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ARTICLE

Cathepsin D improves the prediction of undetected diabetes in patients with myocardial infarction

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ABSTRACT

Background: Newer therapeutic agents for type 2 diabetes mellitus can improve cardiovascular outcomes, but diabetes remains underdiagnosed in patients with myocardial infarction (MI). We sought to identify proteomic markers of undetected dysglycaemia (impaired fasting glucose, impaired glucose tolerance, or diabetes mellitus) to improve the identification of patients at highest risk for diabetes.

Materials and methods: In this prospective cohort, 626 patients without known diabetes underwent oral glucose tolerance testing (OGTT) during admission for MI. Proximity extension assay was used to measure 81 biomarkers. Multivariable logistic regression, adjusting for risk factors, was used to evaluate the association of biomarkers with dysglycaemia. Subsequently, lasso regression was performed in a 2/3 training set to identify proteomic biomarkers with prognostic value for dysglycaemia, when added to risk factors, fasting plasma glucose, and glycated haemoglobin A1c. Determination of discriminatory ability was performed in a 1/3 test set.

Results: In total, 401/626 patients (64.1%) met the criteria for dysglycaemia. Using multivariable logistic regression, cathepsin D had the strongest association with dysglycaemia. Lasso regression selected seven markers, including cathepsin D, that improved prediction of dysglycaemia (area under the receiver operator curve [AUC] 0.848 increased to 0.863). In patients with normal fasting plasma glucose, only cathepsin D was selected (AUC 0.699 increased to 0.704).

Conclusions: Newly detected dysglycaemia, including manifest diabetes, is common in patients with acute MI. Cathepsin D improved the prediction of dysglycaemia, which may be helpful in the a priori risk determination of diabetes as a motivation for confirmatory OGTT.

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Introduction

Cardiovascular disease is a common complication to type 2 diabetes mellitus (DM) (1). Newer therapeutic agents can reduce progression of prediabetes to DM (2). Moreover, these agents have been shown to improve cardiovascular outcomes in patients with manifest DM and high risk for cardiovascular disease, giving renewed incentive to the identification of dysglycaemia in patients with acute myocardial infarction (3,4). European guidelines recommend screening for DM with fasting plasma glucose (FPG) and glycated haemoglobin A1c (HbA1c) (1). In case of continued uncertainty, an oral glucose tolerance test (OGTT) can be offered. OGTT is the only clinical method to identify impaired glucose tolerance and DM in patients with normal FPG. Impaired glucose tolerance and DM are strongly associated with cardiovascular outcome in patients with myocardial infarction (1,5–7). The use of OGTT during hospitalization for acute myocardial infarction has been shown to be reliably related to long-term glucometabolic state (8). However, OGTT is more time-consuming and more expensive than screening with FPG and HbA1c, which may limit its use in clinical practice.

Because the pathophysiological processes of progressive insulin resistance in muscle and liver, pancreatic beta-cell insufficiency, as well as glucolipotoxicity are present long before the onset of manifest DM, measurement of biomarker molecules of these processes may enable earlier diagnosis of dysglycaemia and DM (9). The proximity extension assay, a highly specific proteomics technology, facilitates the identification of novel protein markers as it allows the simultaneous

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Supplemental data for this article can be accessed here.

measurement of large amounts of proteins in small biological samples (10).

The goal of the current study was to use proximity extension assay to identify proteomic biomarkers of dysglycaemia (impaired fasting glucose, impaired glucose tolerance, or DM) in patients admitted with myocardial infarction, and to investigate whether these markers can be used to predict pathological outcome of OGTT in the normoglycaemic subset of patients.

