A urinary peptidomics approach for early stages of cardiovascular disease risk: The African-PREDICT study

Urinary peptidomics and CVD risk

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Abstract

Cardiovascular disease (CVD) affects individuals across the lifespan, with multiple cardiovascular (CV) risk factors increasingly present in young populations. The underlying mechanisms in early cardiovascular disease development are complex and still poorly understood. We therefore employed urinary proteomics as a novel approach to gain better insight into early CVD-related molecular pathways based on a CVD risk stratification approach.

This study included 964 apparently healthy (no self-reported chronic illnesses, free from clinical symptoms of CVD) black and white men and women (aged 20-30 years old) from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) study. Cardiovascular risk factors used for stratification included obesity, physical inactivity, tobacco use, high alcohol intake, hyperglycemia, dyslipidemia and hypertension. Participants were divided into low (0 risk factors), medium (1-2 risk factors) and high (≥3 risk factors) CV risk groups. We analyzed urinary peptidomics by capillary electrophoresis time-of-flight mass spectrometry.

After adjusting for ethnicity, sex and age, 65 sequenced urinary peptides were differentially expressed between the CV risk groups (all q-values≤0.01). These peptides included a lower abundance of collagen type I- and III-derived peptides in the high compared to the low CV risk group. With regard to noncollagen peptides, we found a lower abundance of alpha-1-antitrypsin fragments in the high compared to the low CV risk group (all q-values≤0.01).

Our findings indicate lower abundances of collagen types I and III in the high compared to the low CV risk group, suggesting potential early alterations in the CV extracellular matrix.

Keywords: cardiovascular risk factors, collagen type I, collagen type III, hypertension, peptidomics

Introduction

The early development of cardiovascular diseases (CVDs) is becoming a major health concern, especially in light of the high prevalence of young adults, as well as children, with increased risk factors for CVD [1-3]. Numerous interrelated risk factors contribute to the development of CVD, including hypertension, obesity, elevated cholesterol levels, smoking, excessive alcohol use, diabetes, ethnicity, sex, physical inactivity and increasing age [4-6]. In addition, the mechanisms underlying CVD development have multisystem complexity [7, 8]. New approaches, including proteomics, may provide further insight into CVD-related molecular pathways. Proteomics can detect dynamic changes in protein expression to provide insight into the molecular and cellular processes underlying disease [9] and are therefore able to identify and define novel biomarkers for diagnosis, disease staging, novel therapeutic targets, prediction and ultimately disease prevention [10, 11].

With the use of proteomics, unique molecular patterns have already been identified for various conditions and are able to discriminate between health and disease states. Urinary proteomic analysis enabled defining biomarkers specific to coronary artery disease (CAD) [12, 13], chronic kidney disease (CKD) [14, 15], diabetes [16, 17], diabetic nephropathy [17] and heart failure [18]. However, to the best of our knowledge, no proteomic studies have focused on CV risk factors and the early stages of CVD development in young adults.

We therefore aimed to identify a urinary proteomic profile that would differentiate between low, medium and high cardiovascular risk among young asymptomatic adults to better understand the molecular make-up and pathogenic principles that may be involved in the early stages of CVD development.

Methods

Study population and organizational procedures

The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) is a longitudinal study designed to characterize the development of hypertension over a follow-up period of 10 years. Baseline data were collected from 2013-2017 and included 1202 (aged 20-30 years) healthy adults [19]. This study included baseline data of 964 participants from the African-PREDICT study with complete urinary peptidomics data. Recruitment took place in the North West province of South Africa. Recruited participants were invited to the screening phase to determine if they met the inclusion criteria of the research phase of the African-PREDICT study. The inclusion criteria were HIV-uninfected and clinic normotensive (office brachial blood pressure <140/90 mmHg), men or women aged 20–30 years of self-reported black or white ethnicity with no self-reported chronic illness or use of chronic medication. Participants were required to sign an informed consent form to participate in the screening and research phases of this study. The African-PREDICT study (NWU-00001-12-A1), as well as this substudy (NWU-00495-19-A1), were approved by the Health Research Ethics Committee of the North-West University (ClinicalTrials.gov identifier: NCT03292094).

Anthropometric measurements

An anthropometrist made use of the International Society for the Advancement of Kinanthropometry [20] guidelines to take measurements of the participants. We measured height, weight (Seca, Hamburg, Germany) and waist circumference (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA). Body mass index (BMI) was calculated with the use of the following formula: weight (kg)/height squared (m^2).

