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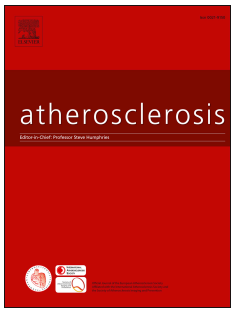


Identification of six cardiovascular risk biomarkers in the young population: A promising tool for early prevention

Paula J. Martínez, Montserrat Baldán-Martín, Juan A. López, Marta Martín-Lorenzo, Aránzazu Santiago-Hernández, Marta Agudiez, Martha Cabrera, Eva Calvo, Jesús Vázquez, Gema Ruiz-Hurtado, Fernando Vivanco, Luis M. Ruilope, María G. Barderas, Gloria Alvarez-Llamas

PII: S0021-9150(19)30004-8

DOI: https://doi.org/10.1016/j.atherosclerosis.2019.01.003

Reference: ATH 15833

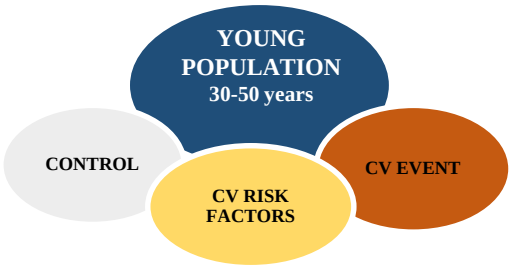
To appear in: *Atherosclerosis*

Received Date: 12 September 2018

Revised Date: 26 December 2018

Accepted Date: 10 January 2019

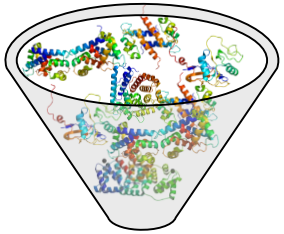
Please cite this article as: Martínez PJ, Baldán-Martín M, López JA, Martín-Lorenzo M, Santiago-Hernández A, Agudiez M, Cabrera M, Calvo E, Vázquez J, Ruiz-Hurtado G, Vivanco F, Ruilope LM, Barderas MG, Alvarez-Llamas * G, Identification of six cardiovascular risk biomarkers in the young population: A promising tool for early prevention, *Atherosclerosis*, https://doi.org/10.1016/j.atherosclerosis.2019.01.003.

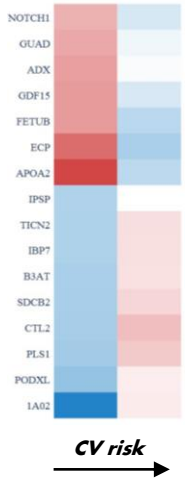


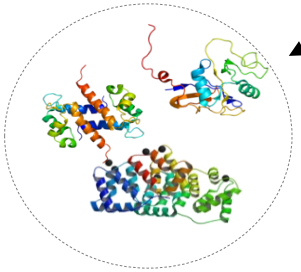
CVrisk estimators have limitations In Young people

Wrong prediction, failed prevention

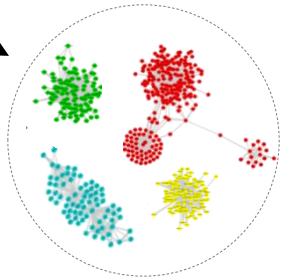
4309 identified proteins

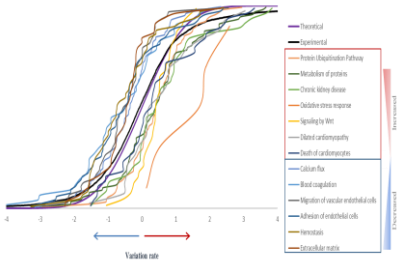






Individual CV Risk markers





Coordinated protein responses
(biological processes)

Identification of six cardiovascular risk biomarkers in the young population: A promising tool for early prevention

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KEYWORDS: Cardiovascular risk, Early prevention, Lifetime risk, Proteomics, Systems biology analysis, Biomarkers.

ABBREVIATIONS: ADX, adrenodoxin; ECP, eosinophil cationic protein; FETUB, fetuin B; GDF15, growth differentiation factor 15; GUAD, guanine deaminase; LTR, lifetime risk;

NOTCH1, neurogenic locus notch homolog protein 1; SBA, system biology analysis; SRM, selected monitoring reaction; TMT, tandem mass tag.

ABSTRACT

Background and aims: The predictive value of traditional CV risk calculators is limited. Novel indicators of CVD progression are needed particularly in the young population. The main aim of this study was the identification of a molecular profile with added value to classical CV risk estimation.

Methods: 81 subjects (30-50 years) were classified in 3 groups according to their CV risk: healthy subjects; individuals with CV risk factors; and those who had suffered a previous CV event. The urine proteome was quantitatively analyzed and significantly altered proteins were identified between patients' groups, either related to CV risk or stablished organ damage. Target-MS and ELISA were used for confirmation in independent patients' cohorts. Systems Biology Analysis (SBA) was carried out to identify functional categories behind CVD.

Results: 4309 proteins were identified, 75 of them differentially expressed. ADX, ECP, FETUB, GDF15, GUAD and NOTCH1 compose a fingerprint positively correlating with lifetime risk estimate (LTR QRISK). Best performance ROC curve was obtained when ECP, GDF15 and GUAD were combined (AUC=0.96). SBA revealed oxidative stress response, dilated cardiomyopathy, signaling by Wnt and proteasome, as main functional processes related to CV risk.