Materials and methods

Cohort characteristics

The Västmanland Myocardial Infarction Study (VaMIS; ClinicalTrials.gov identifier: NCT01452178) is a cohort of consecutive patients admitted for acute myocardial infarction in Västmanland County Hospital, Västerås, Sweden from November 2005 to May 2011 (11). Of 1459 patients admitted with acute myocardial infarction, 201 patients in whom blood samples for biomarker analyses were obtained >72 h after admission were excluded. Other exclusion criteria were dementia or confusion ($n = 81$), other severe diseases ($n = 62$), linguistic difficulties ($n = 57$), or declining participation ($n = 50$), resulting in a final cohort of 1008 patients. For the current analysis, only patients not previously known to suffer from diabetes mellitus with available FPG and 2-h post glucose load values were selected ($n = 626$).

Myocardial infarction was defined according to guidelines at the time of inclusion: a typical rise and/or fall in cardiac troponin I exceeding 0.4 mg/L in combination with ischemic symptoms, new pathological Q waves (evidence of loss of electrically functioning cardiac tissue), ST-segment elevation or depression, or a coronary intervention (12). Written informed consent was obtained from all participants. The Ethics Committee of Uppsala University, Sweden approved the study (Protocol number 2005:169). The study was performed in accordance with the Declaration of Helsinki.

Definition of dysglycaemia

OGTT was performed a median of 3 days (interquartile range 3–5) after admission to the coronary care unit. Levels of FPG and plasma glucose 2 h after oral administration of 75 g glucose were measured from capillary blood with HemoCue 201+ (HemoCue AB, Ängelholm, Sweden). The binary variable 'dysglycaemia' was defined as impaired fasting glucose, impaired glucose tolerance, or DM according to World Health Organization definitions for capillary blood samples (13). Thus, patients met the criteria for dysglycaemia if fasting capillary plasma glucose was ≥6.1 mmol/L or if capillary plasma glucose 2 h after OGTT was ≥8.9 mmol/L.

Blood sampling

Blood samples were taken in 5 mL lithium heparin-coated vacuum tubes. The tubes were centrifuged at 2000 *g* for 10 min (Becton Dickinson and Co., Franklin Lakes, NJ, USA) or 2200 *g* for 10 min (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) at room temperature. Plasma was then reallocated to 5 ml plastic tubes and frozen at −70 °C within 2 h. The plasma samples were stored at −70 °C until analysis. Before analysis, the samples were thawed at room temperature, mixed and centrifuged at 3470 *g* at 4 °C for 15 min, and aliquoted into a microtitre plate using a pipetting robot, the Tecan Freedom EVOlyzer (Tecan, Männedorf, Switzerland).

Proteomics

Measurement of protein biomarkers in plasma was performed using the Olink Proseek Multiplex CVDI 96x96 (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, Uppsala, Sweden, as described previously (10). Of 92 biomarkers, 11 proteins in which <80% of patients had a valid measurement were removed from further analysis (heat shock 27 kDa protein, pappalysin-1, pentraxin-related protein PTX3, beta-nerve growth factor, magnetosome protein, P-selectin glycoprotein ligand 1, melusin, SIR2-like protein 2, interleukin-4, caspase 8, and natriuretic peptide B). The data were adjusted for plate effect to remove any influence of drift in measurements between plates. Values below the limit of detection were imputed as being the limit of detection normalized for the plate.

Statistics

Characteristics were summarized using frequencies and percentages for categorical variables and median and 25th and 75th percentiles for continuous variables. The first part of the analyses consisted of biomarker discovery with a mechanistic purpose. Biomarker discovery was performed using logistic regression models, assessing each biomarker separately with dysglycaemia as the dependent variable with adjustment for age and sex. Adjustment for multiple comparisons was performed with Bonferroni correction. Significant biomarkers were assessed in multivariable models including additional adjustment for smoking status (current smokers versus non-smokers), history of hypertension, family history of first-degree relatives with DM, body mass index, waist circumference, and storage time. Serum creatinine, FPG, and HbA1c were also considered as covariates but were excluded due to the risk of being collider-variables (Supplementary Figure 1, available online). Waist circumference and storage time of biomarkers remained in the models due to clinical relevance. For these analyses, missing values in covariates ($n = 36$ cases with missing values among covariates) were imputed into 20 datasets using multivariate imputation by chained equations with predictive mean matching, including the aforementioned covariates. Imputed values were compared with the recorded values to assess for aberrations. The multivariable models were pooled across the 20 imputed datasets according to Rubin's rule.

