ORIGINAL RESEARCH

Legumain in Acute Coronary Syndromes: A Substudy of the PLATO (Platelet Inhibition and Patient Outcomes) Trial

Ida Gregersen (iD), PhD; Annika E. Michelsen, PhD; Ngoc Nguyen Lunde (iD), PhD; Axel Åkerblom, MD, PhD; Tatevik G. Lakic (iD), MSc; Mona Skjelland, MD, PhD; Karolina Ryeng Skagen, MD, PhD; Richard C. Becker, MD; Johan Lindbäck (iD), PhLic; Anders Himmelmann, MD, PhD; Rigmor Solberg, PhD; Harald T. Johansen (iD), PhD; Stefan K. James, MD, PhD; Agneta Siegbahn, MD, PhD; Robert F. Storey, MD, DM; Frederic Kontny, MD, PhD; Pål Aukrust, MD, PhD; Thor Ueland, PhD; Lars Wallentin, MD, PhD; Bente Halvorsen, PhD

BACKGROUND: The cysteine protease legumain is increased in patients with atherosclerosis, but its causal role in atherogenesis and cardiovascular disease is still unclear. The aim of the study was to investigate the association of legumain with clinical outcome in a large cohort of patients with acute coronary syndrome.

METHODS AND RESULTS: Serum levels of legumain were analyzed in 4883 patients with acute coronary syndrome from a substudy of the PLATO (Platelet Inhibition and Patient Outcomes) trial. Levels were analyzed at admission and after 1 month follow-up. Associations between legumain and a composite of cardiovascular death, spontaneous myocardial infarction or stroke, and its individual components were assessed by multivariable Cox regression analyses. At baseline, a 50% increase in legumain level was associated with a hazard ratio (HR) of 1.13 (95% CI, 1.04–1.21), $P=0.0018$, for the primary composite end point, adjusted for randomized treatment. The association remained significant after adjustment for important clinical and demographic variables (HR, 1.10; 95% CI, 1.02–1.19; $P=0.013$) but not in the fully adjusted model. Legumain levels at 1 month were not associated with the composite end point but were negatively associated with stroke (HR, 0.62; 95% CI, 0.44–0.88; $P=0.0069$), including in the fully adjusted model (HR, 0.57; 95% CI, 0.37–0.88; $P=0.0114$).

CONCLUSIONS: Baseline legumain was associated with the primary outcome in patients with acute coronary syndrome, but not in the fully adjusted model. The association between high levels of legumain at 1 month and decreased occurrence of stroke could be of interest from a mechanistic point of view, illustrating the potential dual role of legumain during atherogenesis and acute coronary syndrome.

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Key Words: acute coronary syndromes ■ ischemic stroke ■ legumain

Atherosclerosis, a progressive pathological process with build-up of intimal plaque in the artery wall, is the main cause of cardiovascular disease. Atherosclerosis is characterized by nonresolving inflammation and both immune and vascular cells express and release an enormous amount of mediators affecting the rate and course of plaque progression, including the development of acute coronary syndrome (ACS) and ischemic stroke.[1]

Legumain, also known as asparagine endopeptidase, is a member of the C13 family of cysteine proteases.[2] It has broad immunoregulatory properties such

CLINICAL PERSPECTIVE

What Is New?

- Legumain has previously been shown to be up-regulated in carotid atherosclerotic plaques and associated with mortality in patients with ST-segment–elevation myocardial infarction.
- In this study, legumain is evaluated as a prognostic biomarker in a large population with acute coronary syndrome.

What Are the Clinical Implications?

- Legumain was associated with worse outcomes in patients with acute coronary syndrome but not in the fully adjusted model.
- Legumain levels at 1 month was negatively associated with occurrence of stroke.
- The association between high levels of legumain at 1 month and decreased occurrence of stroke could be of interest from a mechanistic point of view, illustrating the complex and potential dual role of legumain during acute coronary syndrome and atherogenesis.

