7.6%) were common, with a significant association in terms of regulation ($R^2 = 0.71$, $P < 0.0001$). Regarding the previously defined 160 CAD biomarkers, only five (3.1%) overlapped with our findings, with regulation being significantly associated ($R^2 = 0.96$, $P = 0.0031$). Another confounder of subclinical end-organ damage may be proteinuria or albuminuria. However, none of these 83 peptides was derived from albumin or immunoglobulins, indicating that the observed changes do not appear to be associated with proteinuria.

## Bioinformatics analyses

To gain insights into the molecular mechanisms underlying the observed changes in the urinary proteome/peptidome, bioinformatic analyses were conducted to investigate the 24 parental proteins of the 83 hypertension-associated peptides. The generated protein-protein interaction network consisted of 24 nodes and 84 edges (Fig. 2). Protein–protein interaction enrichment yielded a significant $P$ value of less than $1.0e^{-16}$. Within this network, a major cluster (mainly collagen-driven) comprising 14 nodes and 70 edges (protein-protein interaction enrichment $P < 1.0e^{-16}$) represented proteins involved in extracellular matrix (ECM) remodeling. The second largest cluster (six nodes, six edges, protein-protein interaction enrichment $P = 2.89e^{-07}$) contained proteins involved in the innate immune system (i.e., FGA, FGB, ORM1, PIGR, and GSN).

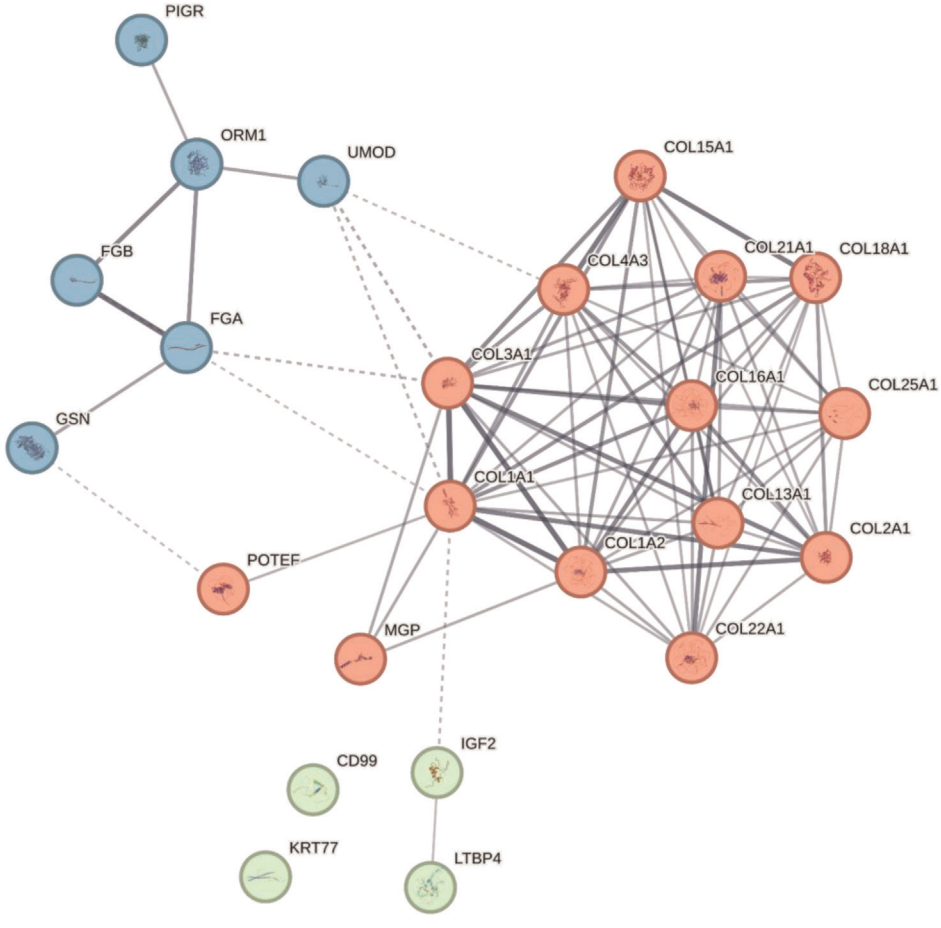


**FIGURE 2** Protein-protein interaction network. The analysis was based on the 24 parental proteins of the 83 hypertension-associated peptides. The colors represent the three identified protein clusters using the STRING database. The red-colored cluster illustrates that predominantly the collagen-interplay appears to potentially be a large component of this condition.