The second part of the analyses evaluated the prognostic value of proteomic markers. To assess whether adding

proteomic biomarkers to established biomarkers and risk factors improved prediction of dysglycaemia, the dataset was randomly split into a training (2/3) and a test set (1/3). Cases with missing values for the established risk factors and FPG or HbA1c were excluded prior to splitting ($n = 44$ for the total population), resulting in 582 eligible patients. In the training set, L1-regularized lasso regression was performed to identify the smallest selection of biomarkers to achieve a risk discrimination comparable to the whole assay, when added to risk factors (age, sex, current smoker, history of hypertension, family history of first-degree relatives with DM, body mass index, waist circumference, and storage time) and established biomarkers (FPG and HbA1c). These covariates were forced into the model, with subsequent ten-fold bootstrapped cross-validation. Discriminatory ability of the model applying the optimum sparse number of biomarkers was evaluated in the hold-out test set using the area under the receiver operator curve (AUC) and pseudo R^2. Likelihood ratio tests were performed to assess goodness of fit before and after addition of the proteomic markers. Finally, these analyses were also performed in a training set of the subset of patients with normal FPG (FPG <6.1 mmol/L), with determination of the discriminatory ability of the model in a test set. In the subset of 415 patients with normal FPG, 26 cases with missing values for the covariates were excluded prior to splitting, resulting in 389 eligible patients. Calculations were performed using IBM SPSS 24 (IBM Corp., Armonk, NY, USA) and R version 3.4.3 (R Foundation for Statistical Computing; 2016, Vienna, Austria).

Results

In total, 826 out of 1008 patients did not have previously known DM (81.9%), of which 626 underwent OGTT (75.8%) (Table 1). Of patients that underwent OGTT, 225 patients (35.9%) had normal glucose tolerance, 55 patients (8.8%) had isolated impaired fasting glucose, 215 patients (34.3%) had impaired glucose tolerance, and 131 patients (20.9%) met the criteria for DM (Table 2). Among the 415 patients with normal FPG, 225 (54.5%) had normal glucose tolerance, 150 (36.1%) had impaired glucose tolerance, and 40 (9.6%) met the criteria for DM.

Using individual logistic models including sex and age, nine biomarkers were significantly associated with dysglycaemia after correction for multiple comparisons. Four markers remained significant after additional multivariable adjustment (Table 3).

Prediction of dysglycaemia

The population was split into a training set, containing 388 patients. With age, sex, smoking status, history of hypertension, family history of first-degree relatives with DM, body mass index, waist circumference, storage time, and established biomarkers (FPG and HbA1c) forced into the model, L1-regularized lasso regression selected cystatin-B, cathepsin D, galanin peptides, galectin 3, interleukin-6 receptor subunit alpha, matrix metalloproteinase-1, and renin. In the training set of patients with normal FPG ($n = 259$), only cathepsin D was selected. Results of discriminatory ability testing for these models was performed in the test sets containing the remaining one-third of these populations (Table 4).

In the total population, the first quartile of cathepsin D (<−0.866), second (−0.866 to −0.205), third (−0.206 to 0.470), and fourth quartiles (>0.470) resulted in dysglycaemia rates of 47.4%, 61.9%, 65.2%, and 81.8%, respectively. Using

Table 1. Characteristics of patients according to availability of OGTT data.