Nonstandard Abbreviations and Acronyms

CABG coronary artery bypass graft
PLATO Platelet Inhibition and Patient Outcomes

as toll-like receptor modulation,[3] processing of antigens for major histocompatibility complex class II presentation,[4] monocyte chemotaxis,[5] induction of Th1 cell responses,[6] and regulation of extracellular matrix remodeling.[7,8] Legumain is expressed in both murine and human atherosclerotic lesions,[5] and in patients with carotid stenosis we found increased legumain levels in plasma and plaques, with the highest expression in lesions from symptomatic patients.[9] Further, it was recently shown that legumain induced vascular remodeling in atherosclerosis-prone ApoE$^{-/-}$ mice by increasing the number of macrophages and vascular smooth muscle cells within the atherosclerotic lesions.[10] Based on these findings, we hypothesized that legumain could be released during plaque destabilization and contribute to myocardial and vascular remodeling following ACS.

To further explore this hypothesis, legumain levels were analyzed in a large population of patients with ACS from the PLATO (Platelet Inhibition and Patient Outcomes) trial, encompassing a broad spectrum of ACS events. Legumain levels were analyzed on admission and after 1 month of follow-up after ACS, together with established prognostic biomarkers, and related to fatal and nonfatal cardiovascular outcomes.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Design and Study Population

The PLATO trial (NCT00391872) was a randomized, placebo-controlled trial including 18 624 patients with ACS. The patients presented with either ST-elevation ACS or non ST-elevation ACS and were randomized to either clopidogrel or ticagrelor treatment in addition to optimal medical therapy, including aspirin, and optional invasive therapy.[11,12] The patients were recruited between October 2006 and July 2008 and were followed for up to 12 months after ACS.

Venous blood samples were obtained from all patients at randomization as part of the main study. In addition, there was a predefined substudy with serial blood sampling conducted at selected sites at discharge and after 1 and 6 months.[11,13] The overall aims of this biomarker substudy program have previously been published.[11–13] Patients with a blood sample at baseline and additional blood sample during 1-month follow-up, with no new cardiovascular event before the date of the 1-month sample, were eligible for inclusion in the current analyses. Informed consent was obtained from all patients included and the trial complied with national and institutional regulatory and ethics committees and the Declaration of Helsinki. A detailed description of Sampling and Laboratory analysis can be found in Data S1.

End Point Definition and Follow-Up

The prespecified primary end point of the present substudy was the composite of cardiovascular death (defined as any cardiovascular cause of death, sudden death, or any death with no clear attributable noncardiovascular cause), spontaneous myocardial infarction (defined as non-procedure-related, nonfatal, MI type 1)[14] or stroke within 1 year of follow-up.[11] Secondary outcomes were procedural MI, stroke and major bleeding not related to coronary artery bypass graft (CABG) surgery, either fatal, intracranial, or requiring ≥ 2 units of blood transfusion or with a drop in hemoglobin of> 5 g/dL.[11] All end points in the PLATO trial were centrally adjudicated by an independent and blinded clinical event adjudication committee, comprising cardiologists or neurologists, in order to subclassify causes of death and to subdivide types of MIs, stroke, or bleeding events.[11,14]

Statistical Analysis

Baseline characteristics and patient demographics were compared between legumain quartile groups using Kruskal-Wallis tests for continuous variables and chi-square tests for categorical variables. The Kruskal-Wallis test was used as it has high power when the normality assumption is not fulfilled and does not lose much power even if the normality assumption holds. Biomarkers were logarithmic-transformed when appropriate. Multivariable regression assessed the relationship between legumain and baseline characteristics, with legumain as the depending variable. We calculated geometric means using the antilogarithms of the model-adjusted means (ie, predicted marginal means), and subsequently compared geometric means between groups (eg, males/females) using ratios. The unadjusted association between legumain quartile groups and clinical outcomes were presented by Kaplan-Meier curves. Cox proportional hazards models were used to investigate the covariate-adjusted association between legumain and the composite end point of cardiovascular death, spontaneous MI, or stroke and secondary outcomes: procedural MI, stroke, and non-CABG major bleeding. Five models, with incremental addition of covariates, were used. Model 0 included legumain and randomized treatment (ticagrelor or clopidogrel). Model 1a added age, sex, body mass index, diabetes mellitus, dyslipidemia, hypertention, chronic renal disease, chronic heart failure, ST-segment–elevation myocardial infarction (STEMI)/non–ST-segment–elevation-ACS at randomization, smoking, type of ACS, aspirin at entry, history of MI, percutaneous coronary intervention, CABG, stroke, or peripheral artery disease. Model 1b included the following covariates in addition to Model 1a: unfractionated heparin, low-molecular-weight heparin, use of glycoprotein IIb/IIIa inhibitor, statin, diuretic, and proton pump inhibitor during hospital stay. Model 1c included the following covariates in addition to Model 1b: hemoglobin, platelets, and white blood cell count. Model 2 further added C-reactive protein; Model 3, cystatin C; Model 4, NT-proBNP (N-terminal-pro-B-type natriuretic peptide) and TnT (troponin T); and Model 5 included all variables in addition to GDF-15 (growth differentiation factor 15). All biomarkers were included as continuous variables after logarithmic transformation. The results were presented as the relative hazard for 50% increase in legumain concentration at baseline. The proportional hazards assumption was assessed by visual inspection of Schoenfeld residual plots. The association between legumain levels and clinical outcomes were illustrated by restricted cubic splines with 4 knots placed at the 5th, 35th, 65th, and 95th sample percentiles.

