Plasma levels of the proprotein convertase furin and incidence of diabetes and mortality

● C. Fernandez[1] (iD), J. Rysä[2], P. Almgren[1], J. Nilsson[1] (iD), G. Engström[1], M. Orho-Melander[1], H. Ruskoaho[3] & O. Melander[1]

From the [1]Department of Clinical Sciences, Lund University, Malmö, Sweden; [2]Faculty of Health Sciences, School of Pharmacy, University of Eastern Finland, Kuopio; and [3]Division of Pharmacology and Pharmacotherapy, Drug Research Program, University of Helsinki, Helsinki, Finland

Abstract. Fernandez C, Rysä J, Almgren P, Nilsson J, Engström G, Orho-Melander M, Ruskoaho H, Melander O (Lund University, Malmö, Sweden; University of Eastern Finland, Finland; University of Helsinki, Helsinki, Finland). Plasma levels of the proprotein convertase furin and incidence of diabetes and mortality. *J Intern Med* 2018; **284**: 377–387.

Background. Diabetes mellitus is linked to premature mortality of virtually all causes. Furin is a proprotein convertase broadly involved in the maintenance of cellular homeostasis; however, little is known about its role in the development of diabetes mellitus and risk of premature mortality.

Objectives. To test if fasting plasma concentration of furin is associated with the development of diabetes mellitus and mortality.

Methods. Overnight fasted plasma furin levels were measured at baseline examination in 4678 individuals from the population-based prospective Malmö Diet and Cancer Study. We studied the relation of plasma furin levels with metabolic and hemodynamic traits. We used multivariable Cox proportional hazards models to investigate the association between baseline plasma furin levels and incidence of diabetes mellitus and mortality during 21.3–21.7 years follow-up.

Results. An association was observed between quartiles of furin concentration at baseline and body mass index, blood pressure and plasma concentration of glucose, insulin, LDL and HDL cholesterol ($|0.11| \leq \beta \leq |0.31|$, $P < 0.001$). Plasma furin (hazard ratio [HR] per one standard deviation increment of furin) was predictive of future diabetes mellitus (727 events; HR = 1.24, CI = 1.14–1.36, $P < 0.001$) after adjustment for age, sex, body mass index, systolic and diastolic blood pressure, use of antihypertensive treatment, alcohol intake and fasting plasma level of glucose, insulin and lipoproteins cholesterol. Furin was also independently related to the risk of all-cause mortality (1229 events; HR = 1.12, CI = 1.05–1.19, $P = 0.001$) after full multivariable adjustment.

Conclusion. Individuals with high plasma furin concentration have a pronounced dysmetabolic phenotype and elevated risk of diabetes mellitus and premature mortality.

Keywords: diabetes mellitus, furin, mortality, population-based cohort, risk prediction.

Introduction

Diabetes mellitus (DM) is associated with risk of premature mortality from vascular disease, several cancers and nonvascular causes not attributed to cancer, including digestive diseases, infectious diseases and neurological disorders, independently of major risk factors [1].

The manuscript has been handled by an external editor, Professor Sam Schulman, Thrombosis Service, McMaster Clinic, HHS – General Hospital 237, Barton Street East, Hamilton, ON, L8L 2X2, Canada.

Furin, a type-1 membrane-bound protease, belongs to the proprotein convertase subtilisin/kexin family (PCSK), which comprises of a total of nine members [2]. Furin cleaves numerous protein and peptide precursors trafficking through the secretory pathway and plays a pivotal role in several physiological processes [3]. Furin is expressed ubiquitously in all mammalian tissues and cell lines examined [4], but the level of expression differs being high in endocrine tissues, intermediate in the liver and gastrointestinal tract and very low in muscle tissues (www.proteinatlas.org) [5].

Previous evidence suggests a connection between furin and metabolic risk. A point mutation at the insulin pro-receptor processing site, later identified as a furin cleavage site [6], was reported in an individual suffering from extreme insulin-resistant diabetes and resulted in the absence of the pro-receptor processing [7]. An association between a polymorphism in the furin gene and decreased triglycerides and increased high-density lipoprotein cholesterol (HDL-C) serum concentration was recently established [8]. Moreover, in a recent epidemiological study, elevated circulating furin levels were demonstrated to be associated with a score for the metabolic syndrome as well as with increased BMI and blood triglyceride concentration [9]. However, there is limited knowledge about the role of furin in the development of DM and risk of premature mortality.

In this study, we measured fasting plasma levels of furin in a large population-based prospective cohort study from Malmö, Sweden and related circulating furin levels to incidence of DM, all-cause and cause-specific mortality during a long-term follow-up.

Materials & methods

Study participants and data collection

The Malmö Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28 449 individuals who attended a baseline examination between 1991 and 1994 [10]. From this cohort, 6103 people were randomly selected and asked to participate in a cardiovascular cohort (MDC-CC), which was designed to study the epidemiology of carotid artery disease [11]. At baseline, all the MDC-CC participants underwent a medical history, physical examination and laboratory assessment. Of the MDC-CC participants, fasting plasma samples were available in 4678 subjects for analysis of circulating furin. Participants with plasma samples had similar age (57.4 ± 5.9 vs. 57.4 ± 5.9; $P = 0.98$), LDL-C (4.2 ± 1.0 vs. 4.1 ± 1.0; $P = 0.51$) and use of anti-hypertensive therapy (16.5% vs. 16.7%, $P = 0.91$) compared to those who lacked plasma ($n = 1425$). However, there were some differences between these two groups in terms of gender (60% women vs. 50.5%, $P < 0.001$), BMI (25.7 ± 3.9 vs. 26.3 ± 4.1; $P < 0.001$), SBP (141.0 ± 18.9 vs. 142.8 ± 19.7; $P = 0.002$), HDL-C (1.4 ± 0.4 vs. 1.3 ± 0.4; $P < 0.001$), glucose (5.1 ± 1.3 vs. 5.5 ± 1.8; $P < 0.001$), prevalent DM (7.6% vs. 9.3%, $P < 0.001$) and smoking status (26% vs. 27%, $P < 0.001$).

