Coronary Artery Disease and Type 2 Diabetes: A Proteomic Study

Diabetes Care 2020;43:843–851 | https://doi.org/10.2337/dc19-1902

Giulia Ferrannini,[1,2] Maria Laura Manca,[3] Marco Magnoni,[4] Felicita Andreotti,[5] Daniele Andreini,[6,7] Roberto Latini,[8] Attilio Maseri,[9] Aldo P. Maggioni,[10] Rachel M. Ostroff,[11] Stephen A. Williams,[11] and Ele Ferrannini[12]

OBJECTIVE

Coronary artery disease (CAD) is a major challenge in patients with type 2 diabetes (T2D). Coronary computed tomography angiography (CCTA) provides a detailed anatomic map of the coronary circulation. Proteomics are increasingly used to improve diagnostic and therapeutic algorithms. We hypothesized that the protein panel is differentially associated with T2D and CAD.

RESEARCH DESIGN AND METHODS

In CAPIRE (Coronary Atherosclerosis in Outlier Subjects: Protective and Novel Individual Risk Factors Evaluation—a cohort of 528 individuals with no previous cardiovascular event undergoing CCTA), participants were grouped into CAD^- (clean coronaries) and CAD^+ (diffuse lumen narrowing or plaques). Plasma proteins were screened by aptamer analysis. Two-way partial least squares was used to simultaneously rank proteins by diabetes status and CAD.

RESULTS

Though CAD^+ was more prevalent among participants with T2D (HbA_{1c} 6.7 ± 1.1%) than those without diabetes (56 vs. 30%, $P < 0.0001$), CCTA-based atherosclerosis burden did not differ. Of the 20 top-ranking proteins, 15 were associated with both T2D and CAD, and 3 (osteomodulin, cartilage intermediate-layer protein 2, and HTRA1) were selectively associated with T2D only and 2 (epidermal growth factor receptor and contactin-1) with CAD only. Elevated renin and GDF15, and lower adiponectin, were independently associated with both T2D and CAD. In multivariate analysis adjusting for the Framingham risk panel, patients with T2D were "protected" from CAD if female ($P = 0.007$), younger ($P = 0.021$), and with lower renin levels ($P = 0.02$).

CONCLUSIONS

We concluded that *1*) CAD severity and quality do not differ between participants with T2D and without diabetes; *2*) renin, GDF15, and adiponectin are shared markers by T2D and CAD; *3*) several proteins are specifically associated with T2D or CAD; and *4*) in T2D, lower renin levels may protect against CAD.

Cardiovascular (CV) disease has steadily represented the leading cause of death in the world for the past 15 years, with coronary artery disease (CAD) and stroke being responsible for a combined 15 million deaths in 2016 (1). Morbidity and mortality of patients with CAD is considerably higher in the presence of diabetes, accounting for ~50% of deaths in this population (2). Therefore, detection of CAD and its risk factors

[1]Department of Medical Sciences, Postgraduate School of Internal Medicine, University of Turin, Turin, Italy
[2]Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden
[3]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
[4]IRCCS Ospedale San Raffaele and Università Vita-Salute San Raffaele, Milan, Italy
[5]Institute of Cardiology, FPG IRCCS, Catholic University Medical School, Rome, Italy
[6]Centro Cardiologico Monzino, IRCCS, Milan, Italy
[7]Cardiovascular Section, Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy
[8]Mario Negri Institute of Pharmacological Research-IRCCS, Milan, Italy
[9]Heart Care Foundation, Florence, Italy
[10]ANMCO Research Center, Heart Care Foundation, Florence, Italy
[11]Clinical Research and Development, SomaLogic Inc., Boulder, CO
[12]CNR Institute of Clinical Physiology, Pisa, Italy

Corresponding author: Ele Ferrannini, ferranni@ifc.cnr.it

Received 24 September 2019 and accepted 31 December 2019

This article contains Supplementary Data online at https://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc19-1902/-/DC1.

is crucial to improve both treatment and prevention, especially in patients with diabetes.

Coronary computed tomography angiography (CCTA) provides a noninvasive, highly sensitive anatomic investigation of the epicardial coronary circulation by visualizing the coronary artery lumen and wall with an intravenous contrast agent (3). CCTA is highly accurate in the early detection of obstructive atherosclerosis, defined by invasive coronary angiography, and therefore is being increasingly included in international guidelines for the diagnostic workup of CAD (4,5). The extent of atherosclerotic burden can be assessed by several CCTA-derived scores, which have demonstrated independent long-term predictive power for adverse cardiac events (i.e., the segment involvement score, the segment stenosis score, and the CT-adapted Leaman score [6]). In patients with diabetes, the prevalence of obstructive, CCTA-detected CAD is nearly 25% (i.e., much higher than in the population without diabetes) (7). Moreover, CCTA provides long-term prognostic information for patients with diabetes, allowing their risk stratification and showing excellent prognosis when no evidence of atherosclerosis is detected (7,8).

Addressing traditional CV risk factors (CVRFs) has proved advantageous in reducing CV mortality in the general population as well as in people with diabetes (9–11). Nevertheless, the relationship between the presence of traditional CVRFs and atherosclerosis development is hardly linear, and CV events can occur independently of traditional CVRF management. Thus, substantial improvement in CAD prevention is still needed, especially in patients with diabetes (12,13). Over the past decade, technology has provided novel tools for the identification of protective and susceptibility factors for both CAD and diabetes. More recently, progress in the technology of proteomics has provided further opportunities for the development of new diagnostic and therapeutic algorithms (14). High-performance platforms can screen a large number of proteins that may serve as biomarkers, eventually to be included in multiparametric models of risk assessment (15). Proteins may also serve as indicators of therapeutic effectiveness and can be targeted directly (16). In the current study, we aimed to identify proteins differentially associated with the presence of type 2 diabetes (T2D) and CCTA-proven CAD by using an expanded proteomic platform and bivariate analysis of associations.