Questionnaires

A general health and demographic questionnaire was completed by each participant. The socioeconomic score of each participant was calculated with the use of a point system adapted from Kuppuswamy's Socioeconomic Status Scale [21] for a South African environment. Participants were scored on three categories, skill level, education level and household income [19], and categorized into low, medium and high socioeconomic groups. Furthermore, each participant also completed a Global Physical Activity Questionnaire [22].

Cardiovascular measurements

Ambulatory blood pressure

Twenty-four-hour ambulatory blood pressure measurements (ABPM) were determined with a Card(X)plore device validated by the British Hypertension Society (Meditech, Budapest, Hungary) [23]. A fitted cuff was used on the participant's nondominant arm that measured blood pressure in 30-minute intervals during daytime (08:00-22:00) and hourly during nighttime (22:00-06:00).

Biochemical analysis

Participants were required to fast for eight hours prior to biological sampling. Biological samples comprised of blood samples taken from the antebrachial vein with the use of a winged infusion set by a registered nurse, as well as an early-morning spot urine sample. Biological samples were taken to an on-site laboratory to be prepared, aliquoted into cryovials and stored in biofreezers (–80 °C) for later analysis. The following basic biochemical measurements were included: serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, high-sensitivity C-reactive protein, gamma-glutamyl transferase, albumin, creatinine, glycated hemoglobin (HbA1c) (Cobas Integra 400 plus, Roche, Basel, Switzerland), serum cotinine (Immulite, Siemens, Erlangen, Germany) and plasma total fibrinogen (Instrumentation Laboratories, Milan, Italy).

Urinary peptidomics

Sample preparation for urinary peptidomics analysis included the dilution of 700 µl of urine with 700 µl of urea 2M solution and 0.1 M of ammonium hydroxide containing 0.02% sodium dodecyl sulfate. Ultracentrifugation was performed with a 20 kDa molecular weight cutoff Centrisart centrifugal ultrafiltration unit (Sarorius, Göttingen, Germany) at 3000 x g for 1 hour at 4 °C [24, 25]. The filtrate was then desalted to remove urea, electrolytes and salts with a PD-10 desalting column (Amersham Bioscience, Buckinghamshire, UK), and peptide elution was achieved with 0.01% aqueous ammonium hydroxide. Then, the samples were lyophilized, stored at 4 °C, and resuspended in high-performance liquid chromatography (HPLC)-grade water to a final concentration of 2 µg/µl before analysis [24, 25]. Capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) was performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) coupled with a microTOF mass spectrometer (Bruker Daltonic, Bremen, Germany) as previously described [26]. For a detailed description of the biochemical analysis of urinary peptidomics, refer to Supplemental Material: **Appendix A: Urinary peptidomics biochemical analysis**. We also included the previously defined urinary proteomic classifier CAD_{238} [12], which consisted of 238 CAD-specific polypeptides that were able to identify patients with CAD with high sensitivity and specificity, as well as classifier CKD_{273} [27], which consisted of 273 CKD-specific polypeptides that were also able to identify CKD patients with high confidence.

Statistical analyses

R version 3.6.0 software (R Foundation for Statistical Computing, Vienna) [28], IBM® SPSS® Statistics version 25 software (IBM Corporation; Armonk, New York, USA) and G*Power version 3.1.9.3 software (Faul, Erdfelder, Lang, & Buchner, 2007) [29] were used to perform statistical analyses.

Participants (n=964) were divided into three groups based on the number of cardiovascular risk factors (**Figure 1**), i.e., zero risk factors, 1-2 risk factors and 3 or more risk factors. The

following factors were chosen to define low, medium and high classifications of early cardiovascular risk: waist/height ratio >0.55 (kg/m) [30]; body mass index ≥30 (kg/m²) [31]; physical inactivity (moderate vigorous intensity exercise<600 MET-min or moderate exercise<150 min and vigorous exercise<75 min) [22]; self-reported smoking or plasma cotinine levels ≥11 (ng/ml) [32]; self-reported alcohol use or gamma-glutamyl transferase levels ≥49 (U/L) [33, 34]; glycated hemoglobin ≥5.7(%) [35]; low-density lipoprotein >3 (mmol/l) [36]; and hypertension (24-hour mean SBP ≥130 mmHg and/or 24-hour mean DBP ≥80 mmHg) [37].