## DISCUSSION

In this study, we attempted to expand our knowledge of the molecular pathophysiology of hypertension from the urinary peptidomics perspective by performing case–control comparisons using a large initial dataset of almost 3000 individuals without end-organ damage. Aiming towards high confidence in the correct labelling of hypertensive and normotensive patients, we excluded participants that did not fulfill the designated criteria for the definition of hypertension. Within a discovery-validation study design, we determined consistent, significant peptide changes between hypertensive and normotensive participants, which were subsequently confirmed in an external cohort matched for relevant clinical and demographic confounders. We identified 83 hypertension-associated peptides of both collagen and noncollagen origin, which were further investigated for correlations with the continuous standardized BP variables and in protein-protein interaction bioinformatics analyses.

Although the molecular mechanisms of hypertension are complex and not completely known, several genome-wide association studies, as reviewed in [28], have provided insights into genetic variants associated with BP, many of them mapping to nonprotein-coding regions but also, in cases, colocalizing with quantitative trait loci associated with the renal expression of specific genes [e.g., Interleukin-1 receptor-associated kinase 1 binding protein 1, microtubule-associated protein 1 B, splice isoforms of NADH dehydrogenase (ubiquinone) complex I, and assembly factor 6]. Additional features and molecular pathways associated with BP have been highlighted following the integration of GWAS with kidney multiomics, including angiotensinogen and angiotensin-converting enzyme, as well as splicing isoforms of mitogen-activated protein kinase–associated protein 1 gene and ubiquitin-conjugating enzyme E2E 3 gene, relevant to sodium reabsorption [28]. Along the same lines, the UMOD genetic locus has been strongly linked to hypertension, with carriers of a certain allele (single nucleotide polymorphism rs13333226) having both lower urinary UMOD levels and lower hypertension risk [29,30].

With regard to relevant proteomic studies, one of the very few was recently conducted by De Beer *et al.* [31] in a population of young individuals. In that study, owing to the small sample size, a limited number of peptides remained significantly different between hypertensive and normotensive individuals after correcting for multiple tests. Comparing the changes observed in that study and in the present study, similarities were observed in groups from the general population, but not in individuals with diabetes. This likely reflects the fact that the participants included in the previous investigation were nondiabetic and of a much younger age.

Because hypertension is a well described risk factor for kidney and cardiovascular diseases, patients with CKD or cardiovascular disease were excluded from our study. However, this approach cannot rule out bias introduced by subclinical organ damage. Therefore, we investigated whether the identified hypertension-associated peptides are linked to kidney or cardiovascular diseases. Most peptides are not associated with hypertension-mediated organ damage in chronic diseases. However, some similarities were observed. This overlap between peptides associated with hypertension and previously defined peptide biomarkers for heart failure, CAD, and CKD might be reflective of shared disease pathophysiological patterns, revolving mainly around chronic inflammation and fibrosis. Consequently, these results indicate that urinary peptides may potentially provide information on chronic diseases earlier than clinical parameters before organ damage is established.

Among the hypertension-associated peptides identified in this study, collagen-derived peptides were the most prominent. Collagens are among the most abundant proteins in the ECM and provide mechanical support to vascular walls. Among the major vascular diseases, ECM remodeling is a hallmark of hypertension, mainly mediated by collagenases or metalloproteinases (MMPs) [32]. Thus, it is unsurprising that collagen fragments are strongly associated with hypertension. The distribution and content of ECM proteins vary depending on the type of vascular wall and its properties [33]. Fibrillar collagen types I and III comprise a notable portion of the vessel collagen localized in the intima, media, and adventitia [33] (in the aorta, type III dominates in the medial layer, whereas type I is more represented in the adventitia [32]). The ratio of these two collagen types affects the mechanical properties of the arterial wall, with an increase in the proportion of collagen type I accounting for the ECM stiffening observed with aging [34]. Most hypertension-associated peptides defined in this study originated from these proteins. Most of these peptides were reduced in hypertension, suggesting attenuated collagen degradation, consequently increasing collagen deposits in these individuals. In addition to the fragments of collagen I and III, differences between the two groups were also documented for the network-forming collagen type IV (COL4A3), potentially because of changes related to the basement membrane [33]. Several processes may occur during hypertensive arterial stiffness, similar to a vicious cycle [32]. Collagen accumulation potentially leads to increased MMP activity, which initiates ECM remodeling. Consequently, a context that is unfavorable for the stability of the relevant structures is generated. The endothelial structure may be disorganized, resulting in infiltration of macrophages and mononuclear cells. This, in combination with the increasing presence of senescent cells in the vasculature due to age-related processes, contributes to chronic inflammation. Collagen synthesis may increase as a compensatory mechanism, leading to the formation of disorganized, cross-linked accumulated collagens, further contributing to vascular stiffness.