RESEARCH DESIGN AND METHODS

Study Design

The Coronary Atherosclerosis in Outlier Subjects: Protective and Novel Individual Risk Factors Evaluation (CAPIRE) study (ClinicalTrials.gov identifier: NCT02157662) is part of the Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI) Outlier Project, jointly promoted by the Heart Care Foundation Onlus, Italian Association of Hospital Cardiologists (ANMCO), and Mario Negri Institute of Pharmacological Research. CAPIRE was designed as a prospective, observational study aimed at identifying possible new mechanisms that promote or protect against atherothrombosis; in its longitudinal phase, participants are being followed for 5 years or longer (17). The study enrolled participants 45–75 years of age, without previous clinical manifestations of ischemic heart disease (acute myocardial infarction, unstable angina, chronic stable angina, previous percutaneous or surgical coronary revascularization, heart failure), who underwent a 64-slice (or superior) CCTA in the outpatient clinics of the 11 participating centers because of suspected CAD. The main indications for CCTA were *1*) uninterpretable, equivocal, or contraindicated functional stress test (44% of patients); *2*) new-onset chest pain syndrome at low-intermediate pretest likelihood of CAD (25% of patients); and *3*) other indication (including evaluation before valve or noncardiac surgery, elevated risk profile, arrhythmias, or atypical symptoms) (31% of patients). Patients with T2D were further selected to be eligible for the CCTA procedure if they were in good clinical condition and stable metabolic control. Exclusion criteria were *1*) CCTA not meeting the quality control criteria; *2*) previous CV events (myocardial infarction, unstable angina, stable or unstable angina, percutaneous or surgical coronary revascularization, heart failure), both clinically evident and confirmed by clinical and conventional diagnostics; *3*) other previous heart disorders documented or identified at CCTA, such as dilated cardiomyopathy (regardless of etiology), obstructive hypertrophic cardiomyopathy, atrial fibrillation, myocarditis, and inflammatory vascular disease; *4*) previous documented acute or chronic peripheral vascular disease (stroke, transient ischemic attack, previous revascularization); *5*) claudication at rest or at low-grade effort); and *6*) active inflammatory or neoplastic disease.

CVRF Definition

The conventional CVRFs that are based on the Adult Treatment Panel III and the 2013 American College of Cardiology/American Heart Association guidelines for CV disease prevention were considered in selecting the study population (18). CVRFs were defined as follows: family history of ischemic heart disease (history of early manifestations of ischemic heart disease in first-degree relatives before 55 years of age for men and 65 years for women), arterial hypertension (history of arterial hypertension, ongoing antihypertensive treatment, or recent observation of blood pressure [BP] values >140/90 mmHg), hypercholesterolemia (total serum cholesterol >200 mg/dL or <200 mg/dL with ongoing lipid-lowering medications), diabetes (fasting plasma glucose levels >126 mg/dL or a 2-h value ≥200 mg/dL by oral glucose tolerance test or isolated elevation of glycated hemoglobin [HbA_{1c}] ≥6.5% or current use of insulin or oral glucose-lowering medications), and cigarette smoking (current cigarette smoking habit or recent abstention within 1 year).

Source data for defining CVRFs were physical examination, medical records, and laboratory tests reported by the participant or documented before CCTA. After enrollment, a centrally performed biomarker profile, including lipid profile and metabolic markers, allowed a refined assessment of CVRFs, such as diabetes and dyslipidemia. According to data in the literature, most patients without any CVRFs or one single risk factor belong to a risk group of <10% of events at 10 years according to the Framingham Heart Study, and patients with three or more CVRFs belong to a risk group of >20% of events at 10 years (18). Diabetes as a single CVRF was excluded because of evidence of its own higher CV risk for the development of CAD and independent association with the atherosclerotic burden (18).

CCTA Analysis

The CCTA data interpretation in CAPIRE was performed with advanced plaque

assessment. Coronary arteries were divided into 16 segments according to the AHA classification (19). Normal coronary arteries were defined as no atherosclerotic plaque detected in any segment within the coronary artery wall or lumen. Lumen stenosis was measured for each coronary artery plaque detected and graded as normal, nonobstructive (<50%), moderate stenosis (50–70%), and severe stenosis (>70%). High-risk plaque features were also assessed and defined as described in a previous report from CAPIRE (20). Plaque length was also recorded. Plaque consistency was assessed using Hounsfield units (HU), and low-attenuation plaque was defined as the presence of any voxel <30 HU. Total plaque volume, defined as the entire volume of coronary plaque (including both calcified and noncalcified plaque) was measured. Low-attenuation plaque volume and noncalcified fibrofatty plaque volume was expressed as the amount of plaque with <30 HU and between 30 and 150 HU, respectively (expressed in mm^3). Noncalcified plaque volume was considered as plaque volume with attenuation density <150 HU (20). Atherosclerosis burden was assessed on a per-patient basis using previously validated CT scores: segment involvement score, segment stenosis score, and CT-adapted Leaman score, as previously reported; the higher quartile was used as the cutoff (6,20,21). Finally, cardiac mass was measured in every patient.

Participant Groups

On the basis of CCTA, participants were grouped into CAD^- (clean coronaries) and CAD^+ (coronary atherosclerosis extended to >5 of the 16 segments according to the AHA classification [19] [segment involvement score >5], with or without coronary stenosis). The five-coronary-segment involvement cutoff was chosen to define CAD on the basis of previously assessed prognostic value and on the results of the COronary CT Angiography EvaluatioN for Clinical Outcomes: An InteRnational Multicenter Registry (CONFIRM) registry study (17,22).

The final population ($n = 528$) consisted of 64 participants with T2D and 464 participants with normal glucose tolerance (NGT). CAD^+ was detected in 180 participants, 36 of whom had T2D; of the 348 participants with CAD^-, 28 had T2D.

Laboratory Determinations

In all participants, a peripheral venous blood sample was taken, mostly under fasting conditions (i.e., in the morning after an overnight, 10- to 12-h fast). Following centrifugation, 0.5-mL aliquots of serum or plasma were stored at −70°C in a dedicated biological bank (SATURNE-1; Mario Negri Institute of Pharmacological Research). All biomarkers were measured in a central laboratory, in a single batch, by personnel unaware of participant characteristics. Serum creatinine, HbA_{1c}, lipids, and plasma metabolites were measured by standard, automated laboratory methods. hs-CRP was measured with an automatic immunoturbidimetric method (Beckman-Coulter, Galway, Ireland). High-sensitivity cardiac troponin T was measured on an automated platform (ECLIA Cobas e 411; Roche Diagnostics, Rotkreuz, Switzerland) with a limit of blank of 3 ng/L. Plasma levels of pentraxin 3 were measured by in-house sandwich ELISA as previously described (21)

Quantification of Proteins in Human Plasma

The method of quantification of proteins by modified aptamers has been previously described (23,24). In brief, each of ~5,000 individual proteins has its own binding reagent made of chemically modified DNA, referred to as modified aptamer. Each plasma sample was incubated with the mixture of modified aptamers to generate modified aptamer-protein complexes. Unbound modified aptamers and unbound or nonspecifically bound proteins were eliminated by two bead-based immobilization steps and competition with unlabeled polyanion. After eluting the modified aptamers from the target protein, the fluorescently labeled modified aptamers were directly quantified on an Agilent hybridization array (Agilent Technologies). Calibrators were included so that the degree of fluorescence was a quantitative reflection of the availability of the three-dimensional shape-charge epitope on each specific protein. Results are expressed as units of fluorescence intensity (F.I.).