All biochemical variables were tested for normality (Q-Q plots). Skewed data (lipids, C-reactive protein, glucose, gamma-glutamyl transferase, cotinine and glycated hemoglobin) were logarithmically transformed. We performed ANOVA and ANCOVA (adjusted for ethnicity) to compare the groups.

All urinary peptide data were logarithmically (log_2) transformed to obtain comparable intensity ranges of peptides. All peptides with >45% missing or undetectable values were excluded from further data analysis, as this would produce unreliable results. Missing data imputation was performed by replacing the missing values with the minimum value of the peptide divided by two (min/2). We performed ANCOVAs (adjusted for ethnicity, sex and age) on the transformed peptidomics data (909 peptides) to compare the peptide levels between the groups. Adjustment for multiple comparisons was applied following the Benjamini–Hochberg approach to lower the false discovery rate (q≤0.05). Only sequenced peptides with a q-value≤0.01 were included in the bar graphs (**Figure 2**). Furthermore, in the total group (n=964), we performed partial correlations (adjusting for age and ethnicity) between the cardiovascular disease risk factors and the urinary peptides with a q-value≤0.01 to assess which risk factors mainly contributed to differences in urinary peptide levels between the CV risk groups.

Results

The general characteristics of the low, medium and high cardiovascular risk groups are described in **Table 1**. The high-risk group was older (mean age 25.4 vs. 23.9 and 24.1; p<0.001) than the medium- and low-risk groups. The low-risk group included a lower fraction of black individuals compared to the medium- and high-risk groups (p<0.006), whereas sex and socioeconomic scores were similar between the groups (all p>0.22).

Cardiovascular risk factors in the low-, medium- and high-risk groups

All metabolic and blood pressure-related risk factors were higher in the high-risk group than in the medium- or low-risk group. Body mass index (with 41.3% obese individuals in the high-risk group) was higher in the high-risk group than in the medium-risk group. A greater proportion of people had hypertension in the high-risk group (by 27%) than in the medium-risk group (5%), and HbA1c was higher in the high-risk group (by 18% above the normal range) than in the other risk groups (7%) (all p<0.001). The remaining risk factors differed significantly between all the groups, including higher cotinine (by 47% above the normal range) and gamma-glutamyl transferase (15% above the normal range in the high-risk group) levels and lower physical inactivity in the high-risk group (by 75%) than in the medium-risk group (41%) (all p<0.001). The high-risk group had a less favorable lipid profile and higher levels of C-reactive protein (with 8% above the normal range (>=10 mg/L)) than the medium-risk group (3%) [38]. With regard to established classifier scores, we found higher levels of the CKD_{273} classifier score in the high-risk group than in the low CV risk group (<0.001).

Urinary peptidomics in the low-, medium- and high-risk groups

In ANCOVAs, 147 sequenced peptides differed significantly between the groups (q-value≤0.05), with 65 peptides with a q-value≤0.01.

Collagen type I- and III-derived peptides

Almost all (except for peptide e12556) COL1A1-derived peptides (represented by 35 peptides) (q-value≤0.01) (**Figure 2**), COL1A2-derived peptides (represented by 10 peptides) (q-value≤0.01), and COL3A1-derived peptides (represented by 5 peptides) (q-value≤0.01) were lower in the high-risk group than in the low-CVD risk group.

Other collagen-derived peptides

Other collagen-derived peptides that differed significantly between the groups included COL2A1, COL4A3, COL6A1, COL10A1, COL11A2, COL16A1, and COL26A1 (all q-values≤0.01) and were lower in the high-risk group than in the low-CVD risk group. Furthermore, COL7A1- and COL11A2-derived peptides were higher in the high-risk group than in the low-CVD risk group (**Figure 2**).

Noncollagen-derived peptides

All noncollagen-derived peptides, including alpha-1-antitrypsin, were lower in the high-risk group than in the low-CVD risk group (all q-values≤0.01). For a detailed description of the ANCOVAs for all urinary peptides (sequenced and nonsequenced peptides), please refer to **Supplemental Material: Appendix B: Analysis of covariance descriptive test for urinary peptides**.