	OGTT ($n = 626$)	No OGTT ($n = 200$)
Age, median years (IQR)	68 (17)	78 (18)
Male gender, % (*n*)	70.0 (438/626)	56.5 (113/200)
Body mass index, median kg/m^2 (IQR)	26.3 (5.1)	25.0 (5.5)
Waist, median cm (IQR)	96 (14)	95 (15)
Current smoker, % (*n*)	22.8 (143/626)	18.6 (37/199)
Hypertension, % (*n*)	49.0 (307/626)	58.0 (116/200)
Hyperlipidaemia, % (*n*)	25.4 (159/625)	21.6 (43/199)
First-degree relatives with diabetes mellitus, % (*n*)	23.7 (144/607)	18.5 (31/168)
Previous myocardial infarction, % (*n*)	18.8 (118/626)	26.5 (53/200)
Previous stroke, % (*n*)	5.1 (32/626)	13.0 (26/200)
Presentation with ST-elevation myocardial infarction, % (*n*)	37.1 (232/626)	27.5 (55/200)
HbA1c, median mmol/mol (IQR)	38 (5)	38 (6)
Serum creatinine, median μmol/L (IQR)	84 (27)	90 (37)
Fasting plasma glucose, median mmol/L (IQR)	5.7 (1.1)	6.0 (1.4)

Hypertension and hyperlipidaemia were determined according to patient history.
HbA1c: glycated haemoglobin A1c; IQR: interquartile range; OGTT: oral glucose tolerance testing.

Table 2. Patient characteristics according to outcome of OGTT.

	Normal glucose tolerance ($n = 225$)	Dysglycaemia ($n = 401$)
Age, median years (IQR)	65 (17)	70 (15)
Male gender, % (*n*)	74.2 (167/225)	67.6 (271/401)
Body mass index, median kg/m^2 (IQR)	26.2 (4.5)	26.3 (5.7)
Waist, median cm (IQR)	94 (12)	97 (14)
Current smoker, % (*n*)	28.0 (63/225)	20.0 (80/401)
Hypertension, % (*n*)	41.3 (93/225)	53.4 (214/401)
Hyperlipidaemia, % (*n*)	23.1 (52/225)	26.8 (107/400)
First-degree relatives with diabetes mellitus, % (*n*)	18.8 (41/218)	26.5 (103/389)
Previous myocardial infarction, % (*n*)	16.9 (38/225)	20.0 (80/401)
Previous stroke, % (*n*)	4.0 (9/225)	5.7 (23/401)
Presentation with ST-elevation myocardial infarction, % (*n*)	38.2 (86/225)	36.4 (146/401)
HbA1c, median mmol/mol (IQR)	36 (4)	39 (6)
Serum creatinine, median μmol/L (IQR)	82 (23)	86 (29)
Fasting plasma glucose, median mmol/L (IQR)	5.3 (0.6)	6.1 (1.1)

See Table 1 for abbreviations and definitions.

Table 3. Association of individual biomarkers with dysglycaemia after adjustment for clinical risk factors, age, and sex.

Biomarker	Odds ratio (95% CI)	*P* value
Cathepsin D	1.61 (1.32–1.97)	4.10×10^{-6}
Tumour necrosis factor-related apoptosis-inducing ligand	0.60 (0.48–0.75)	5.42×10^{-6}
Agouti-related protein	1.50 (1.21–1.87)	3.11×10^{-4}
Interleukin-6	1.45 (1.20–1.76)	1.70×10^{-4}

OR (95% CI) per SD increase in protein abundance.
Adjustment performed for age, sex, smoking status, history of hypertension, family history of first-degree relatives with DM, body mass index, waist circumference, and storage time.

Table 4. Performance of prediction models of dysglycaemia.