Conclusions: A novel urinary protein signature is shown, which correlates with CV risk estimation in young individuals. Pending further confirmation, this six-protein-panel could help in CV risk assessment.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of premature death worldwide despite improvements in outcomes. Cardiovascular (CV) risk is the result of multiple and interacting factors, and different algorithms are available to estimate CV risk in apparently healthy persons in the short-medium term (5-10 years), mainly based on age, gender, race, cholesterol levels, blood pressure, smoking habits and existence of diabetes. The Systematic Coronary Risk Evaluation (SCORE), recommended by the European Society of Cardiology (ESC) prevention guidelines [1], estimates the 10-year risk of a fatal atherosclerotic event, including coronary artery disease (CAD), stroke and abdominal aortic aneurysm. Therefore, CV risk of mortality rather than total CV risk (fatal and nonfatal) is assessed. The ESC guidelines suggest that the risk of total CVD may be calculated from the risk of CVD mortality using a fixed multiplier (3×). Nevertheless, the use of a fixed multiplier to estimate the 10-year total CVD risk from CVD mortality risk is controversial [2,3]. On the other hand, American guidelines define the 10-year risk as the risk of developing a first atherosclerotic CV event as nonfatal myocardial infarction or coronary heart disease death or fatal or nonfatal stroke over a 10-year period [4]. In any case, estimations in the short- medium-term have limitations since the majority of patients with low CV risk over the next 10 years show high risk at long-term if the estimation is calculated along their likely remaining lifetime (Lifetime Risk calculation or LTR QRISK) [5-8]. As age is one of the most contributing factors in these algorithms, CV risk of young population is particularly underestimated. As result, few young individuals reach treatment thresholds for intervention and, consequently, efficient prevention strategies are delayed. This limitation collides with the fact that the atherosclerotic process begins early in life and optimum prevention strategies should start in young population. In agreement, the prevalence and progression of subclinical atherosclerosis in individuals of ≤ 50 years with low

10-year CV risk but high LTR is greater than in individuals with low 10-year and low LTR [9].

Despite the knowledge of main risk factors and the enormous efforts dedicated to improve prevention, the asymptomatic and silent course of atherosclerosis hampers an accurate and individualized CV risk evaluation. Additional strategies and novel tools are needed to be implemented to add further knowledge in molecular subjacent mechanisms taking place in atherosclerosis development. Omics technologies allow identifying significant changes in proteins or metabolites abundance in CVD without pre-selection of molecular targets to be investigated, meaning that no-bias is introduced in the study [10,11]. Our group previously identified proteins and metabolites in urine showing altered response in an acute coronary syndrome and reflecting patient's recovery [12]. Significant alterations directly occurring at arterial tissue were also identified with reflection in plasma [13,14]. And specific molecular fingerprints in urine and plasma were previously identified in hypertensive patients developing albuminuria as those of higher CV risk [15-21]. In those sense, omics not only allow identification of novel markers to improve early diagnosis but also to monitor patient's prognosis [22]. Following these approaches, here we aimed to identify novel urinary targets linked to CV risk in young population (30-50 years) with added value to current estimations based on LTR. When ordinary CV risk factors are elevated in young people, they predict a significant increase in cardio-renal damage at later stages in life. On the other hand, subclinical atherosclerosis is already present in middle-age population and in a small percentage of young people CV events or even death can occur [23]. However, in the great majority of this population CV risk factors are within normal levels. In this work, we recruited young subjects who were classified in three groups so that representing the general population at that age in what refers to CV risk.

PATIENTS AND METHODS

PATIENT SELECTION AND URINE COLLECTION

Urine samples were collected from 81 subjects aged between 30 and 50 years, who were classified according to their CV risk in 3 groups: “control” group (C) (n=32) of healthy subjects with no medication; “factor” group (F) (n=24) including individuals with glomerular filtration rate <100 or albuminuria, and at least one of: arterial hypertension or patients treated with anti-hypertensive medications, hyperglycemia (glucose in blood >110 mg/dL) and/or metabolic syndrome; and “CV event” group (E) (n=25) including non-diabetic individuals who had suffered a nonfatal stroke or acute myocardial infarction (AMI) in the previous 3 years. All subjects included in the study were screened with detailed medical history, physical examination and biochemical profile. Lifetime Risk or LTR QRISK, referred as LTR in the manuscript, was estimated with the calculator https://qrisk.org/lifetime/. The study was approved by the Ethics Committee of the Hospital 12 de Octubre and was conducted according to the principles of the Declaration of Helsinki. All patients signed written informed consent before inclusion. Urine samples were collected in sterile containers, centrifuged at 3000g, 10 min, and stored at -80 °C until analysis.

QUANTITATIVE DIFFERENTIAL ANALYSIS BY TMT

In a first discovery phase, the urine proteome from 30 subjects (n: (C)=10; (F)=8; (E)=12, Table 1) was quantitatively analyzed by isobaric labeling (TMT, Thermo Fisher Scientific) following manufacturer’s instructions, as previously published [24]. Urine proteins were subjected to tryptic digestion and the resulting peptides were TMT-labeled, desalted and fractionated by high-pH reverse phase chromatography. The fractions were analyzed by LC-MS/MS on an Orbitrap Fusion mass spectrometer (Thermo Fisher). All spectra were analyzed

with Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific) using SEQUEST-HT (Thermo Fisher Scientific). Database searching was performed at the Uniprot database containing all sequences from human and contaminants (May 14th, 2016; 70611 entries). Peptide identification was performed using the probability ratio method [25], and the false discovery rate (FDR) was calculated using inverted databases and the refined method [26] with an additional filtering for precursor mass tolerance of 15 ppm [27]. Identified peptides had a FDR equal or lower than 1% FDR. Only those peptides were used to quantify the relative abundance of each protein from reporter ion intensities, and statistical analysis of quantitative data were performed using the WSPP statistical model previously described [28,29]. In this model protein log2-ratios are expressed as standardized variables, i.e., in units of standard deviation according to their estimated variances (Zq values).