Statistical Analysis

Continuous variables are presented as mean ± SD; variables with a skewed distribution are given as median (interquartile range). Continuous variables with a normal distribution were compared using the Student *t* test for independent samples; variables with a skewed distribution (by the Shapiro-Wilk test) were compared by Mann-Whitney *U* tests for independent samples. Proportions were compared using a χ^2 or Fisher exact test, as appropriate. Differences by diabetes (NGT or T2D) and CAD (CAD^+ or CAD^-), each treated as a nominal variable, were analyzed by two-way ANOVA; for this analysis, variables with a skewed distribution were logarithmically transformed for use in ANOVA.

Two-way partial least squares (PLS) was used to rank proteins according to the strength of their separate association with T2D or CAD. This method has been shown to be preferable to random forest or least absolute shrinkage and selection operator regression when the number of predictors (e.g., proteins) is much larger than the number of cases and when there is a high degree of multicollinearity in the data (25). Proteins were ranked according to the Variable Importance in Projection (VIP) score. Multivariate logistic regression was carried out by standard methods. R and SPSS for Mac Os X software were used; the statistical significance threshold level was set at $P < 0.05$.

RESULTS

In the CAPIRE cohort, the percentage of individuals with T2D (12%) is more than double the age-standardized national prevalence (4.9% in 2016, https://www.istat.it/en/archivio/202712), reflecting the expected enrichment of T2D among individuals referred to a cardiology center. The clinical and metabolic phenotype of the participants with T2D included stronger history of hypertension, hypercholesterolemia, and smoking; higher BMI, HbA_{1c}, systolic BP, triglycerides, non-HDL cholesterol, and hs-CRP; and lower HDL cholesterol (Table 1). On the other hand, independently of T2D, the presence of CAD was associated with a higher prevalence of male sex, older age, higher BP, serum creatinine, hs-CRP, and troponin T levels.

Medication use is also shown in Table 1. β-Blockers, ACE inhibitors, angiotensin II receptor blockers (ARBs), and statins were prescribed more frequently in participants with T2D than in participants with NGT. Additionally, the CAD^+ group had a higher frequency of statin use than the CAD^- group.

Table 1—Demographic, clinical, and biochemical characteristics

	CAD$^-$		CAD$^+$				
	NGT (n = 320)	T2D (n = 28)	NGT (n = 144)	T2D (n = 36)	P^1 CAD#	P^2 T2D#	$P^1 \times P^2$#
Sex (% women)	53	57	19	17	<0.0001	NS	NS
Age (years)	58 ± 8	59 ± 9	63 ± 7	65 ± 6	<0.0001	NS	NS
BMI (kg/m^2)	25.7 ± 3.9	28. 5 ± 3.2	27.6 ± 4.5	28.6 ± 4.2	NS	0.0006	NS
Waist girth (cm)	90 ± 18	97 ± 21	93 ± 27	100 ± 20	NS	0.0225	NS
HbA_{1c} (%)	5.3 ± 0.5	6.3 ± 1.1	5.4 ± 0.5	6.7 ± 1.1	0.0003	<0.0001	NS
Glucose (mmol/L)	5.29 ± 1.37	7.02 ± 3.90	5.47 ± 1.83	7.38 ± 3.47	<0.0001	0.0492	NS
OHA/insulin (n)	—	22/3	—	26/3	—	—	—
Familial IHD (%)	29	75	33	50	NS	NS	NS
Hypertension (%)	41	89	60	94	0.0007	<0.0001	NS
Hypercholesterolemia (%)	48	89	55	94	NS	<0.0001	NS
Current smoking (%)	18	39	33	36	NS	0.0338	NS
Cigarettes (n/day)	2.4 ± 5.8	8.8 ± 12.4	5.6 ± 10.0	5.9 ± 14.1	NS	0.0031	0.0072
Systolic BP (mmHg)	126 ± 14	132 ± 18	132 ± 15	136 ± 20	0.0082	0.0108	NS
Diastolic BP (mmHg)	78 ± 8	79 ± 10	81 ± 7	81 ± 8	0.0386	NS	NS
eGFR (mL · min^{-1} · 1.73 m^{-2})	91 ± 12	89 ± 13	85 ± 16	88 ± 14	0.0011	NS	NS
HDL-C (mg/dL)	55 ± 16	46 ± 14	45 ± 12	46 ± 11	0.0013	0.0048	NS
Triglycerides (mg/dL)	105 ± 76	176 ± 129	124 ± 69	134 ± 69	NS	0.0001	0.0041
Non–HDL-C (mg/dL)	148 ± 40	137 ± 38	147 ± 40	121 ± 36	NS	0.0008	NS
hs-CRP (mg/dL)	0.26 ± 0.40	0.41 ± 0.38	0.42 ± 0.70	0.61 ± 1.00	0.0202	0.0241	NS
Troponin T (ng/L)	5.12 ± 3.87	6.05 ± 3.49	8.54 ± 9.97	8.91 ± 5.10	0.0002	NS	NS
β-Blockers, n (%)	67 (21)	12 (43)	34 (24)	17 (47)	NS	0.0009	NS
ACE inhibitors, n (%)	40 (13)	10 (36)	31 (22)	16 (44)	<0.05	0.0002	NS
ARBs, n (%)	31 (10)	7 (25)	28 (19)	11 (31)	NS	0.0210	NS
Statins, n (%)	64 (20)	17 (61)	42 (29)	27 (75)	0.0417	<0.0001	NS
Diuretics, n (%)	20 (6)	3 (11)	16 (11)	6 (17)	NS	NS	NS

Data are mean ± SD unless otherwise indicated. eGFR, estimated glomerular filtration rate; HDL-C, HDL cholesterol; IHD, ischemic heart disease; OHA, oral hypoglycemic agents. #P^1 CAD, effect of CAD; P^2 T2D, effect of diabetes; $P^1 \times P^2$, T2D × CAD interaction.