Total population, test set ($n = 194$)	AUC	Pseudo R^2	Likelihood ratio test, P value[a]
Clinical risk factors and FPG	0.846	0.433	–
Clinical risk factors and HbA1c	0.748	0.218	–
Clinical risk factors, FPG, and HbA1c	0.848	0.438	Reference
Clinical risk factors, FPG, HbA1c, and proteomic markers (cystatin-B, cathepsin D, galanin peptides, galectin 3, interleukin-6 receptor sub-unit alpha, matrix metalloproteinase-1, and renin)	0.863	0.469	5.34×10^{-4}
Patients with normal fasting plasma glucose, test set ($n = 130$)			
Clinical risk factors and FPG	0.687	0.151	–
Clinical risk factors and HbA1c	0.701	0.175	–
Clinical risk factors, FPG, and HbA1c	0.699	0.176	Reference
Clinical risk factors, FPG, HbA1c, and proteomic markers (cathepsin D)	0.704	0.190	1.22×10^{-3}

Clinical risk factors included in the model: age, sex, smoking status, history of hypertension, family history of first-degree relatives with DM, body mass index, waist circumference, and storage time.
[a]Likelihood ratio test assessing change in goodness of fit after addition of proteomic markers to model with clinical risk factors and established markers.
AUC: area under the receiver operator curve; FPG: fasting plasma glucose; HbA1c: glycated haemoglobin A1c.

the same increasing quartiles of cathepsin D, the rates of DM were 9.7%, 16.8%, 16.8%, and 39.6%. In the subset of patients with normal FPG, increasing quartiles of cathepsin D resulted in rates of dysglycaemia of 31.4%, 45.4%, 50.5%, and 63.2%, respectively. Rates of DM for increasing quartiles were 4.2%, 9.3%, 10.1%, and 18.4%.

Discussion

In this cohort of patients with acute myocardial infarction, newly detected dysglycaemia, including manifest DM, was highly prevalent. Multiplex proteomics identified several proteomic markers associated with previously undetected dysglycaemia in this high-risk population. Proteomic biomarkers provided an improved prediction of dysglycaemia when added to clinical risk factors, fasting plasma glucose, and HbA1c. Cathepsin D had the strongest association with dysglycaemia and was the only proteomic marker to predict dysglycaemia in patients with normal fasting plasma glucose. The highest and lowest quartiles of cathepsin D corresponded with high and low risk of DM, potentially aiding in the a-priori risk determination of DM as a motivation for confirmatory OGTT.

Cathepsin D is an aspartic endopeptidase with the primary biological function of protein degradation in an acidic milieu of lysosomes. It has been studied extensively from the perspective of its role in cancer development and as a suggested tumour marker (14). Furthermore, cathepsin D enzymatic activity induces hydrolytic modification of lipoprotein, including low-density lipoprotein, contributing to the accumulation of modified low-density lipoprotein in arterial intima (15). Higher levels of cathepsin D have been observed in diabetes complicated by diabetic ulcers and retinopathy (16,17). Cathepsin D has also been associated with insulin resistance in two community cohorts, although causality could not be shown with Mendelian randomization (18). Additionally, it has been suggested as a marker of β-cell function (19). Animal models support a role for cathepsin D in lysosomal/autophagic-induced cell death as a major driver of β-cell death and dysfunction in response to glucolipotoxicity in type 2 diabetes. In these models, the glucagon-like peptide 1 agonist exendin-4 was shown to protect β-cells from death by increasing autophagic flux and restoring lysosomal function (20). Clinical studies have shown that the glucagon-like peptide 1 agonist liraglutide as an adjunct to diet and exercise reduces the risk of progression from prediabetes to diabetes in obese patients, with improvement of measures of insulin resistance and β-cell function (2). Additionally, glucagon-like peptide 1 agonists reduce cardiovascular and overall mortality in patients with type 2 DM (3). Whether patients with higher cathepsin D levels have a larger benefit of glucagon-like peptide 1 agonists remains unknown.