Main cardiovascular risk factors that correlated with urinary peptides

In partial regression analyses, we identified low-density lipoprotein (LDL), HbA1c, gamma-glutamyl transferase (GGT) and physical activity correlated with the majority of urinary peptides with a q-value≤0.01. Low-density lipoprotein, HbA1c and GGT were inversely correlated, and physical activity was positively correlated with urinary peptides (**Supplemental Material: Appendix C: Partial regression analysis between cardiovascular risk factors and urinary peptides**).

Discussion

In this study, we aimed to identify and compare urinary peptides in adults with low, medium and high CVD risk to aid in the understanding of molecular processes involved in the early development of CVD. We identified 65 sequenced urinary peptides (q-value≤0.01) that were differentially expressed between the low-, medium- and high-cardiovascular risk groups. The majority of peptides that were identified consisted of collagen fragments, which was not surprising since collagen is the most abundant protein in the body [39]. As seen in this study, previous urine-based proteomic studies also revealed that collagen-derived peptides contributed prominently to signatures that define control and various disease groups [13, 40]. In two previous manuscripts, endogenous peptides in urine and plasma were compared [41, 42]. The authors consistently found poor or no correlation when investigating all peptides but significant correlations when only collagen-derived peptides were investigated. These findings gave rise to the hypothesis that a major fraction of the urinary collagen fragments likely result from glomerular filtration of blood and are therefore not kidney derived.

We identified a lower abundance of collagen type I- and III-derived peptides in the high-CV risk group than in the low-CV risk group. Several urinary proteomic studies among patients with arterial stiffness [43], CAD [12], left ventricular diastolic dysfunction [40], chronic kidney disease [44], diabetes and diabetic nephropathy [45] indicated a lower abundance of collagen type I among cases than among controls. A few urine-based proteomic studies on CAD [12] and diabetic nephropathy [44] also found a lower abundance of collagen type III fragments among the cases compared to the controls. Our findings with regard to the abundance of collagen type I- and III-derived peptides indicated the same patterns as seen in the aforementioned studies but in a young population with increased CVD risk and without CVD or overt organ damage. Since the arterial and cardiac extracellular matrix (ECM) are predominantly composed of fibrillar collagen types I and III, which provide tensile strength and structure [46], our results suggest that it may be beneficial to investigate early changes in ECM turnover to identify possible targets for intervention.

Collagen types I and III

A tightly controlled ECM turnover is necessary for normal protein function and relies on the balance between ECM synthesis and degradation [47]. When ECM turnover is disturbed, factors that contribute to the synthesis or degradation of ECM proteins will trigger acute or chronic tissue remodeling, changing the structure and mechanical function of the ECM [47]. Previous studies indicated that transforming growth factor-β (TGF-β) activation is associated with the development of CVD [48, 49]. Under normal physiological circumstances, TGF-β contributes to a healthy vessel wall structure through the control of ECM protein synthesis and deposition [47]. A potential mechanism could be increased activation of TGF-β in the high-CV risk group, which may consequently lead to an increase in type I and III collagen deposition in the ECM and therefore lower excretion of these collagen peptides. Furthermore, with the overall higher blood pressure in the high-risk group, we hypothesize that collagen type I and III degradation may be attenuated as a result of higher mechanical stress exerted on the vascular or cardiac wall [50]. The proposed reduced collagen type I and III degradation in the ECM may be an early altered vascular remodeling process to maintain homeostasis for the higher mechanical strain and workload on the vascular and cardiac walls [51, 52]. Furthermore, in the total population group (n=964), LDL, HbA1c and GGT were inversely correlated, whereas physical activity was positively correlated with collagen type I and III fragments. Research shows that LDL, HbA1c (prediabetes) and GGT promote vascular inflammation [53-55]. Since the high-risk group had higher levels of LDL, HbA1c and GGT, we propose that vascular inflammation may be elevated in the high-risk group compared to the other groups, which may lead to early changes in vascular ECM turnover. Additionally, research has shown that physical activity promotes vascular health, whereas physical inactivity is associated with CVD risk factors such as high blood pressure, lower insulin sensitivity and a less favorable lipid profile [56], which may further promote vascular inflammation. Since physical activity was the lowest in the high-risk group compared to the other risk groups, we suggest that vascular inflammation may be higher in the high-risk group,

which may lead to unhealthy ECM turnover. We therefore propose that the regulation of both collagen types I and III may be altered in the high-CV risk group, which may lead to the development of CVD over time if healthier lifestyle changes are not implemented.