The results of the proteomic analysis are given in Table 2 for the 20 top-ranking proteins selected by the two-way PLS procedure. Fifteen of them were associated with both T2D and CAD (with a significant T2D × CAD interaction for eight of them), while three proteins (osteomodulin, cartilage intermediate-layer protein 2, and serine protease HTRA1) were associated only with T2D, and two other proteins (epidermal growth factor receptor [EGFR] and contactin-1) were selectively associated with CAD. By way of example, Fig. 1 plots the median values of four of the proteins with the highest VIP score; in participants with T2D and CAD, renin levels were more than double—and adiponectin levels were ~30% reduced—compared with participants with NGT without CAD. Of note, renin levels were significantly lower in women than in men and were directly related to age; yet, in a multivariate regression model adjusting for sex and age, renin was still increased in association with both T2D and CAD. Use of ACE inhibitors or ARBs also was independently associated with increased renin signal, yet inclusion in the model of these treatments (or statin, diuretic, and β-blocker treatment) as additional covariates did not change the association of renin with T2D and CAD. Likewise, adiponectin levels were higher in women and increased with age but were significantly reduced in both T2D and CAD (Supplementary Table 1). The physiological relevance of the observed proteomic pattern is supported by individual-level data. For example, one of the proteins uniquely associated with CAD, EGFR, was reciprocally related to hs-CRP and troponin T concentrations (i.e., established CAD markers) (Fig. 2). Likewise, one of the proteins selectively related to T2D, serine protease HTRA1, was directly associated with suboptimal glycemic control, as indexed by HbA_{1c}, and serum triglycerides. Of note, myocardial mass, extent and severity of coronary atherosclerotic involvement (as indexed by lumen stenosis >70% and segment stenosis score), and plaque quality (as indexed by the segment involvement score, plaque volume and length, remodeling index, low-attenuation plaque, spotty calcifications, and noncalcified fibrofatty plaque)—as resolved by CCTA—did not differ between patients with and without T2D (Supplementary Table 2).

In a multivariate logistic model adjusting for the Framingham risk factors (sex, age, hypertension, hypercholesterolemia, and smoking), patients with T2D were "protected" from CAD if they were female (odds ratio [OR] 0.19 [95% CI 0.04–0.70]), younger (OR_{SD} 0.12 [0.012–0.99]), and had lower plasma renin levels ($OR_{ln[F.I.]}$ 0.25 [0.07–0.71]).

CONCLUSIONS

To the best of our knowledge, the current study is the first to investigate ~5,000 plasma proteins in relation to the presence/absence of T2D and CAD and in relation to clinical, metabolic, and angiographic phenotyping. The main conclusion

Table 2—Serum proteins with a VIP >3.8

	CAD$^-$		CAD$^+$				
	NGT	T2D	NGT	T2D	P^1 T2D*	P^2 CAD*	$P^1 \times P^2$*
Growth differentiation factor 15	19.6 ± 7.6	32.5 ± 13.8	24.3 ± 9.5	33.6 ± 12.2	<0.001	<0.001	NS
Renin	15.8 ± 9.6	18.2 ± 11.7	20.7 ± 12.1	35.5 ± 20.5	<0.001	<0.001	0.004
Heparan sulfate 6-*O*-sulfotransferase	1.31 ± 0.21	1.17 ± 0.20	1.18 ± 0.20	1.10 ± 0.18	<0.001	<0.001	NS
Matrix-remodeling–associated protein 8	0.54 ± 0.12	0.43 ± 0.97	0.47 ± 0.10	0.43 ± 0.10	<0.001	NS	0.013
Adiponectin	3.4 ± 1.4	2.4 ± 0.7	2.7 ± 1.1	2.3 ± 0.8	<0.001	0.004	NS
Chondroadherin	8.3 ± 0. 2.6	6.4 ± 2.2	7.4 ± 2.4	5.6 ± 1.6	<0.001	0.013	NS
Osteomodulin	8.4 ± 2.9	5.7 ± 2.1	7.3 ± 2.7	5.6 ± 1.9	<0.001	NS	NS
Cartilage intermediate-layer protein 2	2.2 ± 0.7	1.6 ± 0.5	1.9 ± 0.6	1.6 ± 0.5	<0.001	NS	NS
Serine protease HTRA1	12.0 ± 2.7	14.8 ± 3.2	13.2 ± 3.2	14.5 ± 0.3	<0.001	NS	NS
EGFR	16.1 ± 2.4	15.0 ± 2.3	14.6 ± 2.3	14.4 ± 2.3	NS	0.001	NS
Leucine-rich repeat-containing protein 15	1.3 ± 0.4	1.0 ± 0.3	1.2 ± 0.4	0.98 ± 0.28	<0.001	NS	0.008
Neurocan core protein	4.0 ± 1.5	2.8 ± 1.2	3.3 ± 1.2	3.1 ± 1.1	<0.001	NS	0.008
Anthrax toxin receptor 2	1.6 ± 0.5	1.3 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	0.008	NS	0.007
Tetranectin	4.8 ± 0.7	4.5 ± 0.6	4.5 ± 0.6	4.2 ± 0.5	0.002	<0.001	NS
IGF-binding protein complex subunit	28.8 ± 5.1	26.0 ± 4.9	26.5 ± 5.4	24.6 ± 7.2	<0.001	0.004	NS
Contactin-1	32.8 ± 6.6	30.9 ± 5.9	29.6 ± 5.1	28.4 ± 4.6	NS	0.001	NS
Receptor-type tyrosine-protein phosphatase	5.6 ± 1.8	4.6 ± 1.3	4.7 ± 1.4	4.7 ± 1.3	0.015	NS	0.029
Protein FAM177A1	2.8 ± 0.7	2.6 ± 0.7	2.5 ± 0.7	2.2 ± 0.5	0.005	<0.001	NS
Coiled domain-containing protein 126	1.1 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.001	NS	0.004
Ubiquitin-conjugating enzyme E2 G2	3.2 ± 0.6	3.7 ± 1.0	3.5 ± 0.7	3.6 ± 0.9	0.002	NS	0.017

Data are F.I. units × 10^{-3}. *P^1 CAD, effect of CAD; P^2 T2D, effect of diabetes; $P^1 \times P^2$, T2D × CAD interaction.

is that the majority of "hit" proteins identified by bivariate PLS analysis are shared by the diabetes status and the presence of coronary lesions. Additionally, some interesting proteins are uniquely associated with T2D or CAD, suggesting diverging disease pathways and, possibly, different therapeutic targets.