Systolic (SBP) and diastolic blood pressure (DBP) were measured using a mercury-column sphygmomanometer after 10 min of rest in the supine position. Data on current smoking, use of anti-hypertensive treatment and physical activity during leisure time (PAL) were ascertained from a baseline questionnaire. Alcohol consumption was established from a 7-day food diary [12]. PAL was transformed into a score as previously described [12]. BMI was calculated as weight in kilograms divided by the square of the height in metres.

DM at baseline was defined as a fasting whole blood glucose ≥ 6.1 mmol L^{-1} or self-report of a physician diagnosis or use of diabetes medication.

All participants provided written informed consent, and the study was approved by the Ethics Committee at Lund University, Lund, Sweden.

Laboratory measurements

All laboratory assays were performed on overnight fasted blood samples obtained at the time of the baseline examination. Analyses of plasma lipids, insulin and whole blood glucose were performed according to standard procedures at the Department of Clinical Chemistry, Skåne University Hospital in Malmö. The levels of LDL cholesterol (LDL-C) were calculated according to the Friedewald formula.

Fasting plasma levels of furin were measured, together with 56 other proteins, using Olink Proseek Multiplex proximity extension assay (PEA) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala, Sweden. PEA uses two highly specific oligonucleotide labelled antibodies per protein, which allows the formation of a PCR reporter sequence when both antibodies are bound to the target protein's surface. This sequence is then quantified by real-time quantitative PCR [13]. A goat polyclonal furin antibody was used in the assay that in direct ELISA has less than 1% cross-reactivity with any other human protein. The intra and inter-assay variability for the assay were respectively 5% and 18% (https://www.olink.com/products/document-download-center/). Furin levels were successfully measured in all the samples. Data are presented as arbitrary units (AU).

Follow-up and end-points retrieval

Subjects were followed for first incidence of DM, coronary artery disease, as well as all-cause and cause-specific mortality until the 31st of December 2014. DM was defined as a fasting plasma glucose of ≥ 7.0 mmol L^{-1} or a history of physician diagnosis of DM or being on diabetes medication or having been registered in local or national diabetes registries [14]. Coronary artery disease was defined as coronary revascularization, fatal or nonfatal myocardial infarction, or death due to ischaemic heart disease. Mortality components were defined on the basis of the International Classification of disease – 9th revision (ICD-9) and the ICD-10 as follows: deaths from cancer, ICD-9 codes 140–239 and ICD-10 codes C00-D48; death from cardiovascular disease, ICD-9 codes 390–459 and ICD-10 codes I00-I99; death from other causes, ICD-9 codes 001–139, 240–389, 460–739 and ICD-10 codes A00-B99, D50-89, E00-H95, J00-R99, V00-Y99. End-points were retrieved through record linkage of the personal identification number of each Swedish citizen with Swedish local or national registries as previously described [14, 15].

Statistics

SPSS (version 22.0) was used for all statistical analyses. Due to non-normality, fasting plasma concentration of furin, glucose and insulin were transformed with the natural logarithm. All continuous variables were scaled to multiples of one standard deviation (SD) and centred on zero prior to statistical analysis.

Cross-sectional analyses were performed at baseline using linear regression models adjusting for age and sex with quartiles of furin levels as the independent variable and the respective standardized clinical parameter as dependent variable (i.e. BMI, fasting plasma concentrations of glucose, insulin, LDL-C and HDL-C, SBP, DBP, alcohol intake). The Pearson chi-square test was used to study the association between quartiles of baseline plasma furin concentration and the dichotomous categorical variables, gender, antihypertensive treatment, smoking status and physical activity score.

Kaplan–Meier survival curve was used to describe the rate of primary DM and all-cause of mortality outcome over time in quartiles of baseline furin levels. Multivariable Cox proportional hazards models were adopted to examine the association between fasting plasma levels of furin and each of the outcomes after removal of the prevalent cases for the specific end-point. Of the 4678 participants in whom furin was measured, those without complete data on covariates were excluded from the analyses. Hazard ratios (HRs) for furin were expressed per 1-SD increment of the natural logarithm of furin or per increasing quartiles of furin (quartiles 2 through 4 compared with quartile 1).

In analyses of incident DM, we adjusted in model 1 for age and sex and in model 2 for all the covariates demonstrating a statistically significant association with furin in the aforementioned cross-sectional analyses, which are presented in Table 1 (i.e. age, sex, BMI, SBP, DBP, use of antihypertensive treatment, alcohol intake and fasting plasma concentrations of glucose, insulin, LDL-C and HDL-C at baseline examination). In analyses of all-cause and cause-specific mortality and coronary artery disease incidence, we adjusted in model 1 for age and sex and in model 2 for all the cardiometabolic risk factors demonstrating an association with furin in Table 1 (i.e. age, sex, BMI, SBP, DBP, use of antihypertensive treatment, prevalent DM, alcohol intake and fasting plasma levels of LDL-C and HDL-C at baseline).