Proteins that were identified with at least two peptides and showed homogeneous Zq values within each group (Student´s t-test p <0.05) were selected, being eliminated those proteins that showed differences according to gender. Changes in protein abundances between groups were calculated by comparing their Zq mean values, considering differentially expressed those proteins with differences of mean $Zq \geq \left| 1.5 \right|$.

SYSTEM BIOLOGY ANALYSIS

Functional protein analysis of the whole set of quantified proteins was performed using the Systems Biology Triangle, a novel algorithm specifically developed for the analysis of coordinated protein responses in high-throughput quantitative proteomics experiments [29]. This algorithm correlates the performance of a group of proteins inside of a category (biological process) in terms of their quantitative behavior (relative abundance); thus, changes can be detected in functional biological processes far beyond individual protein responses. As a result of this coordinated behavior, a Z value is assigned to each category (Zcat). To identify the significant biological process altered in disease progressions, F and E groups were

compared to the control group applying Zcat ≥ 2.9 and FDR<0.05. Variations in the abundance of functional categories were visualized by comparing the cumulative frequency (sigmoid) plots of the standardized variable with that of the normal distribution as performed previously [28].

TARGETED PROTEIN ANALYSIS

Confirmation of protein variations in response to CV risk was accomplished by either mass spectrometry using Selected Reaction Monitoring (SRM) (n: (C)=15; (F)=16; (E)=13) or by ELISA (n: (C)=30; (F)=24) in independent cohorts of subjects from those used in the discovery phase (Table 1).

SRM analysis

Targeted SRM analysis was performed as previously described [12,14,15,18,30]. Briefly, urine proteins were digested and proteotypic peptides were analyzed on a 6460QQQ MS connected to ChipCube-nanoLC (Agilent Technologies). SRM transitions were manually inspected and analysis conditions were set-up for each transition. The fragmentor was set to 130 V, dwell time to 20 ms and delta EMV to 600 V (Supplementary Table 1). Peak areas were used for inter-group comparison and statistical analyses were performed by GraphPad Prism 6 (version 6.01). The ROUT method was applied to detect outliers based on the FDR, setting Q value to 5%. Mann-Whitney non-parametric test (95% confidence level) was performed. Univariate and multivariate ROC curves were calculated with Metabolanalyst software using ROC curve based model evaluation (Tester) and Random forest algorithm.

ELISA analysis

Confirmation by ELISA was performed in urine from individual samples (Table 1), following manufacturer's instructions of Human GDF-15 ELISA Kit (Abcam), Human RNASE3/ECP ELISA Kit (Elabscience) and Human GDA ELISA Kit (Elabscience). Standard curves can be

found in Supplementary Fig. 1. Statistical analysis was performed by GraphPad Prism 6 (version 6.01) applying Mann-Whitney non-parametric test (95% confidence level).

Data statement

Datasets are in the process of being deposited.

RESULTS

Characteristics of the study population are compiled in Table 1, corresponding to the 81 recruited subjects classified as control (C), CV risk factor group (F) or those who had suffered a CV acute event (E). As can be seen, all subjects were aged between 30 and 50 years. As expected, main differences observed between groups are those resulting from their CV risk status, e.g. hypertension, glycaemia, lipid profile or pharmacological treatment.

The urine proteome varies with CV risk and stablished damage after an acute event

A total of 4309 proteins were identified by mass spectrometry in urine (Supplementary Table 2), from which 75 proteins showed statistically significant abundance changes (Supplementary Table 3). Sixteen proteins showed differences in abundance between risk factors group (F) and control group (C): 7 increased and 9 decreased in F (Fig. 1A). Analysis of the changes of these proteins along the three populations (Fig. 1B) revealed that most proteins levels tend to recover (towards control values) in those individuals who had suffered an acute event (E). When looking for proteins altered in the event group (E) compared to the factor group (F), 29 proteins significantly varied in abundance: 8 increased and 21 decreased in E (Fig. 2A). These 29 proteins showed minor differences between C and F groups (Fig. 2B), thus mainly reflecting stablished damage for these patients once recovered from the

acute event. Forty-four proteins showed significant alteration between groups E and C (7 increased and 37 decreased in E) (Supplementary Table 3).

Systems biology analysis revealed altered biological processes linked to CV risk estimate

SBA of the whole set of 4309 proteins enabled to identify functional categories with significant alteration in young individuals with CV risk. A functional category involves a group of proteins with coordinated behavior and may arise as significant even if the proteins are not significantly altered individually. As such, this analysis provided added value to the individual protein analysis previously described. Oxidative stress response, dilated cardiomyopathy, chronic kidney disease, cell death of cardiomyocytes, signaling by Wnt and several categories related to protein biosynthesis, proteasome and cytoskeleton, were significantly increased in CV risk factors group (F) with respect to control group (C) (Supplementary Fig. 2 and Supplementary Table 4A). Functional categories related to coagulation, extracellular matrix, calcium flux, hemostasis and vascular system, were significantly decreased in F group (Supplementary Fig. 2 and Supplementary Table 4B).