Proteomics offer unique insights into disease pathways, as proteins are the effectors of all biological processes and their differential expression reflects the intricate interplay between environmental and genetic factors. In fact, the top-ranking proteins in our predictive model are involved in a broad spectrum of processes, including differentiation, extracellular matrix composition, metabolic signaling, and ubiquitination. Reflecting this, for instance, matrix-remodeling–associated protein 8, cartilage intermediate-layer protein 2, chondroadherin, leucine-rich repeat-containing protein 15, and HTRA1 are all engaged in pericellular microenvironment regulation. Moreover, some of the detected proteins are already known to have a pathogenetic role in several clinical conditions; for example, anthrax toxin receptor 2 gene mutation causes hyaline fibromatosis syndrome, a rare recessive autosomal disease. In addition, some of the proteins detected in the current study have already been studied in both CAD and T2D by investigations using traditional assay methods.

In our analysis, GDF15 emerged as the highest-ranking protein associated with both CAD and T2D (Fig. 1). GDF15 is a cytokine widely expressed in macrophages, vascular smooth muscle cells, adipocytes, cardiomyocytes, endothelial cells, fibroblasts, and brain tissues in response to inflammation, oxidative stress, and hypoxia. In patients with established CAD, GDF15 concentrations are independently associated with age, diabetes, smoking, hs-CRP levels, and renal impairment, all features of our CAD$^+$ subgroup. In addition—unlike cardiac troponin, hs-CRP, and natriuretic peptides—circulating GDF15 levels remain stable over time, therefore representing an optimal biomarker of chronic CAD burden. Among 14,577 patients with stable CAD participating in the Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy Trial (STABILITY), GDF15 was found to be independently associated with CV, non-CV, and cancer mortality. Furthermore, GDF15 concentrations were lowest in patients with NGT, intermediate in patients with prediabetes, and highest in patients with T2D (26). Strengthening this finding, the Malmö Diet and Cancer-Cardiovascular Cohort, in which 4,360 participants with normoglycemia were followed for 19 years, recently showed that GDF15 may be useful for the identification of people with a risk of incident diabetes (27). High GDF15 levels might be a target for further investigations and/or strengthen the indication for preventive measures.

The second-highest ranking protein was renin, with more than twofold higher levels in patients with both T2D and CAD than in participants with normoglycemia without CAD. Growing evidence points to plasma renin activity, and the attendant activation of the renin-angiotensin-aldosterone system (RAAS), as a marker of CV risk in patients with hypertension (28). Most drugs targeting the RAAS, either directly or indirectly, have shown a favorable effect in reducing morbidity and mortality of patients with CAD and/or heart failure (28). Moreover, in animal models of hypertension, direct renin inhibition improves insulin sensitivity and secretion, skeletal muscle glucose uptake, and glucose tolerance (29). Our data on lower renin levels in women than in men are in line with epidemiological studies, an effect attributed to estrogens and

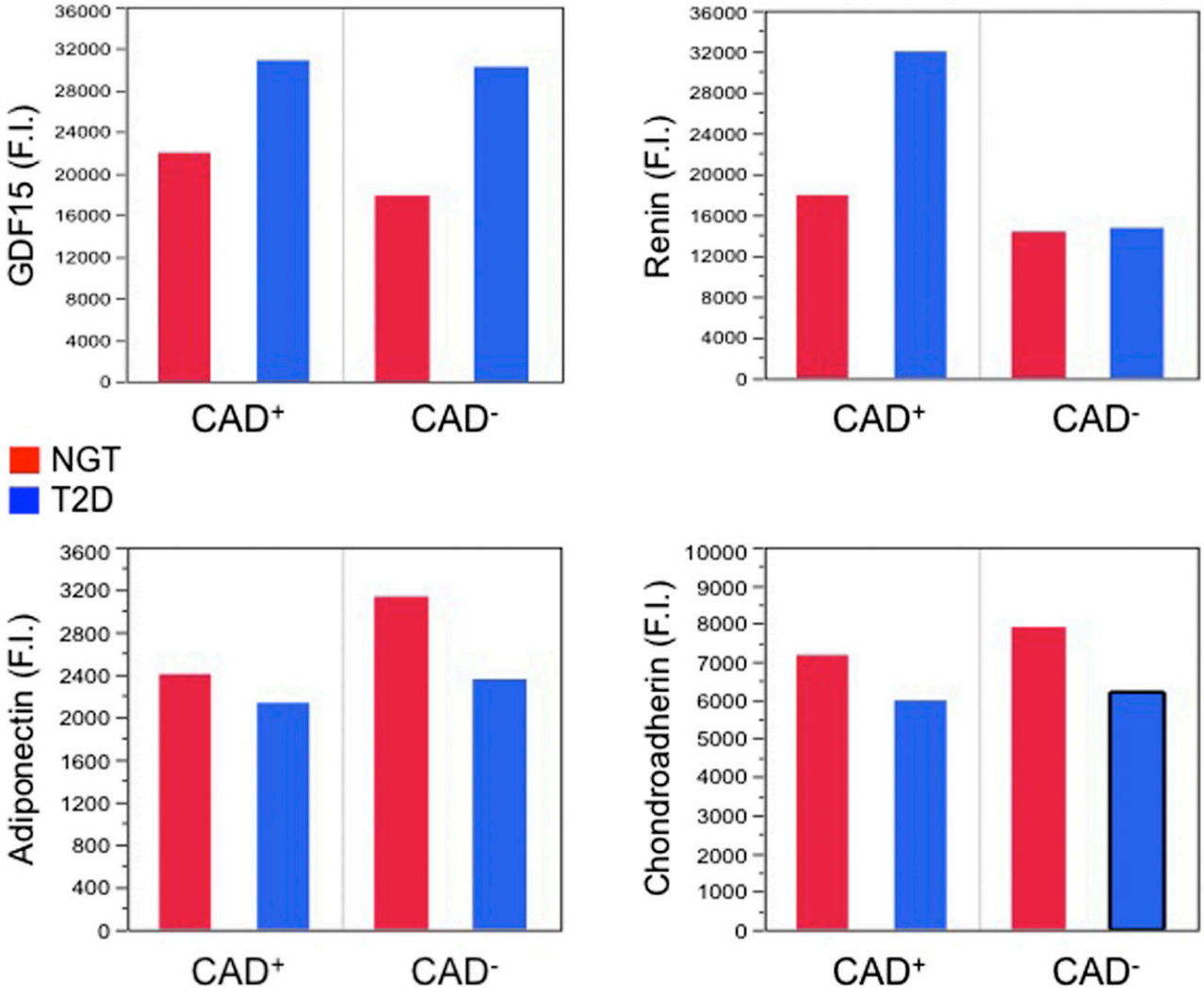

Figure 1—GDF15, renin, adiponectin, and chondroadherin signals in participants with T2D and participants with NGT with or without CAD. Bars are median group values. Statistical analysis is presented in Table 2.

associated with favorable lipid and glucose profiles, less visceral adipose tissue, and lower BP in premenopausal women (29). Consistent with the above, the current study identified a younger female with T2D but free of CAD. Importantly, the association of renin with T2D and CAD was independent of the use of RAAS blockers, which raise renin levels. In future studies, this finding should be confirmed by direct assay of plasma renin concentrations.