Noncollagen peptides

Regarding the identification of noncollagen peptides, we found a lower abundance of alpha-1-antitrypsin fragments in the high-CV risk group than in the low-CV risk group. Alpha-1-antitrypsin is a serine proteinase inhibitor and acute phase protein with anti-inflammatory properties [57]. Although we found higher levels of inflammatory markers, such as C-reactive protein, in the high-CV risk group than in the low-CV risk group, it is possible that alpha-1-antitrypsin may carry out a cardioprotective role in the high-CV risk group by binding to the endothelium to limit inflammatory pathways and vascular injury [57].

Perspectives

The peptides that are differentially expressed between low, medium and high early cardiovascular risk may reflect early changes and possible cardioprotective pathways, such as altered ECM remodeling and inflammatory responses involved in the development of cardiovascular disease. Even though this hypothesis-generating study focused on single peptides, many of the sequenced peptides identified in our study correspond with the previously identified and well-established CKD_{273} and CAD_{238} classifiers, including fragments of collagen types I and III and alpha-1-antitrypsin [12, 58]. Therefore, the analyses of single peptides in young adults with increased CVD risk may contribute to our understanding of the mechanism involved in the early development of CVD. Hence, the possible hypotheses represented by the identified biomarkers should not be underestimated.

Strengths and limitations

This study consisted of a young (aged 20-30 years) and healthy black and white population without overt organ damage. To the best of our knowledge, this study is the first to identify an

altered abundance of collagen types I- and III-related peptides among young asymptomatic adults with high compared to low CVD risk that may indicate an altered ECM turnover. It is beyond the scope of this descriptive study to explain the mechanisms of different collagen turnover and whether it is a cause or a consequence of other pathological processes. This study does not have TGF-β data, and more research should be conducted to test our hypotheses about the possible involvement of TGF-β and urinary peptides in an altered ECM.

Conclusion

To conclude, we found a significantly lower abundance of collagen types I and III and alpha-1-antitrypsin in the high compared to the low-CVD risk groups. Our results may reflect potential cardioprotective pathways involved in the circulatory ECM and inflammatory responses due to the presence of multiple cardiovascular risk factors even at younger ages.

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Conflicts of Interest

Harald Mischak is the co-founder and co-owner of Mosaiques Diagnostics. All other authors have no conflicts of interest to declare.

References

1. Gooding HC, Gidding SS, Moran AE, Redmond N, Allen NB, Bacha F, *et al*. Challenges and Opportunities for the Prevention and Treatment of Cardiovascular Disease Among Young Adults: Report From a National Heart, Lung, and Blood Institute Working Group. J Am Heart Assoc 2020; 9: e016115. DOI:10.1161/JAHA.120.016115.
2. Clark CJ, Alonso A, Spencer RA, Pencina M, Williams K and Everson-Rose SA. Predicted long-term cardiovascular risk among young adults in the national longitudinal study of adolescent health. Am J Public Health 2014; 104: e108-e115. 2014/10/16. DOI: 10.2105/AJPH.2014.302148.
3. Kruger R, Litwin M and Climie RE. Editorial: Determinants and Impact of Early Vascular Aging in Children and Adolescents. Front Pediatr 2022; 10. Editorial. DOI: 10.3389/fped.2022.871524.
4. Carnethon MR, Pu J, Howard G, Albert MA, Anderson CAM, Bertoni AG, *et al*. Cardiovascular Health in African Americans: A Scientific Statement From the American Heart Association. Circ. J 2017; 136: e393-e423. DOI:10.1161/CIR.0000000000000534.
5. Gao Z, Chen Z, Sun A and Deng X. Gender differences in cardiovascular disease. Med Novel Technol Devices 2019; 4: 100025. DOI: 10.1016/j.medntd.2019.100025.
6. Hajar R. Risk Factors for Coronary Artery Disease: Historical Perspectives. Heart Views 2017; 18: 109-114. DOI: 10.4103/heartviews.heartviews_106_17.
7. Doran S, Arif M, Lam S, Bayraktar A, Turkez H, Uhlen M, *et al*. Multi-omics approaches for revealing the complexity of cardiovascular disease. Briefings Bioinf 2021; 22. DOI: 10.1093/bib/bbab061.
8. MacLellan WR, Wang Y and Lusis AJ. Systems-based approaches to cardiovascular disease. Nat Rev Cardiol 2012; 9: 172-184. DOI: 10.1038/nrcardio.2011.208.