A urinary fingerprint composed by six proteins varies with CV risk in the young population

Our last goal was to identify molecular indicators associated with CV risk estimate in young population. Thus, we focused on those proteins significantly increased in urine from individuals with CV risk factors (F) with respect to healthy controls (C) (Fig. 1A, Supplementary Table 3). Adrenodoxin (ADX), eosinophil cationic protein (ECP), fetuin B (FETUB), growth differentiation factor 15 (GDF15), guanine deaminase (GUAD) and neurogenic locus notch homolog protein 1 (NOTCH1) proteins were analyzed for

confirmation by a different technical approach (SRM) and in a different group of individual samples (Table 1). Increased abundance in the CV risk group (F) was confirmed for the 6 proteins (Fig. 3A). Area under the curve (AUC) values from individual and multivariate ROC curves showed best performance when ECP, GDF15 and GUAD are combined resulting in AUC=0.96 (Fig. 3B). Predicted class probabilities graph also shows good separation between patients' groups (Fig. 3C). By ELISA, significant variation was found for these three proteins in the risk factors group (F) *versus* control group (C), thus confirming again previous data in a different cohort of subjects (Fig. 4). Supplementary Table 5 shows the correlations of the identified proteins with parameters used to estimate CV risk.

The urinary biomarker panel correlates with lifetime risk

Correlation between proteins abundance and LTR was investigated. Significant correlation was found for the 6 proteins as shown in Supplementary Fig. 3 (Spearman correlation values: ADX r = 0.4889 p <0.0001; ECP r = 0.6221 p <0.0001; FETUB r = 0.4762 p <0.0001; GDF15 r = 0.5570 p <0.0001; GUAD r = 0.5549 p <0.0001; and NOTCH1 r = 0.6493 p <0.0001), showing a positive correlation in all cases. It means that both the LTR and the values of these 6 proteins in the urine of the patients increase while increasing their CV risk estimate showing a pattern represented by the correlation coefficient that could be considered as adequate in any biological background.

DISCUSSION

The need to overcome current limitations of CV risk available estimations, particularly in young population, prompts the identification of novel indicators with added value to existing algorithms. In this sense, the ultimate goal to reduce CVD mortality is to introduce novel and easily quantifiable molecular targets in the clinic which can serve to monitor general population at early stages of atherosclerosis development [23]. The actual proteomic

strategies offer the possibility to identify thousands of proteins in a biological context while identifying most relevant variations linked to a certain pathological status, not only in individual proteins but also in biological processes [29].

The urinary proteome correlates with CV risk estimate

This study shows how the urinary proteome reflects specific protein changes which are modulated by the status of CV risk or existing damage. Different sub-sets of proteins were identified with altered urine levels either in subjects with CV risk factors, or in those who had suffered an acute CV event despite of being fully recovered, as recently reported by our group in plasma [24]. Following quantitative analysis of the whole urinary proteome by different technical approaches and in different patients' cohorts, we highlight six proteins with significantly increased urinary levels in individuals with CV risk factors compared to healthy subjects: ADX, ECP, FETUB, GDF15, GUAD and NOTCH1. It was found significant and positive correlation between the 6 proteins and LTR. The variation in these proteins decreases after an acute nonfatal event, showing their association with CV risk more than organ damage. With regard to the latter, their reduced levels in the event group could be attributed to the fact that these patients have been accordingly managed following the event and perform a better lifestyle, thus reducing their CV risk. Interestingly, we developed a novel target mass spectrometry assay based on SRM methodology. SRM high throughput and automation capacity allows significantly shortening the analysis time compared to non-mass spectrometry-based approaches, thus improving the cost-effectiveness compared to conventional procedures.

Oxidative stress, inflammation and CV risk

In line with previous findings from our group in hypertensive patients who develop albuminuria [19,21,30] SBA showed oxidative stress response as a significantly increased functional category in individuals with CV risk factors with respect to healthy controls. Oxidative stress is known to be involved in the pathogenesis of CVD as myocardial infarction, atherosclerosis, cardiac hypertrophy or congestive heart failure [31,32]. GUAD is an aminohydrolase enzyme that converts guanine to xanthine in the uric acid cycle, in which also participates xanthine oxidase, one of the major source of ROS in the human heart, since it produces O_2^- and H_2O_2 while catalyzing the conversion of hypoxanthine to xanthine and xanthine to uric acid [33]. Hyperuricemia has been considered a key risk factor for development of gout, renal dysfunction, hypertension, hyperlipidemia, diabetes and obesity [33,34]. An increase in GUAD gene expression was associated with the development of hypertensive cardiac hypertrophy or diastolic heart failure, in a study with spontaneously hypertensive rats [35]. Moreover, uric acid levels, even below the clinical threshold for hyperuricemia, are associated with increased CV risk by increasing oxidative stress, promoting endothelial dysfunction and enhancing inflammation [36]. GDF15 is expressed and secreted by macrophages, cardiomyocytes, vascular smooth muscle cells, adipocytes and endothelial cells, in response to inflammation, oxidative stress, hypoxia or mechanical stress [37,38]. GDF15 has a CV protective function, since it regulates signaling pathways essential for cardioprotection [39]. It has been shown to be strongly associated with future CV events and complements stablished risk predictors in CVD progression and prognosis [40-42].

In the inflammatory context of CVD, macrophages increase the expression of CCL5/RANTES chemokine, which could attract activated eosinophils through its CCR3 receptor. Several studies have shown an association between eosinophils and CVD [43,44]. ECP is a marker of eosinophil activity and degranulation, which has been proposed as biomarker of coronary atherosclerosis [45] and risk marker for ischemic stroke [46]. It has

been described a protective role of ECP under oxidative stress, as it inhibits ROS-induced apoptosis in cardiomyocytes via PI3K-Akt pathway [47].