Tests for interactions were performed by including prevalent DM, standardized furin levels, and their multiplicative factor adjusting for age, sex, BMI, SBP, DBP, use of antihypertensive treatment, alcohol intake and fasting plasma levels of LDL-C and HDL-C at baseline.

A 2-sided *P* value of <0.05 was considered statistically significant.

Results

Association between plasma furin levels and clinical parameters

The baseline characteristics of the study participants are listed in Table 1. The fasting levels of plasma furin at baseline examination were associated significantly and very strongly with all metabolic (BMI, plasma concentrations of glucose, insulin, LDL-C and HDL-C) ($|0.11| \leq \beta \leq |0.31|$, $P < 0.001$) and hemodynamic (SBP, DBP, antihypertensive treatment) ($\beta = 0.15$, $P < 0.001$) traits investigated. Participants in the lowest quartile of furin were younger, leaner, had lower levels of

Table 1 *Clinical characteristics of the MDC-CC participants at baseline examination according to quartiles of furin*

	Furin Q1 4.6–6.1 (AU) ($n = 1169$)	Furin Q2 6.1–6.5 (AU) ($n = 1170$)	Furin Q3 6.5–6.8 (AU) ($n = 1170$)	Furin Q4 6.8–8.3 (AU) ($n = 1169$)	β (SE) or χ^2 (df)	P^a
Age (years)	55.71 ± 5.88	57.38 ± 5.78	58.19 ± 5.91	58.63 ± 5.80	0.96 ± 0.08	<0.001
Sex, No. (% women)	752 (64.3)	658 (56.2)	712 (60.9)	686 (58.7)	17.22 (3)	0.001
BMI (kg m^{-2})	23.89 ± 3.09	25.21 ± 3.35	25.97 ± 3.72	27.78 ± 4.36	0.31 ± 0.01	<0.001
Fasting glucose (mmol L^{-1})	4.82 ± 1.00	5.07 ± 1.41	5.16 ± 1.20	5.46 ± 1.42	0.18 ± 0.01	<0.001
Fasting insulin (μIU mL^{-1})	5.0 (3.0–7.0)	6.0 (4.0–8.0)	7.0 (4.0–9.0)	9.0 (6.0–13.0)	0.29 ± 0.01	<0.001
Fasting LDL-C (mmol L^{-1})	3.91 ± 0.93	4.17 ± 0.96	4.24 ± 0.97	4.35 ± 1.02	0.11 ± 0.01	<0.001
Fasting HDL-C (mmol L^{-1})	1.51 ± 0.38	1.41 ± 0.37	1.38 ± 0.37	1.27 ± 0.33	−0.20 ± 0.01	<0.001
SBP (mm Hg)	135.37 ± 17.99	138.56 ± 17.75	143.41 ± 19.16	146.60 ± 18.68	0.15 ± 0.01	<0.001
DBP (mm Hg)	84.51 ± 8.84	85.65 ± 8.79	87.51 ± 9.54	89.18 ± 9.17	0.15 ± 0.01	<0.001
Antihypertensive medication use, No. (%)	117 (10)	163 (13.9)	220 (18.8)	274 (23.4)	86.52 (3)	<0.001
Smoker, No. (%)	294 (25.1)	293 (25)	321 (27.5)	319 (27.4)	3.24 (3)	0.36
Prevalent diabetes, No. (%)	28 (2.4)	44 (3.8)	38 (3.2)	77 (6.6)	30.13 (3)	<0.001
Alcohol intake (g day^{-1})	9.73 ± 10.38	10.04 ± 11.67	10.22 ± 11.97	10.52 ± 13.31	0.035 ± 0.012	0.004
Physical activity, No. (%)^b	269 (24.4)	258 (23.8)	276 (25.9)	268 (25.6)	1.72 (3)	0.63

Values are displayed as mean ± SD, median and 25–75% interquartile range, or frequency in per cent.
β coefficients represent the standardized change of a clinical parameter per quartile of furin.
AU, arbitrary units; DBP, diastolic blood pressure; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; SBP, systolic blood pressure.
[a]*P* values were obtained from linear regressions adjusted for age and sex for the continuous variables or from Pearson chi-square tests for the binary variables. [b]Physical activity was defined as the top quartile (i.e. coded 1) versus the lowest three quartiles (i.e., coded 0) of the physical activity score.

fasting circulating glucose and insulin, a more favourable lipid profile and lower blood pressure compared with participants in the highest quartile of furin (Table 1).

Furin levels were elevated in the participants with DM at baseline ($n = 187$) compared to those without diabetes ($n = 4491$) (6.65 arbitrary units (AU) ± 0.5 vs. 6.46 (AU) ± 0.5 and $P < 0.001$).