A statement of statistical significance implies a P value of <0.05 and there were no adjustments for multiple comparisons. All statistical analyses were performed with SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Legumain at Admission in Relation to Baseline Characteristics

Compared to the total PLATO population, the baseline characteristics of the current substudy showed a similar pattern except for more frequent STEMI, less frequent diabetes mellitus, and lower high-sensitivity TnT and NT-proBNP (Table S1). Legumain levels at admission were available in 4883 patients with a median (Q1-Q3) of 2.78 (1.97–3.86) ng/mL. Baseline characteristics by legumain quartile groups are presented in Table 1. In multivariable analysis of baseline characteristic, the strongest correlations with legumain were age, STEMI, use of glycoprotein IIb/IIIa inhibitor, GDF-15, and platelet count ($P<0.001$ for all; Table S2).

Association of Baseline Legumain Levels With Clinical Outcomes

Of the 4883 patients included, the primary composite end point (cardiovascular death/spontaneous MI/stroke) was observed in 421 individuals, with an event rate of 8.6%. Baseline legumain levels (with hazard ratio [HR] per 50% increase of legumain) were associated with the primary composite end point (HR, 1.13; 95% CI, 1.04–1.21; $P=0.0018$) after adjusting for randomized treatment, Model 0 (Table 2). Kaplan-Meyer estimates per quartile of baseline legumain levels are presented in Figure 1A, showing a positive association with the primary composite end point. Restricted cubic spline curves for legumain at baseline against different outcomes are shown in Figure 1B. In multivariable Cox regression analyses (Table 2), the association between baseline legumain and the primary composite end point remained associated after adjustment for important clinical and demographic variables (Model 1a; HR, 1.13; 95% CI, 1.05–1.22; $P=0.0021$) and the use of medication (ie, statins, diuretics, use of glycoprotein IIb/IIIa inhibitor, Model 1b; HR, 1.10; 95% CI, 1.02–1.19; $P=0.013$). However, these associations were attenuated after further adjustment for hemoglobin, platelets, white blood cell count, C-reactive protein, cystatin C, NT-proBNP, TnT, and GDF-15 (Model 1c-5; HR 1.08; 95% CI, 0.99–1.17; $P=0.0747$). There were no associations between baseline legumain levels and the randomized treatment regimen (ie, clopidogrel or ticagrelor, Figure S1) on any end point (Table 2).

Table 1.   Baseline Characteristics of Study Participants According to Legumain Quartiles (N=4883)