9. Aslam B, Basit M, Nisar MA, Khurshid M and Rasool MH. Proteomics: Technologies and Their Applications. J Chromatogr Sci 2017; 55: 182-196. DOI: 10.1093/chromsci/bmw167.

10. Pejchinovski M, Hrnjez D, Ramirez-Torres A, Bitsika V, Mermelekas G, Vlahou A, *et al*. Capillary zone electrophoresis on-line coupled to mass spectrometry: a perspective application for clinical proteomics. PROTEOMICS–clinical applications 2015; 9: 453-468. DOI: 10.1002/prca.201400113.

11. Gramolini AO and Emili A. Uncovering early markers of cardiac disease by proteomics: avoiding (heart) failure! Expert Rev Proteomics 2005; 2: 631-634. DOI: 10.1586/14789450.2.5.631.

12. Delles C, Schiffer E, von Zur Muhlen C, Peter K, Rossing P, Parving HH, *et al*. Urinary proteomic diagnosis of coronary artery disease: identification and clinical validation in 623 individuals. J Hypertens 2010; 28: 2316-2322. DOI: 10.1097/HJH.0b013e32833d81b7.

13. Zimmerli LU, Schiffer E, Zürbig P, Good DM, Kellmann M, Mouls L, *et al*. Urinary proteomic biomarkers in coronary artery disease. Mol Cell Proteomics 2008; 7: 290-298. DOI: 101074/mcp.M700394-MCP200.

14. Verbeke F, Siwy J, Van Biesen W, Mischak H, Pletinck A, Schepers E, *et al*. The urinary proteomics classifier chronic kidney disease 273 predicts cardiovascular outcome in patients with chronic kidney disease. Nephrol Dial Transplant 2019. DOI: 10.1093/ndt/gfz242.

15. Øvrehus MA, Zürbig P, Vikse BE and Hallan SI. Urinary proteomics in chronic kidney disease: diagnosis and risk of progression beyond albuminuria. Clin Proteomics 2015; 12: 21-21. DOI: 10.1186/s12014-015-9092-7.

16. Mischak H, Kaiser T, Walden M, Hillmann M, Wittke S, Herrmann A, *et al*. Proteomic analysis for the assessment of diabetic renal damage in humans. Clin Sci 2004; 107: 485-495. DOI: 10.1042/CS20040103.

17. Rossing K, Mischak H, Dakna M, Zürbig P, Novak J, Julian BA, *et al*. Urinary proteomics in diabetes and CKD. J Am Soc Nephrol 2008; 19: 1283-1290. DOI: 10.1681/ASN.2007091025.

18. Zhang ZY, Ravassa S, Nkuipou-Kenfack E, Yang WY, Kerr SM, Koeck T, *et al*. Novel Urinary Peptidomic Classifier Predicts Incident Heart Failure. J Am Heart Assoc 2017; 6: e005432. DOI: DOI:10.1161/JAHA.116.005432.

19. Schutte AE, Gona PN, Delles C, Uys AS, Burger A, Mels CM, *et al*. The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT): Design, recruitment and initial examination. Eur J Prev Cardiol 2019; 26: 458-470. DOI: 10.1177/2047487318822354.

20. Stewart A, Marfell-Jones M, Olds T and Ridder DH. International standards for anthropometric assessment. ISAK 2011: 50-53. ISBN: 0-620-36207-3.

21. Patro BK, Jeyashree K and Gupta PK. Kuppuswamy's socioeconomic status scale 2010—the need for periodic revision. Indian J Pediatr 2012; 79: 395-396. DOI: 10.1007/s12098-011-0517-7.

22. Bull FC, Maslin TS and Armstrong T. Global physical activity questionnaire (GPAQ): nine country reliability and validity study. JPAH 2009; 6: 790-804. DOI: 10.1123/jpah.6.6.790.

23. Barna, I., A. Keszei, and A. Dunai. Evaluation of Meditech ABPM-04 ambulatory blood pressure measuring device according to the British Hypertension Society protocol. Blood Press Monit 1998. 3(6): p. 363-8.