Fig. 1 *Association between baseline furin levels and incidence of diabetes mellitus in the MDC-CC. The Kaplan–Meier plot shows cumulative percentages of diabetes mellitus events during follow-up according to the quartiles of fasting plasma furin levels at baseline examination (Q1; lowest and Q4; highest). The numbers at risk are shown at 5-year intervals.*
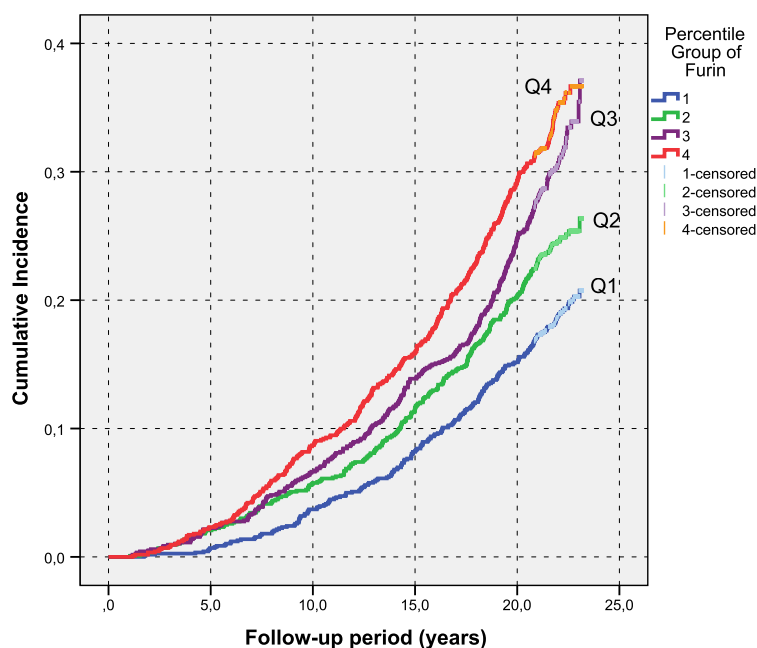
No. at risk					
First quartile	1141	1123	1083	1012	894
Second quartile	1126	1082	1021	922	794
Third quartile	1132	1065	986	852	698
Fourth quartile	1091	990	872	716	544

Furin and risk of diabetes mellitus

Amongst 4490 participants free from DM at baseline examination, 750 developed new-onset DM during a median follow-up time of 21.3 years [25–75% interquartile range (IQR) 16.2–22.3]. The cumulative incidence rate of DM according to quartiles of baseline fasting plasma concentration of furin was higher for subjects in the upper quartiles (Fig. 1). A higher fasting value of furin (HR/SD increment of natural logarithm of fasting furin) was associated with increased risk of DM after age and sex adjustment (model 1) (HR = 1.82, CI = 1.68–1.96, *P* < 0.001) (Table 2). The association remained significant after further adjustment for BMI, SBP, DBP, use of antihypertensive treatment, alcohol intake and fasting plasma levels of glucose, insulin, LDL-C and HDL-C at baseline (model 2), with a multivariable-adjusted HR of 1.24 (95% CI, 1.14–1.36, *P* < 0.001) per one SD increment of furin for risk of future DM (Table 2). There was a linear relationship between plasma furin concentration and the risk of incident DM in both model 1 and 2 (*P* for trend < 0.001), and the top versus bottom quartile of furin in the fully adjusted

model was associated with a HR of 1.72 (95% CI, 1.33–2.23) (Table 2).

Furin and risk of all-cause mortality

A total of 1263 participants died during a median follow-up time of 21.7 years [IQR, 20.9–22.4]. The cumulative incidence rate of all-cause mortality in quartiles of baseline fasting plasma concentration of furin is presented in Fig. 2 and was higher for participants with high plasma concentration of furin. Increasing furin levels associated significantly with elevated all-cause mortality during follow-up after age and sex adjustment (model 1) (*P* < 0.001) (Table 3). After further adjustment for BMI, SBP, DBP, use of antihypertensive treatment, alcohol intake, prevalent diabetes mellitus and fasting plasma concentrations of LDL-C and HDL-C at baseline (model 2), the association remained significant (*P* = 0.001). Each one SD increase of baseline furin was associated with a multivariable-adjusted HR of 1.12 (95% CI, 1.05–1.19) and top versus bottom quartile of furin was associated with a HR of 1.37 (95% CI, 1.14–1.64, *P* for trend <0.001) for risk of all-cause mortality (Table 3).

Table 2 *Incidence of diabetes mellitus in relation to baseline levels of furin in the MDC-CC*

	Diabetes mellitus ($n = 750/3740$) Model 1[a]	Diabetes mellitus ($n = 727/3670$) Model 2[b]
Overall		
HR[c]	1.82 (1.68–1.96)	1.24 (1.14–1.36)
P-value	<0.001	<0.001
Quartiles		
HR[d] Q1	1.0 (referent)	1.0 (referent)
HR[d] Q2	1.50 (1.15–1.96)	1.21 (0.93–1.58)
HR[d] Q3	2.49 (1.95–3.18)	1.56 (1.21–2.02)
HR[d] Q4	4.38 (3.47–5.54)	1.72 (1.33–2.23)
P for trend[e]	<0.001	<0.001

[a]Model 1 was adjusted for age and sex. [b]Model 2 was adjusted for age, sex, BMI, systolic blood pressure, diastolic blood pressure, use of antihypertensive treatment, alcohol intake and fasting plasma levels of glucose, insulin, LDL cholesterol and HDL cholesterol at baseline. In model 2, individuals without complete data on covariates were excluded from the analysis. [c]Hazard Ratios (HR) are expressed per 1-SD increment of log-transformed furin. 95% confidence intervals of the HRs are reported in parenthesis. [d]Hazard Ratios (HR) are expressed as categories of furin quartiles using quartile 1 as reference. 95% confidence intervals of the HRs are reported in parenthesis. [e]*P* for trend across quartiles of furin in a linear regression model.