Related to FETUB, serum levels were significantly higher in patients with CAD when it was compared to healthy controls, being proposed as a potential biomarker for CAD [48]. Besides, expression level of FETUB was significantly increased in patients with AMI compared with stable angina subjects, suggesting that serum FETUB is involved in the development of AMI by influencing atherosclerotic plaque rupture [49]. Our data are in alignment with these observations, as FETUB increases in young individuals with CV risk factors. With the same trend, we identified NOTCH1. Endothelial NOTCH1 acts as an antagonist of endothelial cell activation preventing inflammation in the aorta, and circulating lipids were shown to decrease NOTCH1 expression and signaling in human aortic endothelial cells [50]. With these evidences a reduction of endothelial NOTCH1 has been proposed as a predisposing factor of initiation of atherosclerosis. However, expression and activation of NOTCH1 in podocytes, which is mostly silenced in the glomeruli of normal mature human kidney, has been correlated with development of proteinuria, glomerulosclerosis and kidney dysfunction [51-54].

Cardio-renal damage and urinary pattern

The cross-talk between the heart and the kidneys is clearly evidenced [55]. Observational and clinical data showed that acute/chronic worsening of kidney function directly contributes to acute/chronic cardiac disease and *viceversa*, deriving in the simultaneous presence of the cardio-renal damage [56]. In early stages, chronic kidney disease (CKD) develops silently and asymptomatic, which enormously complicates early diagnosis and intervention. Surprisingly, although none of our patients showed albuminuria, systems biology analysis detected an alteration in the behavior of CKD-related proteins, arising as a category significantly

increased in the CV risk factor group compared to healthy controls. Interestingly, this pattern is not coincidental with the one that we described in hypertensive subjects chronically RAS suppressed [15,22,30]. This indicates that the proteomic urinary pattern changes during long-term evolution of cardio-renal damage. Sustained activation of Notch and Wnt signaling in podocytes were shown to be causally related to albuminuria development in genetically modified animal models, being albuminuria a strong and independent indicator of increased CV risk and supporting our findings shown here in a young population [57]. Wnt signaling was also identified in individuals with CV risk in agreement with an upregulation of Wnt signaling described in atherosclerosis and cardiometabolic diseases [58-60].

Limitations

The study fulfilled the requirements of an omics study in terms of group size and technical workflow [61]. Protein variations identified in a first discovery stage were confirmed by different technical approaches and in different patients' cohorts. The main limitation could be the relatively low number of patients from a clinical point of view and prior to translation to clinical practice, further studies (e.g. multicentric, wider cohorts) should follow. However, the strength of the correlations found in this trial enhances the possibility that the required larger multicenter cohorts to further confirm their potential use in CV risk stratification could simply confirm our data. Future prospective trials with clinical CV endpoints would be needed to address if the proteins here shown are risk markers up and above traditional risk estimates, reflecting risks factors, organ damage or response to organ damage. The role of these proteins may be different in primary and secondary prevention.

Conclusions

We have identified ADX, ECP, FETUB, GDF15, GUAD and NOTCH1, as urinary proteins linked to CV risk estimate in young individuals with traditional CV risk factors. This finding correlates with LTR constituting a potential tool to improve the accuracy of CV risk estimation in young population.

CONFLICT OF INTERESTS

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

FINANCIAL SUPPORT

ISCIII co-supported by FEDER grants (PI14/01650, PI14/01917, PI14/01841, PI16/01334, IF08/3667-1, FI12/00126, CPII15/00027, CP15/00129, PT13/0001/0013, PI17/01093, PI17/01193, PRB3 (IPT17/0019 ISCIIIS-GEFI/ERDF, REDinREN (RD12/0021/0001, RD16/0009)), Fundación SENEFRO, Fundación Íñigo Álvarez de Toledo and Fundación Conchita Rábago de Jiménez Díaz. Results are lined up with the Spanish initiative on the Human Proteome Project.

AUTHOR CONTRIBUTIONS

PJM, MBM, JAL and MA performed the experiments. PJM, MBM, JAL, MML, ASH, JV participated in data analysis/interpretation, Fig.s and literature search. MC and EV

participated in clinical data collection. GRH, FV, LMR, MGB and GAL designed the study and contributed to data interpretation, and manuscript drafting.

ACKNOWLEDGEMENTS

The authors acknowledge Lucía Guerrero and Maria Cruz Casal (Hospital 12 de Octubre) for her participation in samples collection and patients' classification.

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FIG. LEGENDS

Fig. 1. Urine proteins showing significantly altered levels in subjects with CV risk factors compared to healthy controls: a link to CV risk.

(A) Proteins with significant variation in F with respect to C. Differences in abundance between groups are represented in Zq average by color code: decreased (blue, from -4 to 0), increased (red, from 0 to +4). (B) Variation of these proteins, showing a recovery towards the "healthy" status in most cases. C: control group, E: CV event group, F: CV risk factor group.

Fig. 2. Urine proteins showing altered levels in individuals recovered from an acute event compared to subjects with CV risk factors: a link to established organ damage.

(A) Proteins with significant variation in E with respect to F. Differences in abundance between groups are represented in Zq average by color code: decreased (blue, from -4 to 0), increased (red, from 0 to +4). (B) Protein variation, showing protein responses to stablished CV damage despite of full recovery after a CV event. C: control group, E: CV event group, F: CV risk factor group.

Fig. 3. Confirmation by target mass spectrometry and evaluation of clinical sensitivity and specificity.