The inverse correlation between adiponectin and both CAD and T2D is consistent with substantial literature that has unraveled its antioxidant, anti-inflammatory, antiatherosclerotic, and insulin-sensitizing effects. Indeed, adiponectin is now considered a key protective regulator of vascular homeostasis, glucose metabolism, and lipid oxidation (30). Of note, meta-analyses have failed to clearly establish its predictive role in CAD, reflecting the multitude of factors that influence its circulating levels (30). To our knowledge, while the association between T2D and adiponectin is well established (31), no previous study has tested adiponectin against anatomical evidence of CAD as done here. Collectively, our data support a protective role of adiponectin against both CAD and T2D.

Encouragingly, one of the proteins selectively associated with T2D, serine protease HTRA1, correlated directly with both HbA_{1c} levels and serum triglycerides, which are hallmarks of the diabetic phenotype. With regard to its specific function, serine protease HTRA1 has been suggested to play a role in vascular abnormalities and angiogenesis (32). It is involved in the inhibition of transforming growth factor-β signaling, in programmed cell death, and in modulating the EGFR/Akt pathway (33). Moreover, this protease—protective against both CAD and diabetes in our data—contains an IGF-binding domain potentially interacting with other IGF-binding protein complex subunits, which also ranked high in our model. To the best of our knowledge, this is the first report linking serine protease HTRA1 with a clinical metabolic disease.

One of the proteins selectively associated with CAD is EGFR, a tyrosine kinase widely studied as a therapeutic target in cancer biology and more recently in relation to CV disease (34). However, the clinical impact of EGFR activation for cardiac function and atherogenesis remains to be elucidated with targeted clinical studies, since it acts as a

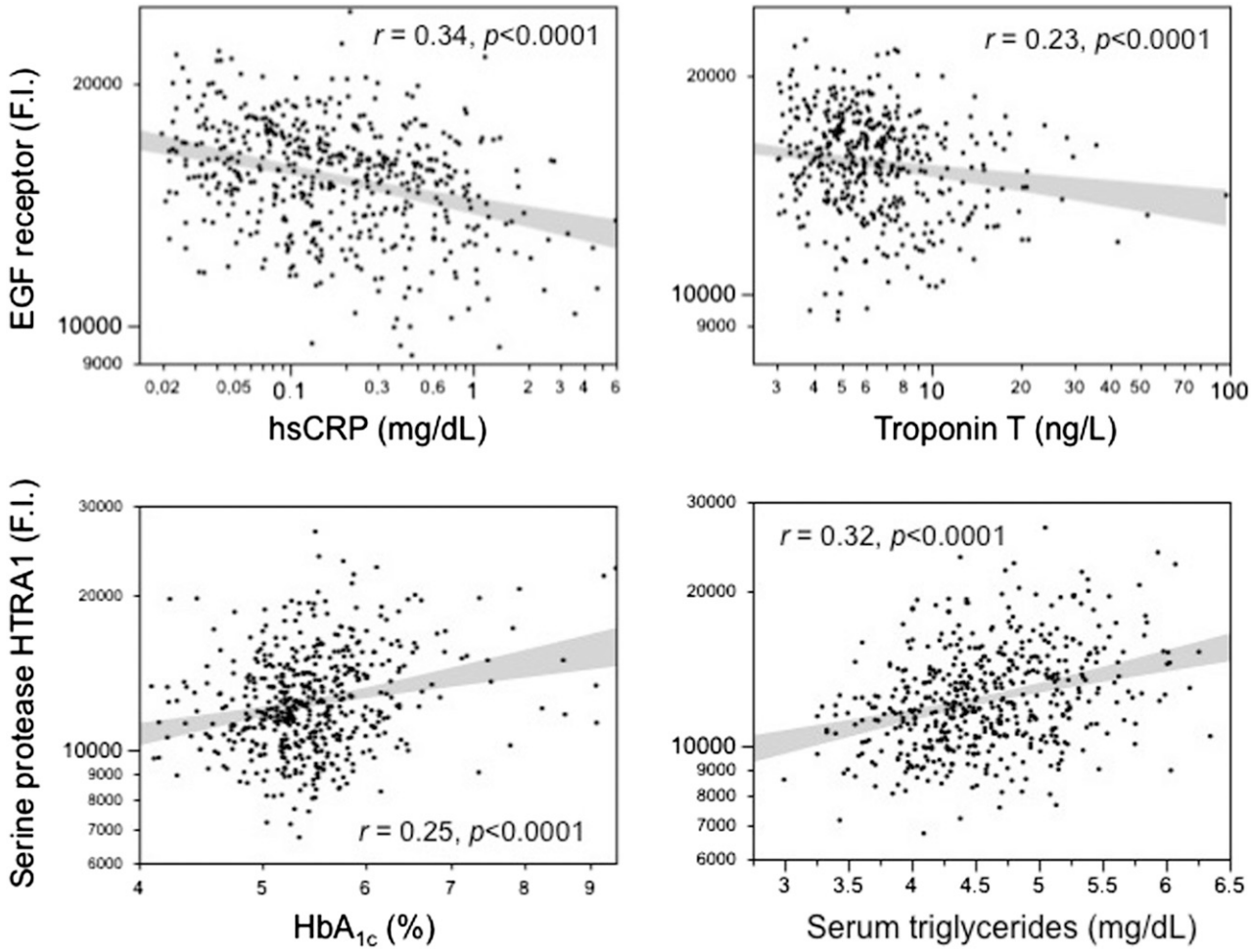

Figure 2—Association of EGFR with markers of CAD (hs-CRP and high-sensitivity cardiac troponin T) and of serine protease HTRA1 with markers of diabetes (HbA_{1c} and triglycerides).

crossroad to many signaling pathways and its levels are influenced by diverse concurrent factors (34,35). In our model, EGFR was found to be higher in the CAD^- group and, consistently, inversely related to hs-CRP and troponin T, well-established CAD markers. This association may contribute to improving our understanding of its role in clinical settings.