Furin and risk of cause-specific mortality

In the next step, we investigated how plasma furin levels relate to the main causes of death. After adjustment for baseline age and sex (model 1), each SD increment of furin was associated with HRs of 1.14 (95% CI, 1.04–1.24) for death from cancer, 1.31 (95% CI, 1.17–1.46) for death from cardiovascular causes and 1.13 (95% CI, 1.01–1.26) for death from noncardiovascular causes not attributed to cancer (Table 3). However, furin levels did not associate with total cancer incidence in an age and sex-adjusted model (1210 events; HR, 1.04; 95% CI, 0.98–1.10; *P* = 0.25).

These HRs were reduced after full adjustment for conventional risk factors for cardiovascular disease that is age, sex, BMI, SBP, DBP, use of antihypertensive treatment, alcohol intake, prevalent diabetes mellitus and fasting plasma concentrations of LDL-C and HDL-C at baseline (model 2). In the fully adjusted models, furin levels only remained significantly associated with cancer

mortality (HR = 1.13, 95% CI, 1.03–1.25, *P* = 0.013) (Table 3). In a next step, we explored whether plasma furin levels were associated with coronary artery disease incidence (545 events during a median follow-up time of 21.4 years [IQR, 18.6–22.3]). Each SD increment of furin was associated with a HR of 1.28 (95% CI, 1.17–1.40; *P* < 0.001) for the risk of future coronary artery disease adjusting for age and sex. However, after full adjustment for the conventional cardiovascular disease risk factors, this association was not statistically significant (530 events; HR, 1.10; 95% CI, 1.00–1.22; *P* = 0.05).

As furin levels were higher in the participants with DM at baseline examination, we tested if the aforementioned associations were dependent on the DM status at baseline. There was a significant interaction between prevalence of DM and furin levels on the outcomes of all-cause mortality, cardiovascular death and coronary artery disease ($P_{interaction}$ = 0.033, 0.007 and 0.008, respectively) but not for death from cancer and noncardiovascular noncancer cause ($P_{interaction}$ = 0.49 and 0.71, respectively). Stratifying for DM status at baseline showed that the positive association between furin and coronary artery disease remained significant after full adjustment in participants without DM despite a smaller sample size (Table 4) but not in subjects with DM at baseline (Table S1). Furthermore, the association between furin and cardiovascular mortality was strengthened in the fully adjusted model in participants without diabetes and reached statistical significance.

Discussion

To our knowledge, this is the first epidemiological study on fasting plasma concentration of furin in relation to risk of future DM and mortality. We here show that higher fasting plasma levels of furin in a middle-aged population were associated with increased incidence of DM as well as all-cause and cause-specific mortality during long-term follow-up.

Although furin is a membrane protein, earlier work indicates the existence of a secreted form of furin. It has been previously shown that cells transfected with a recombinant furin gene secrete a soluble truncated furin protein with similar enzymatic activity as the membrane-bound protein [16]. Furthermore, it is established that furin is partially shed from most cells [17]. Plasma furin concentration has also recently been measured in healthy
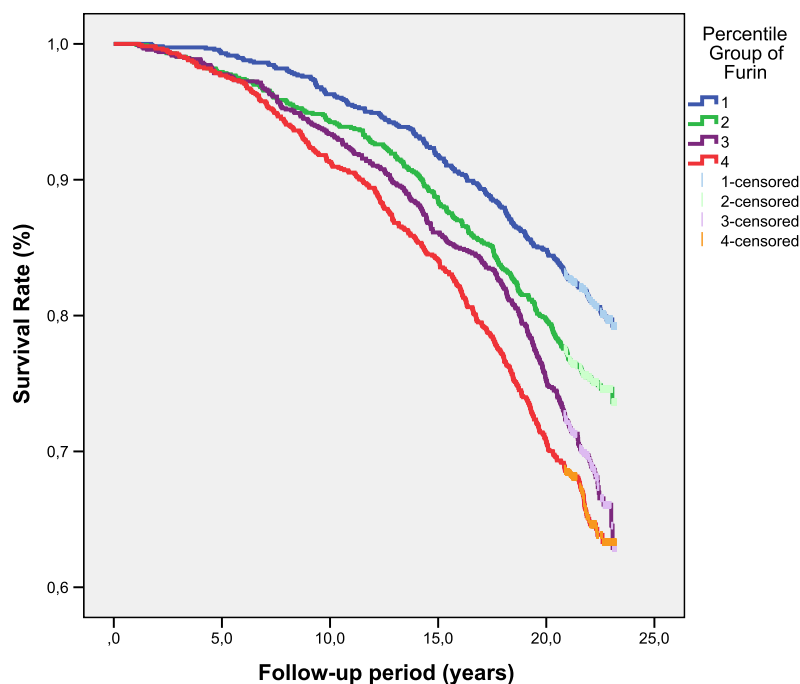
Fig. 2 *Association between baseline furin levels and all-cause mortality in the MDC-CC. The Kaplan–Meier plot shows the survival rate during follow-up according to the quartiles of fasting plasma furin levels at baseline examination (Q1; lowest and Q4; highest). Follow-up time is displayed at 5-year intervals.*

No. at risk					
First quartile	1161	1153	1118	1066	985
Second quartile	1163	1139	1096	1028	927
Third quartile	1160	1134	1083	999	872
Fourth quartile	1161	1134	1060	976	821

individuals in two small studies [9, 18]. Here, we were able to measure furin levels in the circulation in a large number of individuals from a middle-aged general population.