(A) Target analysis by selected reaction monitoring (SRM) of proteins associated with CV risk. (B) Multivariate receiver operating curve (ROC) and (C) predicted class graph for the CV risk proteins ECP, GDF15 and GUAD. AUC, area under the curve; CI, confidence interval; ADX, adrenodoxin; ECP, eosinophil cationic protein; FETUB, fetuin B; GDF15, growth differentiation factor 15; GUAD, guanine deaminase; NOTCH1, neurogenic locus notch homolog protein 1. Mann-Whitney non-parametric test (95% confidence level) was performed. *p <0.05, ***p <0.001, ****p <0.0001.

Fig. 4. ELISA analysis of proteins associated with CV risk.

Mann-Whitney non-parametric test (95% confidence level) was performed. *$p < 0.05$. ECP, eosinophil cationic protein; GDF15, growth differentiation factor 15; GUAD, guanine deaminase.

Table 1. Baseline clinical data of different cohorts expressed as mean ± S.D. or percentages.

	DISCOVERY PHASE			SRM CONFIRMATION			ELISA CONFIRMATION	
	C	**F**	**E**	**C**	**F**	**E**	**C**	**F**
n	10	8	12	15	16	13	30	24
Age (years)	44 ± 5	44 ± 6	45 ± 5	42 ± 5	44 ± 5	45 ± 4	42 ± 5	44± 5
Sex (male), %	60	50	67	13	88	92	53	75
Glycaemia (mg/dl)	79 ± 7	104 ± 45	100 ± 24	80 ± 8	95 ± 22	117 ± 54	80 ± 8	98 ± 32
eGFR (ml/min/1.73m^2)	91 ± 8	97± 21	98 ± 27	95 ± 11	85 ± 9	98 ± 15	94 ± 10	89 ± 15
Systolic blood pressure (mmHg)	113 ± 10	131 ± 8	122 ± 16	111± 8	139± 13	122± 22	112± 9	136 ± 12
Diastolic blood pressure (mmHg)	73 ± 8	84 ± 10	75 ± 10	70 ± 8	90 ± 8	77 ± 14	71 ± 8	88 ± 9
Metabolic syndrome, %	10	13	0	0	81	15	3	54
Total cholesterol (mg/dl)	197 ± 30	207 ± 36	158 ± 36	192 ± 41	208 ± 35	138 ± 41	196 ± 8	208 ± 35
HDL cholesterol (mg/dl)	70 ± 19	53 ± 12	43 ± 10	73 ± 16	38 ± 9	40 ± 11	71 ± 17	43 ± 12
LDL cholesterol (mg/dl)	112 ± 32	131 ± 34	90 ± 33	102 ± 31	135 ± 33	73 ± 40	108± 31	134 ± 33
Triglycerides (mg/dl)	88 ± 50	140 ± 121	121 ± 72	80 ± 29	220 ± 72	105 ± 35	85 ± 39	193 ± 99
Uric acid (mg/dl)	5 ± 1.5	5 ± 0.9	6 ± 1.4	4 ± 0.9	7 ± 1.7	6 ± 1.4	5 ± 1.2	6 ± 1.7
Current smoking, %	0	25	50	27	25	69	17	25
Antihypertensives, %	0	25	8	0	50	54	0	42
Lipid-lowering agents, %	0	13	8	0	25	46	0	21
LTR QRISK	24 ± 8	34 ± 8	_	21 ± 3	47 ± 7	_	23 ± 6	42 ± 10

C: control group, E: CV event group, eGFR: estimated glomerular filtration rate, F: CV risk factor group.

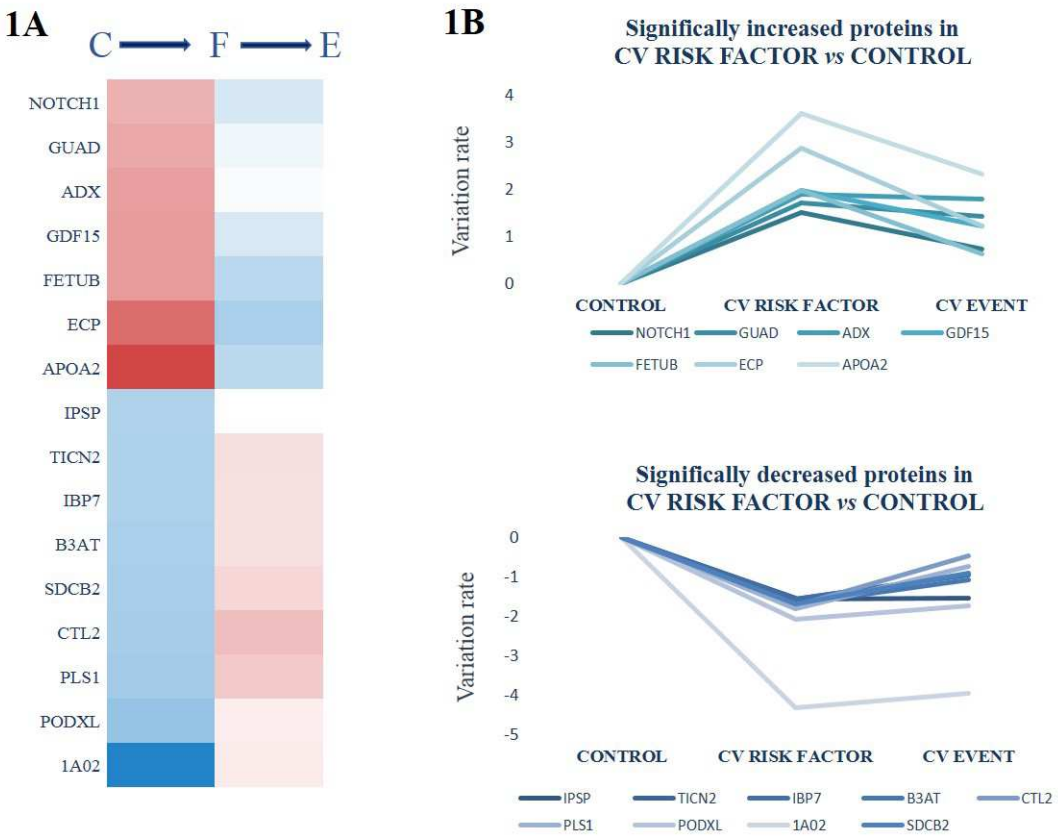
1A
C → F → E
NOTCH1
GUAD
ADX
GDF15
FETUB
ECP
APOA2
IPSP
TICN2
IBP7
B3AT
SDCB2
CTL2
PLS1
PODXL
1A02
1B
Significally increased proteins in
CV RISK FACTOR vs CONTROL
Variation rate
4
3
2
1
0
CONTROL
CV RISK FACTOR
CV EVENT
NOTCH1
GUAD
ADX
GDF15
FETUB
ECP
APOA2
Significally decreased proteins in
CV RISK FACTOR vs CONTROL
Variation rate
0
-1
-2
-3
-4
-5
CONTROL
CV RISK FACTOR
CV EVENT
IPSP
TICN2
IBP7
B3AT
CTL2
PLS1
PODXL
1A02
SDCB2