Characteristics*	Q1 <1.97 ng/mL n=1219	Q2 1.97–2.78 ng/mL n=1225	Q3 2.78–3.86 ng/mL n=1218	Q4 >3.86 ng/mL n=1221	P Value†
Demographics					
Age, y	63 (54–71)	63 (54–71)	63 (54–71)	61 (53–69)	0.0017
Female	385 (31.6%)	395 (32.2%)	357 (29.3%)	330 (27.0%)	0.0204
Weight, kg	80 (70–90)	80 (70–90)	81 (71–91)	80 (70–90)	0.0223
Body mass index, kg/m^2	27.3 (24.9–30.2)	27.5 (24.8–30.5)	27.7 (25.2–30.9)	27.8 (25.2–31.0)	0.0116
Risk factors					
Habitual smoker	437 (35.8%)	437 (35.7%)	422 (34.6%)	485 (39.7%)	0.0491
Hypertension	759 (62.3%)	808 (66.0%)	816 (67.0%)	845 (69.2%)	0.0033
Dyslipidemia	509 (41.8%)	513 (41.9%)	538 (44.2%)	497 (40.7%)	0.3617
Diabetes mellitus	228 (18.7%)	245 (20.0%)	294 (24.1%)	332 (27.2%)	<.0001
Medical history					
Angina pectoris	505 (41.4%)	571 (46.6%)	604 (49.6%)	598 (49.0%)	0.0002
Myocardial infarction	189 (15.5%)	240 (19.6%)	267 (21.9%)	269 (22.0%)	<.0001
Congestive heart failure	51 (4.2%)	54 (4.4%)	81 (6.7%)	98 (8.0%)	<.0001
Percutaneous coronary intervention	124 (10.2%)	155 (12.7%)	159 (13.1%)	169 (13.8%)	0.0379
Coronary artery bypass graft	46 (3.8%)	59 (4.8%)	67 (5.5%)	70 (5.7%)	0.1115
Transient ischemic attack	23 (1.9%)	23 (1.9%)	42 (3.4%)	25 (2.0%)	0.0252
Nonhemorrhagic stroke	33 (2.7%)	43 (3.5%)	45 (3.7%)	44 (3.6%)	0.5106
Peripheral arterial disease	59 (4.8%)	71 (5.8%)	97 (8.0%)	108 (8.8%)	0.0002
Chronic renal disease	49 (4.0%)	42 (3.4%)	44 (3.6%)	37 (3.0%)	0.6110
Thrombolysis in myocardial infarction risk score	4 (2–5)	4 (2–5)	4 (2–5)	4 (3–5)	<.0001
Global Registry of Acute Coronary Events risk score	136 (121–153)	133 (117–150)	134 (117–151)	133 (115–151)	0.0185
Type of acute coronary syndrome ST-elevation myocardial infarction	710 (58.2%)	558 (45.6%)	459 (37.7%)	458 (37.5%)	<0.0001
In hospital medication					
Aspirin	1206 (98.9%)	1205 (98.4%)	1192 (97.9%)	1198 (98.1%)	0.2012
Unfractioned heparin	736 (60.4%)	657 (53.6%)	638 (52.4%)	628 (51.4%)	<0.0001
Low-molecular-weight heparin	600 (49.2%)	676 (55.2%)	694 (57.0%)	672 (55.0%)	0.0008
Fondaparinux	12 (1.0%)	21 (1.7%)	19 (1.6%)	21 (1.7%)	0.3897
Bivalirudin	14 (1.1%)	15 (1.2%)	19 (1.6%)	27 (2.2%)	0.1281
Glycoprorein IIb/IIIa inhibitor	426 (34.9%)	318 (26.0%)	281 (23.1%)	246 (20.1%)	<.0001
Beta blocker	1081 (88.7%)	1059 (86.4%)	1060 (87.0%)	1054 (86.3%)	0.2800
Angiotensin-converting inhibitor and/or angiotensin receptor blocker	1043 (85.6%)	1069 (87.3%)	1069 (87.8%)	1056 (86.5%)	0.3992
Cholesterol lowering (statin)	1166 (95.7%)	1162 (94.9%)	1131 (92.9%)	1104 (90.4%)	<0.0001
Ca-inhibitor	251 (20.6%)	263 (21.5%)	255 (20.9%)	259 (21.2%)	0.9575
Diuretic	408 (33.5%)	436 (35.6%)	473 (38.8%)	530 (43.4%)	<0.0001
Proton pump inhibitor	598 (49.1%)	537 (43.8%)	506 (41.5%)	485 (39.7%)	<0.0001
Biomarkers					
Hemoglobin	140 (130–149)	140 (130–149)	143 (132–153)	143 (133–153)	<0.0001
Platelets	223 (190–262)	227 (191–270)	236 (202–276)	246 (207–292)	<0.0001
White blood cells	9.6 (7.4–11.9)	9.1 (7.4–11.3)	9.1 (7.3–11.3)	9.5 (7.5–11.6)	0.0123
Neutrophils	7.0 (5.0–9.4)	6.6 (4.9–8.7)	6.6 (4.8–8.7)	6.7 (4.9–9.0)	0.0010
Monocytes	0.4 (0.2–0.6)	0.4 (0.2–0.6)	0.4 (0.3–0.6)	0.5 (0.3–0.7)	<0.0001