Consistent with our results, in a recent study of 138 participants from the Västerbotten Intervention Programme, a positive association between plasma furin levels and BMI was established [9]. We also identified a positive association between plasma furin levels and plasma concentrations of glucose, insulin and LDL-C and a negative association with HDL-C. The association between circulating furin and LDL-C could be mediated via PCSK9, as hepatic membrane-bound furin has been established as the key inactivating protease of PCSK9 [19]. We also showed a positive association between furin levels and both SBP and DBP, in line with previous findings showing an association between polymorphisms in the furin gene and blood pressure [20, 21].

A key finding of our study was that plasma furin levels associated with future DM independently of all traditional DM risk factors. Regarding potential mechanisms, as furin is responsible for the maturation of the insulin pro-receptor [6], one could speculate that more furin in the circulation reflects a compensatory mechanism to increase the synthesis of active insulin receptors. Another possible mechanism of action of furin in DM development may be via pancreatic β-cells as furin has been demonstrated to control the proliferation and differentiation of pancreatic β-cell lines and to be involved in the maturation of insulin secretory granules [22, 23].

We identified an elevation of furin several years before the onset of DM. Furthermore, furin levels remained elevated in overt DM, which is in agreement with a recent study where patients with DM had a tendency to higher furin plasma levels compared to healthy subjects [18]. Furthermore, the aforementioned study established that a strong increase in plasma furin concentration occurred in patients with both overt DM and cardiovascular disease compared to controls.

Because DM predicts premature mortality of virtually all causes [1], we tested in a next step if

Table 3 *Incidence of all-cause and cause-specific mortality in relation to baseline levels of furin in the MDC-CC*

	All-cause death (n = 1263/3382) Model 1[a]	All-cause death (n = 1229/3352) Model 2[b]	Cancer death (n = 542/4099) Model 1[a]	Cancer death (n = 527/4050) Model 2[b]	Cardiovascular death (n = 371/4272) Model 1[a]	Cardiovascular death (n = 361/4218) Model 2[b]	Non-cancer, Non-cardiovascular death (n = 347/4298) Model 1[a]	Non-cancer, Non-cardiovascular death (n = 338/4243) Model 2[b]
Overall								
HR[c]	1.18 (1.12–1.25)	1.12 (1.05–1.19)	1.14 (1.04–1.24)	1.13 (1.03–1.25)	1.31 (1.17–1.46)	1.12 (0.99–1.26)	1.13 (1.01–1.26)	1.09 (0.96–1.24)
P-value	<0.001	0.001	0.005	0.013	<0.001	0.08	0.032	0.17
Quartiles								
HR[d] Q1	1.0 (referent)	1.0 (referent)	1.0 (referent)	1.0 (referent)	1.0 (referent)	1.0 (referent)	1.0 (referent)	1.0 (referent)
HR[d] Q2	1.13 (0.95–1.35)	1.11 (0.93–1.33)	1.06 (0.82–1.38)	1.08 (0.82–1.41)	1.06 (0.75–1.50)	0.96 (0.67–1.36)	1.34 (0.97–1.85)	1.35 (0.97–1.88)
HR[d] Q3	1.39 (1.17–1.64)	1.29 (1.08–1.53)	1.32 (1.02–1.69)	1.32 (1.02–1.72)	1.69 (1.23–2.32)	1.34 (0.96–1.87)	1.22 (0.88–1.70)	1.16 (0.82–1.64)
HR[d] Q4	1.56 (1.32–1.84)	1.37 (1.14–1.64)	1.43 (1.12–1.83)	1.42 (1.08–1.86)	1.79 (1.31–2.45)	1.22 (0.87–1.73)	1.55 (1.13–2.13)	1.47 (1.04–2.08)
P for trend[e]	<0.001	<0.001	0.001	0.004	<0.001	0.09	0.018	0.09

[a]Model 1 for all-cause and cause-specific mortality was adjusted for age and sex. [b]Model 2 for all-cause and cause-specific mortality was adjusted for: age, sex, BMI, systolic blood pressure, diastolic blood pressure, use of antihypertensive treatment, alcohol intake, prevalent diabetes mellitus and fasting plasma concentrations of LDL cholesterol and HDL cholesterol at baseline. In model 2, individuals without complete data on covariates were excluded from the analysis. [c]Hazard Ratios (HR) are expressed per 1-SD increment of log-transformed furin. 95% confidence intervals of the HRs are reported in parenthesis. [d]Hazard Ratios (HR) are expressed as categories of furin quartiles using quartile 1 as reference. 95% confidence intervals of the HRs are reported in parenthesis. [e]*P* for trend across quartiles of furin in a linear regression model.