Figure 1

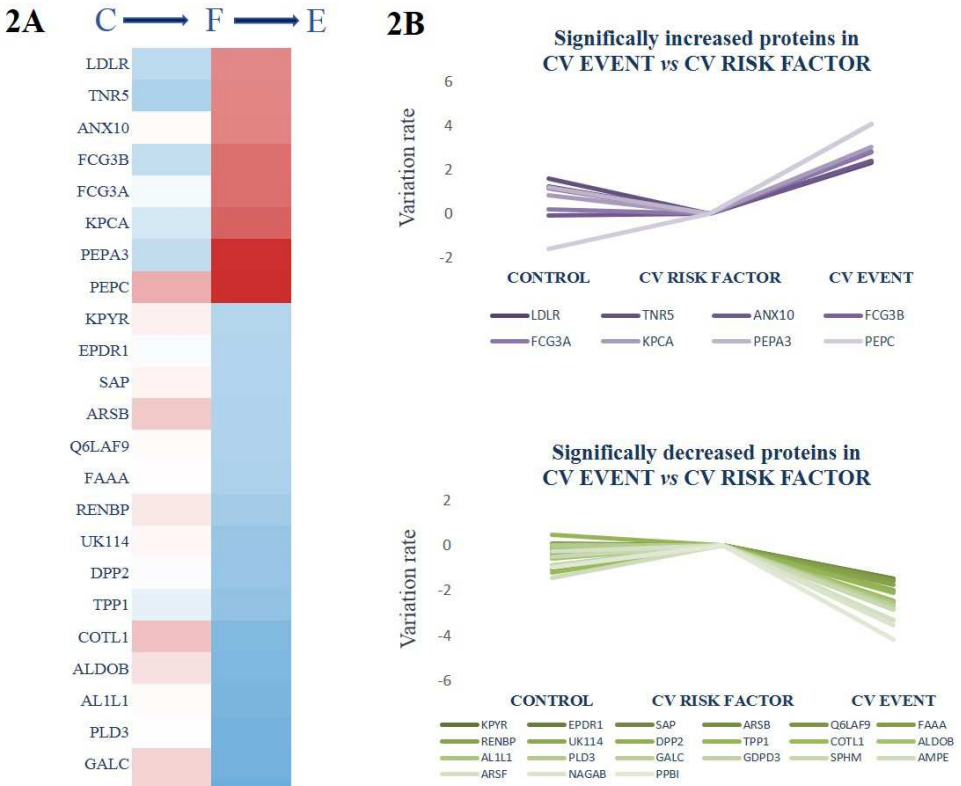
2A
C → F → E
LDLR
TNR5
ANX10
FCG3B
FCG3A
KPCA
PEPA3
PEPC
KPYR
EPDR1
SAP
ARSB
Q6LAF9
FAAA
RENBP
UK114
DPP2
TPP1
COTL1
ALDOB
AL1L1
PLD3
GALC
GDPD3
SPHM
AMPE
ARSF
NAGAB
PPBI
2B
Significally increased proteins in CV EVENT vs CV RISK FACTOR
Variation rate
6
4
2
0
-2
CONTROL
CV RISK FACTOR
CV EVENT
LDLR
TNR5
ANX10
FCG3B
FCG3A
KPCA
PEPA3
PEPC
Significally decreased proteins in CV EVENT vs CV RISK FACTOR
Variation rate
2
0
-2
-4
-6
CONTROL
CV RISK FACTOR
CV EVENT
KPYR
EPDR1
SAP
ARSB
Q6LAF9
FAAA
RENBP
UK114
DPP2
TPP1
COTL1
ALDOB
AL1L1
PLD3
GALC
GDPD3
SPHM
AMPE
ARSF
NAGAB
PPBI