(*Continued*)

Table 1. Continued

Characteristics*	Q1 <1.97 ng/mL n=1219	Q2 1.97–2.78 ng/mL n=1225	Q3 2.78–3.86 ng/mL n=1218	Q4 >3.86 ng/mL n=1221	P Value†
Lymphocytes	1.7 (1.3–2.2)	1.8 (1.3–2.3)	1.8 (1.4–2.3)	1.9 (1.4–2.4)	<0.0001
Troponin T, ng/L	124.0 (35.1–420.0)	152.5 (43.5–468.5)	180.0 (37.4–604.0)	195.0 (42.8–692.0)	<0.0001
N-terminal pro-B-type natriuretic peptide, pmol/L	280.0 (97.0–865.0)	398.0 (129.0–998.0)	478.5 (149.5–1248)	546.0 (192.5–1565)	<0.0001
Cystatin C, mg/L	0.78 (0.63–0.94)	0.81 (0.66–0.99)	0.83 (0.69–1.00)	0.86 (0.71–1.06)	<0.0001
Growth differentiation factor 15, ng/mL	1454 (1076–1992)	1508 (1136–2082)	1535 (1158–2148)	1699 (1229–2471)	<0.0001
C-reactive protein, mg/L	2.6 (1.2–5.8)	3.2 (1.5–7.7)	3.7 (1.6–9.3)	5.4 (2.1–14.0)	<0.0001
Interleukin-6, ng/mL	2.9 (1.7–5.2)	3.1 (1.9–6.7)	3.6 (2.0–8.1)	4.2 (2.2–9.5)	<0.0001

*Continuous variables are expressed median (interquartile range). Categorical variables are expressed as frequency (%).
†P values from the chi-square test (categorical variables) or Kruskal-Wallis test (continuous variables).

Association of Legumain Levels at Follow-Up With Clinical Outcomes

The distribution of legumain levels was higher at discharge compared with baseline, followed by a slight decline reaching steady state levels at 1 month with similar levels at 6 months (Figure 2A), with no differences in levels between the treatment groups (Figure 2B). Follow-up measurements at 1 month were available from 3927 patients of whom 228 (event rate 5.8%) suffered a primary composite end point (cardiovascular death/spontaneous MI/stroke). The numbers of strokes, procedural MIs, and non-CABG-related major bleeds (secondary end points) were 34 (0.9%), 25 (0.6%) and 69 (1.8%), respectively. Legumain at 1 month follow-up (with HR per 50% increase of legumain) was not statistically significantly associated with the composite primary outcome, procedural MI, or non-CABG-related major bleeds but was negatively associated with stroke (HR, 0.62; 95% CI, 0.44–0.88; P=0.0069; Model 0, adjusted for randomized treatment). The association between legumain levels at 1 month and different outcomes is shown in Figure S2. In multivariable Cox regression analyses this association with stroke remained statistically significant when adjusting for all covariates, including C-reactive protein, cystatin C, NT-proBNP, TnT, and GDF-15, that are shown to have a significant prognostic power in this population[15] (HR, 0.57; 95% CI, 0.37–0.88; P=0.0114 [Model 5, Table 3]).

DISCUSSION

Legumain has previously been shown to be upregulated in carotid atherosclerotic plaques, with the highest levels in those with symptomatic lesions.[9] Moreover, legumain levels are shown to be associated with complex coronary lesions,[16] and we have recently shown that low legumain levels were associated with

mortality (univariate analyses) in a small population of patients with STEMI (n=272).[17] The present study is, however, to the best of our knowledge, the first study that evaluates legumain as a prognostic biomarker in a large population with ACS (n=4883). Although baseline legumain levels were significantly associated with the primary end point after adjusting for important demographic and clinical factors (eg, age, sex, body mass index, diabetes mellitus) and use of medications, this association was not significant in the full model adjusting for biomarkers including C-reactive protein, TnT, cystatin C, GDF-15, and NT-proBNP. These findings suggest that although legumain is upregulated in patients with ACS, it does not give additional prognostic information beyond the established biomarkers.