24. Mavrogeorgis E, Mischak H, Latosinska A, Siwy J, Jankowski V and Jankowski J. Reproducibility Evaluation of Urinary Peptide Detection Using CE-MS. Molecules 2021. 26(23): p. 7260. DOI: 10.3390/molecules26237260.

25. Mischak H, Vlahou A and Ioannidis JP. Technical aspects and inter-laboratory variability in native peptide profiling: The CE–MS experience. Clin Biochem 2013; 46: 432-443. DOI: 10.1016/j.clinbiochem.2012.09.025.

26. Albalat A BV ZP, Siwy J, Mullen W. High-resolution proteome/peptidome analysis of body fluids by capillary electrophoresis coupled with MS. Methods Mol Biol 2013;984:153-65. DOI: 10.1002/pmic.200600230.

27. Good DM, Zürbig P, Argilés À, Bauer HW, Behrens G, Coon JJ, *et al*. Naturally Occurring Human Urinary Peptides for Use in Diagnosis of Chronic Kidney Disease. Mol Cell Proteomics 2010; 9: 2424-2437. DOI: 10.1074/mcp.M110.001917.

28. R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

29. Faul FEE, Lang AG and Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 2007; 39: 175-191. DOI: 10.3758/bf03193146.

30. Yoo EG. Waist-to-height ratio as a screening tool for obesity and cardiometabolic risk. Korean J Pediatr 2016; 59: 425. DOI: 10.3345/kjp.2016.59.11.425.

31. Weir CB, Jan A. BMI Classification Percentile And Cut Off Points. In: StatPearls. StatPearls Publishing, Treasure Island (FL); 2021. PMID: 31082114.

32. Kim S. Overview of cotinine cutoff values for smoking status classification. Int J Environ Res Public Health 2016; 13(12):1236. DOI: 10.3390/ijerph13121236.

33. Du Buisson K, Kramer S. Ampath medical surveillance guideline. 2014-2015. URL: https://www.medichem.org/information/Ampath%202014.pdf.

34. Puukka K, Hietala J, Koivisto H, Anttila P, Bloigu R and Niemelä O. Age-related changes on serum ggt activity and the assessment of ethanol intake. Alcohol Alcohol 2006 Sep 1;41(5):522-7. DOI: 10.1093/alcalc/agl052.

35. American Diabetes Association. Classification and diagnosis of diabetes: standards of medical care in diabetes—2018. Diabetes care 2018; 41: S13-S27. DOI: 10.2337/dc18-S002.

36. Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, *et al*. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to

reduce cardiovascular risk. Russ J Cardiol 2020; 25: 3826. DOI: 10.1093/eurheartj/ehz455.

37. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M. *et al*. 2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). Eur Heart J 2018; 39: 3021-3104. DOI: 10.1093/eurheartj/ehy339.

38. Nehring SM, Goyal A and Patel BC. C Reactive Protein. In: StatPearls. Treasure Island (FL): StatPearls. 2021.

39. Shoulders MD and Raines RT. Collagen structure and stability. Annu Rev Biochem 2009; 78: 929-958. DOI: 10.1146/annurev.biochem.77.032207.120833.

40. Kuznetsova T, Mischak H, Mullen W and Staessen JA. Urinary proteome analysis in hypertensive patients with left ventricular diastolic dysfunction. Eur Heart J 2012; 33: 2342-2350. DOI: 10.1093/eurheartj/ehs185.

41. Magalhaes P, Pontillo C, Pejchinovski M, Siwy J, Krochmal M, Makridakis M, et al. Comparison of urine and plasma peptidome indicates selectivity in renal peptide handling. Proteomics Clin Appl, 2018. 12(5): p. 1700163.

42. He T, Pejchinovski M, Mullen W, Beige J, Mischak H, Jankowski V. Peptides in Plasma, Urine, and Dialysate: Toward Unravelling Renal Peptide Handling. Proteomics Clin Appl, 2021. 15(1): p. 2000029.

43. McNulty M, Mahmud A, Spiers P and Feely J. Collagen type-I degradation is related to arterial stiffness in hypertensive and normotensive subjects. J Hum Hypertens 2006; 20: 867-873. DOI: 10.1038/sj.jhh.1002015.

44. Alkhalaf A, Zürbig P, Bakker SJ, Bilo HJ, Cerna M, Fischer C, *et al*. Multicentric validation of proteomic biomarkers in urine specific for diabetic nephropathy. PloS one 2010; 5: e13421. DOI: 10.1371/journal.pone.0013421.