Three other biomarkers (AgRP, TRAIL, and interleukin-6) were strongly associated with dysglycaemia in the current material. AgRP is a powerful orexigenic (appetite-inducing) peptide involved in the regulation of energy homeostasis. Acute activation of AgRP neurons causes insulin resistance through impairment of insulin-stimulated glucose uptake into brown adipose tissue. AgRP neurons integrate numerous signals of the periphery, including levels of glucose, insulin, and ghrelin (21). Inhibition of P2Y6 signalling in AgRP neurons has recently been shown to reduce food intake and improve systemic insulin sensitivity in obese mice, potentially providing a novel therapeutic target for the treatment of obesity and DM (22). To our knowledge, AgRP has not previously been associated with impaired glucose tolerance and DM in patients with myocardial infarction. TRAIL, a type II transmembrane protein and member of the tumour necrosis factor-ligand family, has been studied extensively in the setting of DM and obesity (23). Lower levels of TRAIL were associated with dysglycaemia, which is in accordance with a previous study comparing healthy controls with patients with newly detected but unmedicated diabetes. Initiation of treatment was found to elevate levels of TRAIL measured after 6 months (24). Interleukin-6 is a pleiotropic cytokine involved in the immune system, metabolism, and numerous other functions. It has previously been identified as a predictor of type 2 DM and associated cardiovascular events (25).

Proteomic markers improved the prediction of dysglycaemia, selecting cathepsin D, cystatin-B, galanin peptides, galectin-3, interleukin-6 receptor sub-unit alpha, matrix metalloproteinase-1, and renin. However, cathepsin D was the only useful proteomic marker for the prediction of dysglycaemia in patients with normal FPG. Indicated by the lower prediction model AUC, it is more difficult to predict a pathological OGTT in patients with normal FPG. Both HbA1c and FPG had limited performance in these patients, which is in accordance with previous observations (26). As such, OGTT remains an essential test to identify reduced glucose tolerance and DM in patients with myocardial infarction and normal FPG. However, higher quartiles of cathepsin D showed a consistent increasing prevalence of dysglycaemia and manifest DM, which may be helpful to identify patients at high a-priori risk of DM. As the proteomic technology does not permit an absolute quantification of the biomarkers, additional laboratory analyses of absolute values are needed to confirm these results, and to determine whether cathepsin D can be used to guide decision-making for the need of further confirmatory testing with OGTT. Increased precision of the measurement of cathepsin D could increase the predictive value. However, cathepsin D assays are currently limited by lack of standardization and are associated with higher costs than OGTT. Several other limitations should be noted. The current findings are based on a single cohort with limited size including Caucasian, predominantly male, patients with acute myocardial infarction. As such, findings need to be validated in other cohorts, as well as other settings such as in primary care. The proximity extension assay chip focused on proteins associated with cardiovascular disease and/or inflammation. An assay targeted directly at diabetes candidate proteins may have revealed additional findings. Another limitation is storage of plasma samples for up to 10 years before being analysed, so we cannot exclude the possibility that different protein stabilities might have influenced the analysis. Storage time is known to influence concentrations of certain proteins in proteomic analyses, but how this influenced the cathepsin D concentrations is unclear (27). However, the plasma samples were collected and stored consistently, which should have minimized any pre-analytical bias, and analyses were corrected for storage time. As the blood samples were taken within 72 h after acute myocardial infarction, we do not know to what extent, or which, biomarkers exhibited an acute phase expression.

In conclusion, dysglycaemia is very common in patients with acute MI, and proteomic biomarkers improve the prediction of undetected dysglycaemia over clinical risk factors and established biomarkers. Cathepsin D showed the most promise of these proteomic biomarkers due to its strong association with dysglycaemia and its predictive ability in patients with normal FPG.

Disclosure statement

E.H. has received institutional research grants from Amgen, Sanofi; consulting fees from Amgen, NovoNordisk, Sanofi; and speaker fees from AstraZeneca, Amgen, Boehringer Ingelheim, NovoNordisk, Sanofi. J.Ä. has received lecturing fees from AstraZeneca. The remaining authors report no conflicts of interest.

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