The role of legumain in atherogenesis and acute cardiovascular events is at present not clear. Legumain is shown to induce vascular smooth muscle cells migration and atherosclerotic vascular remodeling, driving atherosclerotic plaque development.[10] However, our previous findings illustrate that legumain also may have plaque stabilizing and anti-atherogenic properties.[17] Further, whereas legumain has been reported to promote an inflammatory M1 phenotype and foam cell formation in macrophages,[10] we have recently shown that legumain also can induce an anti-inflammatory macrophage phenotype.[17] Furthermore, legumain has been shown to mediate effects of M2 macrophages in a mouse model of obstructive nephropathy[18] and to promote pulmonary artery hypertension through induction of transforming growth factor β[19]. Although transforming growth factor β signaling could be harmful in fibrotic disorders, it could potentially stabilize the plaque phenotype in atherosclerotic lesions. Interestingly, we have shown that legumain is released from platelets and macrophages and colocalized with these cells in carotid atherosclerotic plaques as well as in thrombi from patients with STEMI and patients with ischemic stroke.[17] This suggests that legumain is

Table 2. Effect of Baseline Legumain on Outcomes (N=4883)

	Cardiovascular Death/Spontaneous MI/Stroke			Stroke[†]			Procedural MI			Non-CABG-Related Major Bleeds		
	N (%)[‡]	HR (95% CI)[§]	P Value[‖]	N (%)[‡]	HR (95% CI)[§]	P Value[‖]	N (%)[‡]	HR (95% CI)[§]	P Value[‖]	N (%)[‡]	HR (95% CI)[§]	P Value[‖]
Model 0*	421 (8.6)	1.13 (1.04–1.21)	0.0018	59 (1.2)	0.97 (0.80–1.18)	0.7711	94 (1.9)	0.96 (0.82–1.12)	0.5733	185 (3.8)	1.00 (0.90–1.12)	0.9641
Model 1a*	419 (8.6)	1.13 (1.05–1.22)	0.0021	59 (1.2)	0.96 (0.78–1.18)	0.6951	93 (1.9)	0.92 (0.79–1.09)	0.3388	184 (3.8)	1.02 (0.91–1.14)	0.7645
Model 1b*	419 (8.6)	1.10 (1.02–1.19)	0.013	59 (1.2)	0.93 (0.76–1.15)	0.5215	93 (1.9)	0.94 (0.80–1.11)	0.4621	184 (3.8)	1.03 (0.91–1.15)	0.6510
Model 1c*	375 (8.5)	1.08 (0.99–1.17)	0.0747	53 (1.2)	0.97 (0.77–1.21)	0.7718	86 (1.9)	0.91 (0.77–1.08)	0.2945	159 (3.6)	1.02 (0.90–1.16)	0.7248
Model 2*	350 (8.7)	1.06 (0.97–1.16)	0.2166	47 (1.2)	0.91 (0.72–1.15)	0.4312	79 (2.0)	0.86 (0.72–1.03)	0.1110	150 (3.7)	1.03 (0.90–1.18)	0.6482
Model 3*	350 (8.7)	1.06 (0.97–1.15)	0.2314	47 (1.2)	0.91 (0.72–1.15)	0.4374	79 (2.0)	0.86 (0.72–1.03)	0.1083	150 (3.7)	1.03 (0.90–1.18)	0.6500
Model 4*	348 (8.7)	1.02 (0.94–1.12)	0.6211	47 (1.2)	0.93 (0.73–1.18)	0.5275	79 (2.0)	0.86 (0.72–1.03)	0.1008	149 (3.7)	1.03 (0.90–1.17)	0.7141
Model 5*	348 (8.7)	1.01 (0.93–1.10)	0.7995	47 (1.2)	0.93 (0.73–1.18)	0.5265	79 (2.0)	0.85 (0.71–1.02)	0.0896	149 (3.7)	1.02 (0.89–1.16)	0.7956