Figure 2

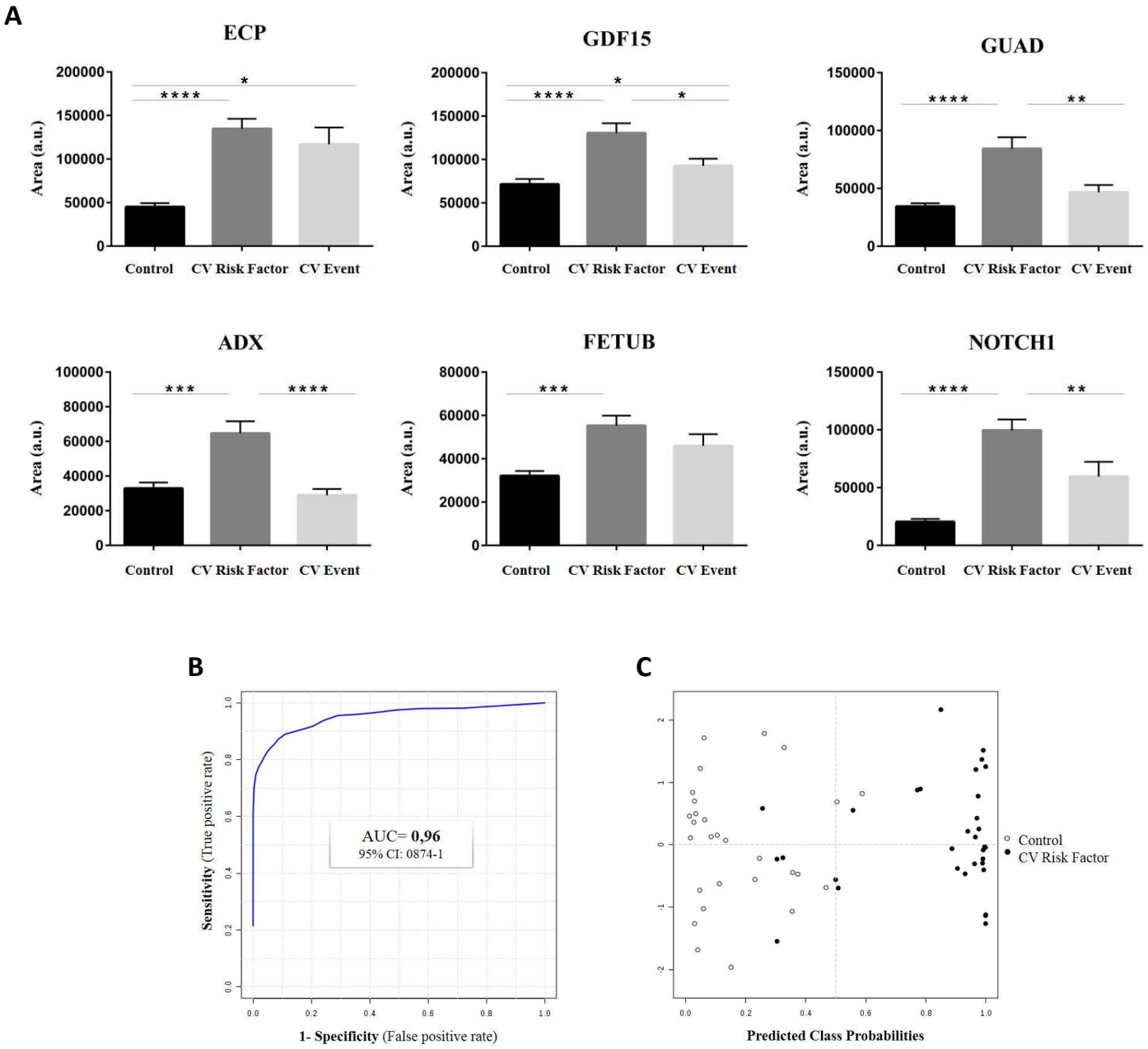
A
ECP
GDF15
GUAD
ADX
FETUB
NOTCH1
Area (a.u.)
Control
CV Risk Factor
CV Event
B
Sensitivity (True positive rate)
1- Specificity (False positive rate)
AUC= 0,96
95% CI: 0874-1
C
Predicted Class Probabilities
Control
CV Risk Factor

Figure 3

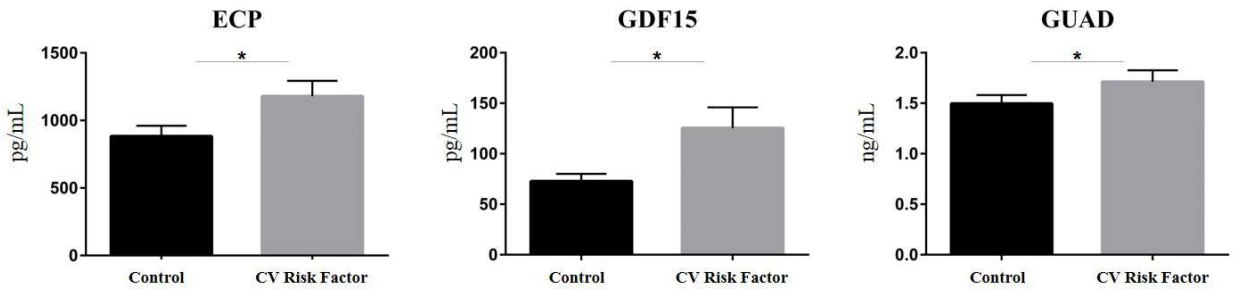
ECP
pg/mL
1500
1000
500
0
*
Control
CV Risk Factor
GDF15
pg/mL
200
150
100
50
0
*
Control
CV Risk Factor
GUAD
ng/mL
2.0
1.5
1.0
0.5
0.0
*
Control
CV Risk Factor

Figure 4

HIGHLIGHTS

-Cardiovascular risk of young/middle-age population is underestimated.

-The urinary proteome reflects changes modulated by CV risk or existing damage.

-Six proteins compose a fingerprint in asymptomatic individuals with CV risk factors.

-This tool would improve the accuracy of CV risk estimation and prevention criteria.