Table 4 *Incidence of cardiovascular disease-related mortality and of coronary artery disease in relation to baseline levels of furin in participants without diabetes*

	Cardiovascular death ($n = 301/3987$) Model 1[a]	Cardiovascular death ($n = 293/3940$) Model 2[b]	Coronary artery disease ($n = 459/3780$) Model 1[a]	Coronary artery disease ($n = 447/3737$) Model 2[b]
Overall				
HR[c]	1.31 (1.16–1.48)	1.19 (1.04–1.37)	1.28 (1.16–1.42)	1.17 (1.05–1.30)
P-value	<0.001	0.011	<0.001	0.005
Quartiles				
HR[d] Q1	1.0 (referent)	1.0 (referent)	1.0 (referent)	1.0 (referent)
HR[d] Q2	1.04 (0.71–1.52)	0.99 (0.67–1.45)	1.40 (1.06–1.86)	1.32 (0.99–1.76)
HR[d] Q3	1.81 (1.29–2.55)	1.55 (1.09–2.21)	1.55 (1.17–2.06)	1.31 (0.98–1.76)
HR[d] Q4	1.74 (1.23–2.47)	1.40 (0.96–2.04)	1.91 (1.45–2.53)	1.49 (1.10–2.01)
P for trend[e]	<0.001	0.015	<0.001	0.018

[a]Model 1 was adjusted for age and sex. [b]Model 2 was adjusted for: age, sex, BMI, systolic blood pressure, diastolic blood pressure, use of antihypertensive treatment, alcohol intake and fasting plasma concentrations of LDL cholesterol and HDL cholesterol at baseline. In model 2, individuals without complete data on covariates were excluded from the analysis. [c]Hazard Ratios (HR) are expressed per 1-SD increment of log-transformed furin. 95% confidence intervals of the HRs are reported in parenthesis. [d]Hazard Ratios (HR) are expressed as categories of furin quartiles using quartile 1 as reference. 95% confidence intervals of the HRs are reported in parenthesis. [e]*P* for trend across quartiles of furin in a linear regression model.

baseline plasma furin levels relate to death from any cause. Participants with elevated furin levels at baseline were observed to be at higher risk of death compared to individuals with lower furin levels. Furthermore, the association between circulating furin and total mortality was independent of conventional cardiovascular disease risk factors. This suggests that high fasting furin concentration is part of a prediabetic phenotype with high risk of premature mortality.

It is known from previous studies that furin is linked to the cardiovascular system. For instance, inactivation of the furin gene in mouse results in embryonic lethality and failure of the heart tube to fuse and undergo looping morphogenesis [24]. Furin has also been shown to be involved in human atherosclerosis as indicated by the increase of furin mRNA levels in atherosclerotic plaques [25]. Moreover, a recent study identified a genetic association between a polymorphism in the furin gene and risk of coronary artery disease [26]. We now took those findings further and identified a positive association between plasma furin levels and cardiovascular mortality and coronary artery disease. We could also show that the association between furin levels and cardiovascular mortality and coronary artery disease is dependent upon the DM status of the

participants. Furthermore, part of the relationship between plasma furin levels and cardiovascular mortality and coronary artery disease could be mediated through other risk factors for cardiovascular disease and/or confounded by these, as the associations were weakened after full adjustment for these risk factors, which in turn were all strongly correlated with furin levels.

Although it is also known that furin promotes many cancer-related processes including cell proliferation, migration and invasion [2, 27], we did not find an association with total cancer incidence. However, we report in the present study for the first time that increased plasma furin levels in a middle-aged cohort are associated with elevated cancer mortality during long-term follow-up. Furin's role is pluripotent [3], which is also demonstrated by the fact that aside death from cancer and cardiovascular cause, we also observed a positive association of furin levels with death from noncardiovascular conditions not attributed to cancer. However, especially noncardiovascular noncancer mortality is a heterogeneous group of causes of death, many of which could in fact be influenced by CVD and cancer.

Our study has some limitations that deserve clarification. First, we do not have information on

whether DM is of type I or II in our cohort. However, we have assumed that the number of incident type I DM cases is extremely low, given the participants' mean age of 57.4 ± 5.9 years at the baseline examination. Second, furin has been involved in autoimmunity [28] but we do not have any data regarding the autoimmunity status in our cohort. Therefore, we cannot study if autoimmunity contributes to the premature mortality associated with high furin nor if there is an autoimmune-related pathogenesis in the association between high furin levels and DM incidence. Third, we acknowledge that the sample size is low for some of the end-points. Fourth, our cohort may not be fully representative of the background population as we were lacking some samples. However, the missing participants only had a rather modestly more unfavourable metabolic profile than the included participants. Our study also has some strengths. First, our study had an extensive follow-up of individuals through registers with no loss of follow-up. Second, selection bias may be a minor issue considering the population-based prospective design of our study, which also allowed us to study several end-points.

In conclusion, individuals with high plasma furin concentration have a pronounced dysmetabolic phenotype and elevated risk of diabetes mellitus and premature mortality. Further mechanistic studies are now warranted.

Conflict of Interest Disclosures

The authors declare that they have no competing interests.

Authors Contributions

CF contributed to study concept and design, statistical analyses, interpretation of data and drafted the manuscript. OM contributed to study concept and design, acquisition of data, statistical analyses as well as interpretation of data. JR and HR contributed to study concept and design and interpretation of data. JN, GE, MOM contributed to acquisition of data and interpretation of data. PA contributed to statistical analyses and interpretation of data. All authors made intellectual contributions to drafting and/or revising the manuscript and approved the final version. CF is the guarantor of this work and had full access to all the data in the study and take responsibility for the integrity for the data and the accuracy of the data analysis.

References

1 Seshasai SRK, Kaptoge S, Thompson A *et al.* Diabetes mellitus, fasting glucose, and risk of cause-specific death. *New Engl J Med* 2011; **364:** 829–41.

2 Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Bio* 2002; **3:** 753–66.

3 Nakayama K. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J* 1997; **327:** 625–35.

4 Schalken JA, Roebroek AJM, Oomen PPCA *et al.* Fur gene-expression as a discriminating marker for small-cell and nonsmall cell lung carcinomas. *J Clin Invest* 1987; **80:** 1545–9.