45. Snell-Bergeon JK, Maahs DM, Ogden LG, Kinney GL, Hokanson JE, Schiffer E, *et al*. Evaluation of urinary biomarkers for coronary artery disease, diabetes, and diabetic kidney disease. Diabetes Technol Ther 2009; 11: 1-9. DOI: 10.1089/dia.2008.0040.

46. del Monte-Nieto G, Fischer JW, Gorski DJ, Harvey RP, and Kovacic JC. Basic Biology of Extracellular Matrix in the Cardiovascular System, Part 1/4: JACC Focus Seminar. J Am Coll Cardiol 2020; 75: 2169-2188. DOI: 10.1016/jacc.2020.03.024.

47. LeBar K and Wang Z. Extracellular Matrix in Cardiac Tissue Mechanics and Physiology: Role of Collagen Accumulation. In: Madhurapantula, R. S. , P.R.O., J. O., Loewy, Z. , editors. Extracellular Matrix - Developments and Therapeutics [Internet]. London: IntechOpen; 2021 [cited 2022 May 13]. Available from: https://www.intechopen.com/chapters/75606 DOI: 10.5772/intechopen.96585.

48. Aihara K-i, Ikeda Y, Yagi S*,* Akaike M and Matsumoto T. Transforming growth factor-β1 as a common target molecule for development of cardiovascular diseases, renal insufficiency and metabolic syndrome. Cardiol Res Prac*t* 2010;2011:175381. DOI: 10.4061/2011/175381.

49. Ruiz-Ortega M, Rodríguez-Vita J, Sanchez-Lopez E, Carvajal G and Egido J. TGF-β signaling in vascular fibrosis. Cardiovasc Res 2007; 74: 196-206. DOI: 10.1016/j.cardiores.2007.02.008.

50. Bishop JE and Lindahl G. Regulation of cardiovascular collagen synthesis by mechanical load. Cardiovasc Res 1999; 42: 27-44. DOI: 10.1016/s0008-6363(99)00021-8.

51. Mels CM, Delles C, Louw R and Schutte AE. Central systolic pressure and a nonessential amino acid metabolomics profile: the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension. J Hypertens 2019; 37: 1157. DOI: 10.1097/HJH.0000000000002040.

52. De Beer D, Mels CM, Schutte AE, Louw R, Delles C and Kruger R. Left ventricular mass and urinary metabolomics in young black and white adults: The African-

PREDICT study. Nutr Metab Cardiovasc 2020; 30: 2051-2062. DOI: 10.1016/j.numecd.2020.06.004.

53. Catapano AL, Pirillo A and Norata GD. Vascular inflammation and low-density lipoproteins: is cholesterol the link? A lesson from the clinical trials. Br J Pharmacol, 2017. 174(22): p. 3973-3985.

54. Luc K, Schramm-Luc A, Guzik TJ and Mikolajczyk TP. Oxidative stress and inflammatory markers in prediabetes and diabetes. J Physiol Pharmacol, 2019. 70(6).

55. Bradley R. Gamma glutamyltransferase (GGT) as a biomarker of atherosclerosis. Biomarkers in cardiovascular disease. Dordrecht, Netherlands: Springer, 2016: p. 673-702.

56. Nystoriak MA and Bhatnagar A, *Cardiovascular Effects and Benefits of Exercise.* Front Cardiovasc Med, 2018. **5**: p. 135.

57. Lubrano V and Balzan S. Role of oxidative stress-related biomarkers in heart failure: galectin 3, α1-antitrypsin and LOX-1: new therapeutic perspective? Mol Cell Biochem 2020; 464: 143-152. DOI: 10.1007/s11010-019-03656-y.

58. Argiles A, Siwy J, Duranton F, Gayrard N, Dakna M, Lundin U, *et al*. CKD273, a new proteomics classifier assessing CKD and its prognosis. Plos one 2013; 8: e62837. DOI: 10.1371/journal.pone.0062837.

Table legends

Table 1: Characteristics of the cardiovascular risk groups.

Figure legends

Figure 1: Stratification of low-, medium- and high-risk groups according to cardiovascular risk factors.

Figure 2: Urinary peptide level comparison between the low, medium and high early cardiovascular risk groups.