Model 0 includes legumain and randomized treatment. Model 1a includes legumain, age, sex, body mass index, diabetes mellitus, dyslipidemia, hypertension, chronic renal disease, chronic heart failure, ST elevation myocardial infarction/non ST elevation-acute coronary syndrome at randomization, smoking, type of acute coronary syndrome, aspirin at entry, randomized treatment, previous MI/peripheral artery disease/CABG/ percutaneous coronary intervention/nonhemorrhagic stroke. Model 1b includes the following covariates in addition to Model 1a: unfractionated heparin, low-molecular-weight heparin, use of glycoprotein IIb/IIIa inhibitor, statin, diuretic, and proton pump inhibitor during hospital stay. Model 1c includes the following covariates in addition to Model 1b: hemoglobin, platelets, and white blood cells. Model 2 includes the following covariates in addition to Model 1c: C-reactive protein. Model 3 includes the following covariates in addition to Model 2: cystatin C. Model 4 includes the following covariates in addition to Model 3: N-terminal pro–B-type natriuretic peptide and troponin T. Model 5 includes the following covariates in addition to Model 4: growth differentiation factor 15. All biomarkers are logarithmic transformed. CABG indicates coronary artery bypass graft; HR, hazard ratio; and MI, myocardial infarction.

*Multivariable Cox regression models.

[†]Stroke is a subset of cardiovascular death/Spontaneous MI/Stroke, procedural MI and non-CABG bleed are not.

[‡]Incidence during follow-up, (no. events / no. of subjects) × 100%

[§]The HR is per 50% increase of legumain at 1 month.

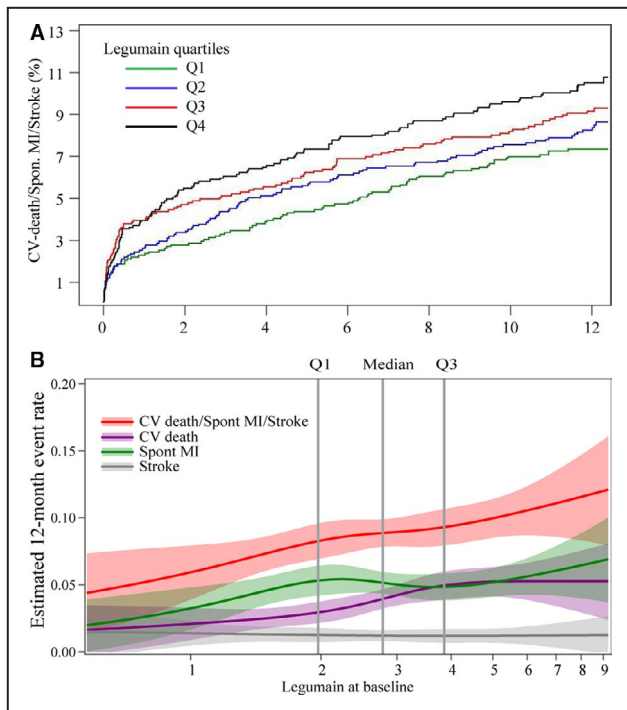[‖]P value for the effect of legumain at 1 month.

Figure 1. Kaplan-Meier estimated event rates of the primary outcome (composite of cardiovascular [CV] death, spontaneous myocardial infarction [MI], and stroke) per quartile of baseline legumain level during 12 months follow-up.

A, Cubic spline curves for legumain at baseline (ng/mL) against the primary and secondary outcomes (**B**).

operating at the site of acute cardiovascular events, but based on its dual role in inflammation, the net effects of this complex molecule are at present not clear. In fact, the lack of independent prognostic power of legumain in relation to the primary end point in the present ACS population may reflect its complex role in atherogenesis that most probably also depend on costimuli within the microenvironment.