Tetranectin, which is actively involved in fibrinolysis as a plasminogen activation enhancer, has been suggested as a biomarker in stable and acute CAD, both in general populations and in T2D (36). Accordingly, in our screen, tetranectin was inversely associated with both T2D and CAD. Also, we found cartilage intermediate-layer protein 2, which may have a role in both cardiac extracellular matrix remodeling and insulin resistance (37). An altered level of enzyme heparan sulfate 6-*O*-sulfotransferase, the third highest-ranking protein in our model, previously showed a direct correlation with albuminuria in participants with diabetes (38).

To qualify our findings, it is important to consider that the T2D group included in CAPIRE is not a random sample of a typical T2D population—as the anthropometrics and metabolic characteristics would indicate—on several accounts. First, patients were selected for absence of previous CV events but presence of suspected CAD and an indication for CCTA. Second, patients with T2D had to be older than 45 years and in good glycemic control (as attested by their HbA_{1c} and fasting glucose levels); in fact, only six of them were on insulin treatment, the rest being on oral antihyperglycemic agents or diet alone. Finally, as participants were grouped into CAD^+ and CAD^- a posteriori (i.e., after the CCTA), the CV risk profile of patients with T2D with or without CAD was not very different (i.e., familial ischemic heart disease, hypertension, hypercholesterolemia, etc.) (Table 1). This has two corollaries. On the one hand, it helps justify the finding of similar quantitative and qualitative involvement of the coronary vasculature between patients with T2D and participants without diabetes (Supplementary Table 1); in less selected cohorts, coronary atherosclerosis, when present, is more diffuse and more severe in individuals with diabetes than in those without diabetes (39). On the other hand, individuals with T2D (and NGT) with a high CV risk profile who have reached their 60s with anatomically clean coronaries offer the opportunity to begin to search for potentially protective features. Efforts to phenotype study participants are key when performing proteomic studies, particularly with advanced methodology of

recent introduction, such as aptamer-based technology, for which there are limited data available for replication studies.

There are several limitations in our study. First, being a cross-sectional investigation, it cannot prove causality. Second, the number of participants is relatively small, especially in the T2D group. CAPIRE was not an intervention trial population but a highly selected cohort that was based on CCTA and designed to yield a sizeable number of outlier participants (i.e., at the opposite extremes for the presence of CAD and traditional CVRFs) with the specific purpose of generating hypotheses rather than hypothesis-driven outcomes. To achieve this goal, the anatomical phenotype of participants had to be described meticulously. Thus, CCTA was the main entry criterion.

Nonetheless, we believe this to be the first proteomic analysis of a T2D population in relation to coronary phenotyping; accuracy is supported by plausibility with published data and by significant individual-level data correlations. Our model highlights the potential role of GDF15, renin, serine protease HTRA1, EGFR, and other top-ranking proteins in the development of T2D and/or CAD. Further investigation, including specific mechanistic studies, is required to validate our findings. In addition, direct quantitation of the more promising protein biomarkers could contribute to a deeper understanding of their clinical significance and potential therapeutic implications. Also, follow-up events in CAPIRE will help with understanding whether screening proteomics can sizably improve CAD prediction in participants with and without diabetes or even reduce the indication for CCTA.

Funding. Funding was provided by the Heart Care Foundation of the Italian Association of Hospital Cardiologists, Florence, Italy.

Duality of Interest. F.A. reports receiving consultancy/speaker fees from Amgen, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb/Pfizer, and Daiichi Sankyo. A.P.M. reports receiving fees, outside the present work, from Bayer, Fresenius, and Novartis for participation in study committees. R.M.O. and S.A.W. are employees of SomaLogic Inc. E.F. reports receiving consultancy/speaker fees, outside the present work, from Boehringer Ingelheim, Eli Lilly, AstraZeneca, and Sanofi. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. G.F. and E.F. researched data and wrote the manuscript. M.L.M. carried out all statistical analyses. M.M., F.A., D.A., R.L., A.M., A.P.M., R.M.O., and S.A.W. reviewed/edited the manuscript and contributed to the discussion. R.L. supervised the routine laboratory analyses. R.M.O. and S.A.W. were responsible for the proteomics. E.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. World Health Organization. Global Health Estimates 2016: Disease Burden by Cause, Age, Sex, by Country and by Region, 2000–2016 [Internet], 2018. Available from https://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html. Accessed 20 January 2020

2. American Diabetes Association. 9. Cardiovascular disease and risk management: *Standards of Medical Care in Diabetes—2018*. Diabetes Care 2018;41(Suppl. 1):S86–S104

3. Mark DB, Berman DS, Budoff MJ, et al.; American College of Cardiology Foundation Task Force on Expert Consensus Documents. ACCF/ACR/AHA/NASCI/SAIP/SCAI/SCCT 2010 expert consensus document on coronary computed tomographic angiography: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents. Circulation 2010;121:2509–2543

4. Knuuti J, Wijns W, Saraste A, et al.; ESC Scientific Document Group. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes [published correction appears in Eur Heart J 2019:ehz825]. Eur Heart J 2019:ehz425

5. Fihn SD, Gardin JM, Abrams J, et al.; American College of Cardiology Foundation. 2012 ACCF/AHA/ACP/AATS/PCNA/SCAI/STS guideline for the diagnosis and management of patients with stable ischemic heart disease: executive summary: a report of the American College of Cardiology Foundation/American Heart Association task force on practice guidelines, and the American College of Physicians, American Association for Thoracic Surgery, Preventive Cardiovascular Nurses Association, Society for Cardiovascular Angiography and Interventions, and Society of Thoracic Surgeons. Circulation 2012;126:3097–3137

6. Mushtaq S, De Araujo Gonçalves P, Garcia-Garcia HM, et al. Long-term prognostic effect of coronary atherosclerotic burden: validation of the computed tomography-Leaman score. Circ Cardiovasc Imaging 2015;8:e002332

7. Budoff MJ, Raggi P, Beller GA, et al.; Imaging Council of the American College of Cardiology. Noninvasive cardiovascular risk assessment of the asymptomatic diabetic patient: the Imaging Council of the American College of Cardiology. JACC Cardiovasc Imaging 2016;9:176–192

8. Andreini D, Pontone G, Mushtaq S, et al. Prognostic value of multidetector computed tomography coronary angiography in diabetes: excellent long-term prognosis in patients with normal coronary arteries. Diabetes Care 2013;36:1834–1841

9. Piepoli MF, Hoes AW, Agewall S, et al.; ESC Scientific Document Group. 2016 European Guidelines on cardiovascular disease prevention in clinical practice: the Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts) Developed with the special contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR). Eur Heart J 2016;37:2315–2381