5 Uhlen M, Bjorling E, Agaton C *et al.* A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol Cell Proteomics* 2005; **4:** 1920–32.

6 Bravo DA, Gleason JB, Sanchez RI, Roth RA, Fuller RS. Accurate and efficient cleavage of the human insulin proreceptor by the human proprotein-processing protease furin – characterization and kinetic-parameters using the purified, secreted soluble protease expressed by a recombinant baculovirus. *J Biol Chem* 1994; **269:** 25830–7.

7 Yoshimasa Y, Seino S, Whittaker J *et al.* Insulin-resistant diabetes due to a point mutation that prevents insulin proreceptor processing. *Science* 1988; **240:** 784–7.

8 Ueyama C, Horibe H, Yamase Y *et al.* Association of FURIN and ZPR1 polymorphisms with metabolic syndrome. *Biomed Rep* 2015; **3:** 641–7.

9 Harlid S, Myte R, Van Guelpen B. The metabolic syndrome, inflammation, and colorectal cancer risk: an evaluation of large panels of plasma protein markers using repeated, prediagnostic samples. *Mediators Inflamm* 2017; **2017:** 4803156.

10 Berglund G. The Malmo diet and cancer study design, biological bank and biomarker program. *J Intern Med* 1993; **233:** 39–40.

11 Persson M, Hedblad B, Nelson JJ, Berglund G. Elevated Lp-PLA(2) levels add prognostic information to the metabolic syndrome on incidence of cardiovascular events among middle-aged nondiabetic subjects. *Arterioscl Throm Vas* 2007; **27:** 1411–6.

12 Hellstrand S, Sonestedt E, Ericson U *et al.* Intake levels of dietary long-chain PUFAs modify the association between genetic variation in FADS and LDL-C. *J Lipid Res* 2012; **53:** 1183–9.

13 Assarsson E, Lundberg M, Holmquist G *et al.* Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS ONE* 2014; **9:** e95192.

14 Enhorning S, Hedblad B, Nilsson PM, Engstrom G, Melander O. Copeptin is an independent predictor of diabetic heart disease and death. *Am Heart J* 2015; **169:** 549.

15 Ludvigsson JF, Andersson E, Ekbom A *et al.* External review and validation of the Swedish national inpatient register. *BMC Public Health* 2011; **11:** 1–16.

16 Vidricaire G, Denault JB, Leduc R. Characterization of a secreted form of human furin endoprotease. *Biochem Bioph Res Co* 1993; **195:** 1011–8.

17 Plaimauer B, Mohr G, Wernhart W, Himmelspach M, Dorner F, Schlokat U. 'Shed' furin: mapping of the cleavage determinants and identification of its C-terminus. *Biochem J* 2001; **354:** 689–95.

18 Fathy SA, Hamid FFA, Zabut BM, Jamee AF, Ali MAM, Abu Mustafa AM. Diagnostic utility of BNP, corin and furin as biomarkers for cardiovascular complications in type 2 diabetes mellitus patients. *Biomarkers* 2015; **20:** 460–9.

19 Essalmani R, Susan-Resiga D, Chamberland A *et al.* In vivo evidence that furin from hepatocytes inactivates PCSK9. *J Biol Chem* 2011; **286:** 4257–63.

20 Ehret GB, Munroe PB, Rice KM *et al.* Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. *Nature* 2011; **478:** 103–9.

21 Li NF, Luo WL, Juhong Z *et al.* Associations between genetic variations in the FURIN gene and hypertension. *BMC Med Genet* 2010; **11:** 124.

22 Kayo T, Sawada Y, Suda M *et al.* Proprotein-processing endoprotease furin controls growth of pancreatic beta-cells. *Diabetes* 1997; **46:** 1296–304.

23 Louagie E, Taylor NA, Flamez D *et al.* Role of furin in granular acidification in the endocrine pancreas: identification of the V-ATPase subunit Ac45 as a candidate substrate. *P Natl Acad Sci USA* 2008; **105:** 12319–24.

24 Roebroek AJM, Umans L, Pauli IGL *et al.* Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase furin. *Development* 1998; **125:** 4863–76.

25 Turpeinen H, Raitoharju E, Oksanen A *et al.* Proprotein convertases in human atherosclerotic plaques: the overexpression of FURIN and its substrate cytokines BAFF and APRIL. *Atherosclerosis* 2011; **219:** 799–806.

26 Deloukas P, Kanoni S, Willenborg C *et al.* Large-scale association analysis identifies new risk loci for coronary artery disease. *Nat Genet* 2013; **45:** 25–U52.

27 Jaaks P, Bernasconi M. The proprotein convertase furin in tumour progression. *Int J Cancer* 2017; **141:** 654–63.

28 Ranta N, Valli A, Gronholm A *et al.* Proprotein convertase enzyme FURIN is upregulated in primary Sjogren's syndrome. *Clin Exp Rheumatol* 2018; [Epub ahead of print].

Correspondence: Céline Fernandez or Olle Melander, Department of Clinical Sciences, Hypertension and Cardiovascular Disease, Clinical Research Center, Box 50332, 202 13 Malmö, Sweden. (fax: +4640391222; e-mails: celine.fernandez@med.lu.se (CF) or olle.melander@med.lu.se (OM)).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Incidence of cardiovascular disease related mortality and of coronary artery disease in relation to baseline levels of furin in participants with diabetes.■