The molecular changes observed at the level of urinary peptides extend beyond collagens. MGP is a vascular calcification inhibitor that is activated by vitamin K through γ-glutamate carboxylation and serine phosphorylation [35]. The protein is highly expressed in the epithelium of Bowman's capsule and proximal tubules [36] and is also secreted by vascular smooth muscle cells and chondrocytes in the arterial tunica media [37], suggesting a role in vascular structures. Increased plasma levels of inactive MGP forms are positively associated with arterial stiffness and various

forms of cardiovascular disease [38–42], predictive of cardiovascular mortality [43], and negatively associated with eGFR [44]. We identified a consistently increased urinary abundance of the two MGP peptides in the hypertensive group, which may be the result of increased degradation and hence reduced abundance in the body, resulting in attenuated protection from vascular calcification.

A significant increase in UMOD peptide levels was observed in patients with hypertension. UMOD is the most abundant protein in urine and is secreted by epithelial cells lining the thick ascending limb of Henle's loop, modulating the sodium-potassium-two-chloride transporter [45]. Consistent with our findings, a causal relationship between hypertension and UMOD levels has been suggested based on Mendelian randomization studies [46].

In a recent study, urinary peptides were shown to predict responses to BP medication [47]. Using the identified peptide changes, along with the predicted impact of intervention on urinary peptides [5], might help personalize intervention and ultimately select the most appropriate therapy.

### Limitations

Our study has several limitations. First, the study was based on previously collected datasets generated using different scientific designs. Second, based on data availability, only a case–control design was implemented and no information on the prognostic value of the identified hypertension-associated peptides could be obtained. In addition, the BP measurement was based on an unstandardized methodology, which may have contributed to the weak peptide associations observed with continuous BP.

### Conclusion

Our findings provide insights into the molecular alterations underlying hypertension. Prominent deregulation of collagen type I and III peptides associated with hypertension likely indicates vascular ECM remodeling. Differences in the abundance of collagen peptides in the basement membranes were also observed. Peptides from proteins involved in vascular calcification, sodium homeostasis, and sodium-potassium transport were also significantly different. These findings suggest that changes in the vessel wall, tubular sodium transport, and other subtle mechanisms occur early in the development of hypertension, and more importantly, are mirrored by changes in urinary peptides. The corresponding proteomic profile is largely different from that observed in patients with established kidney and/or cardiovascular disease. Given the multifactorial nature of hypertension, urinary proteomics is unlikely to be a diagnostic tool for hypertension, however, the data presented here may shed new light on the early mechanisms of hypertension development. The changes observed in urinary peptides, particularly those derived from various collagen subtypes, point toward early vascular changes and thus provide insight into pathophysiology and may potentially direct the use of existing treatments or help develop new ones. These peptide changes may also correlate with tissue and virtual histology of the vessel wall, arterial stiffness, and other biomechanical properties of the arteries.

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### Conflicts of interest

H.M. is the founder and co-owner of Mosaiques Diagnostics (Hannover, Germany). E.M., J.S., and A.L. are employed in Mosaiques Diagnostics. P.R. has received grants from Astra Zeneca, Bayer, and Novo Nordisk and honoraria (to Steno Diabetes Center Copenhagen) from Astra Zeneca, Abbott, Bayer, Boehringer Ingelheim, Eli Lilly, Novo Nordisk, Gilead, and Sanofi. The other authors declare no conflicts of interest.

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