10. Oellgaard J, Gæde P, Rossing P, et al. Reduced risk of heart failure with intensified multifactorial intervention in individuals with type 2 diabetes and microalbuminuria: 21 years of follow-up in the randomised Steno-2 study. Diabetologia 2018;61:1724–1733

11. Rawshani A, Rawshani A, Franzén S, et al. Risk factors, mortality, and cardiovascular outcomes in patients with type 2 diabetes. N Engl J Med 2018;379:633–644

12. Rao Kondapally Seshasai S, Kaptoge S, Thompson A, et al.; Emerging Risk Factors Collaboration. Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med 2011;364:829–841

13. Sarwar N, Gao P, Seshasai SR, et al.; Emerging Risk Factors Collaboration. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies [published correction appears in Lancet 2010;376:958]. Lancet 2010;375:2215–2222

14. Lindsey ML, Mayr M, Gomes AV, et al.; American Heart Association Council on Functional Genomics and Translational Biology, Council on Cardiovascular Disease in the Young, Council on Clinical Cardiology, Council on Cardiovascular and Stroke Nursing, Council on Hypertension, and Stroke Council. Transformative impact of proteomics on cardiovascular health and disease: a scientific statement from the American Heart Association. Circulation 2015;132:852–872

15. Ganz P, Heidecker B, Hveem K, et al. Development and validation of a protein-based risk score for cardiovascular outcomes among patients with stable coronary heart disease. JAMA 2016;315:2532–2541

16. López-Villar E, Martos-Moreno GÁ, Chowen JA, Okada S, Kopchick JJ, Argente J. A proteomic approach to obesity and type 2 diabetes. J Cell Mol Med 2015;19:1455–1470

17. Magnoni M, Andreini D, Gorini M, et al.; CAPIRE Study Group. Coronary atherosclerosis in outlier subjects at the opposite extremes of traditional risk factors: rationale and preliminary results of the Coronary Atherosclerosis in outlier subjects: Protective and novel Individual Risk factors Evaluation (CAPIRE) study. Am Heart J 2016;173:18–26

18. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). JAMA 2001;285:2486–2497

19. Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery

Disease, Council on Cardiovascular Surgery, American Heart Association. Circulation 1975; 51(Suppl.):5–40
20. Andreini D, Magnoni M, Conte E, et al.; CAPIRE Investigators. Coronary plaque features on CTA can identify patients at increased risk of cardiovascular events [published ahead of print on 14 August 2019]. JACC Cardiovasc Imaging DOI: 10.1016/j.jcmg.2019.06.019
21. Andreini D, Pontone G, Mushtaq S, et al. A long-term prognostic value of coronary CT angiography in suspected coronary artery disease. JACC Cardiovasc Imaging 2012;5:690–701
22. Min JK, Dunning A, Lin FY, et al.; CONFIRM Investigators. Age- and sex-related differences in all-cause mortality risk based on coronary computed tomography angiography findings results from the International Multicenter CONFIRM (Coronary CT Angiography Evaluation for Clinical Outcomes: an International Multicenter Registry) of 23,854 patients without known coronary artery disease. J Am Coll Cardiol 2011;58:849–860
23. Gold L, Ayers D, Bertino J, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. PLoS One 2010;5:e15004
24. Vaught JD, Bock C, Carter J, et al. Expanding the chemistry of DNA for in vitro selection. J Am Chem Soc 2010;132:4141–4151
25. Palermo G, Piraino P, Zucht H-D. Performance of PLS regression coefficients in selecting variables for each response of a multivariate PLS for omics-type data. Adv Appl Bioinform Chem 2009;2:57–70
26. Desmedt S, Desmedt V, De Vos L, Delanghe JR, Speeckaert R, Speeckaert MM. Growth differentiation factor 15: a novel biomarker with high clinical potential. Crit Rev Clin Lab Sci 2019; 56:333–350
27. Bao X, Borné Y, Muhammad IF, et al. Growth differentiation factor 15 is positively associated with incidence of diabetes mellitus: the Malmö Diet and Cancer-Cardiovascular Cohort. Diabetologia 2019;62:78–86
28. Bangalore S, Fakheri R, Wandel S, Toklu B, Wandel J, Messerli FH. Renin angiotensin system inhibitors for patients with stable coronary artery disease without heart failure: systematic review and meta-analysis of randomized trials. BMJ 2017;356:j4
29. White MC, Fleeman R, Arnold AC. Sex differences in the metabolic effects of the renin-angiotensin system. Biol Sex Differ 2019;10:31
30. Woodward L, Akoumianakis I, Antoniades C. Unravelling the adiponectin paradox: novel roles of adiponectin in the regulation of cardiovascular disease. Br J Pharmacol 2017;174:4007–4020
31. Li S, Shin HJ, Ding EL, van Dam RM. Adiponectin levels and risk of type 2 diabetes: a systematic review and meta-analysis. JAMA 2009; 302:179–188
32. Klose R, Adam MG, Weis E-M, et al. Inactivation of the serine protease HTRA1 inhibits tumor growth by deregulating angiogenesis. Oncogene 2018;37:4260–4272
33. Skorko-Glonek J, Zurawa-Janicka D, Koper T, et al. HtrA protease family as therapeutic targets. Curr Pharm Des 2013;19:977–1009
34. Forrester SJ, Kawai T, O'Brien S, Thomas W, Harris RC, Eguchi S. Epidermal growth factor receptor transactivation: mechanisms, pathophysiology, and potential therapies in the cardiovascular system. Annu Rev Pharmacol Toxicol 2016;56:627–653
35. Makki N, Thiel KW, Miller FJ Jr. The epidermal growth factor receptor and its ligands in cardiovascular disease. Int J Mol Sci 2013;14:20597–20613
36. Rahim MAA, Rahim ZHA, Ahmad WAW, Bakri MM, Ismail MD, Hashim OH. Inverse changes in plasma tetranectin and titin levels in patients with type 2 diabetes mellitus: a potential predictor of acute myocardial infarction? Acta Pharmacol Sin 2018;39:1197–1207
37. Wu T, Zhang Q, Wu S, et al. CILP-2 is a novel secreted protein and associated with insulin resistance. J Mol Cell Biol 2019;11:1083–1094
38. Teumer A, Tin A, Sorice R, et al.; DCCT/EDIC. Genome-wide association studies identify genetic loci associated with albuminuria in diabetes. Diabetes 2016;65:803–817
39. Natali A, Vichi S, Landi P, Severi S, L'Abbate A, Ferrannini E. Coronary atherosclerosis in Type II diabetes: angiographic findings and clinical outcome. Diabetologia 2000;43:632–641