Samples taken after 1 month were available from 3927 patients. Whereas legumain levels at this time point were not associated with the primary composite end point, legumain had a negative association with stroke, also in the fully adjusted model. Although there were few patients who suffered a stroke following 1 month (n=34) and biomarkers giving prognostic information when assessed after 1 month, and not at baseline, could be difficult to use in the clinic, this intriguing observation is of interest from a mechanistic point of view. Although the reason for these seemingly contradictory findings is at present not clear, they could reflect the pleiotropic effects of legumain, potentially promoting both plaque stabilizing and destabilizing effects. Future studies should elucidate the dual effects of legumain on macrophages and the triggers for these apparently divergent effects and if these effects are of particular relevance to ischemic stroke.

Limitations

The current study provides deeper insights to the role of legumain in a large population with ACS, but has some limitations. The PLATO trial comprises a broad population with ACS, but patients requiring dialysis or with recent significant bleeding were not eligible. Furthermore, as mortality was lower in the group randomized to ticagrelor, a survival bias with ticagrelor may have been present. Also, as legumain could exert its effect locally, for example inside an atherosclerotic plaque, the circulating levels might not reflect its functions in vivo. Further studies are needed to clarify this relationship and if legumain is suitable to study from an epidemiologic point of view.
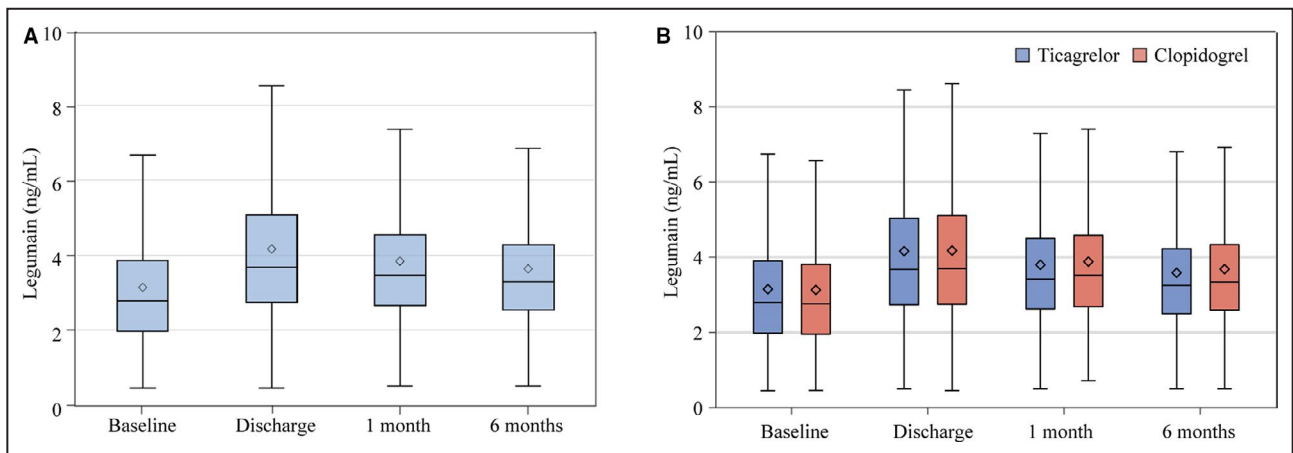

Figure 2. Serum legumain levels (ng/mL) at baseline, discharge, 1 and 6 months in the whole patient group (A) and according to treatment groups, clopidogrel or ticagrelor (B).

Presented as median and interquartile range.

Table 3. Effect of Legumain Levels at 1 Month Follow-Up on Subsequent Outcomes (N=3927)

	Cardiovascular Death/Spontaneous MI/Stroke			Stroke†			Procedural MI			Non-CABG-Related Major Bleeds		
	N (%)‡	HR (95% CI)§	P Value‖	N (%)‡	HR (95% CI)§	P Value‖	N (%)‡	HR (95% CI)§	P Value‖	N (%)‡	HR (95% CI)§	P Value‖
Model 0*	228 (5.8)	0.89 (0.77–1.03)	0.1084	34 (0.9)	0.62 (0.44–0.88)	0.0069	25 (0.6)	1.11 (0.72–1.70)	0.6328	69 (1.8)	0.97 (0.75–1.25)	0.8140
Model 1a*	228 (5.8)	0.91 (0.79–1.05)	0.1790	34 (0.9)	0.59 (0.40–0.87)	0.0077	25 (0.6)	1.08 (0.69–1.68)	0.7395	69 (1.8)	1.01 (0.78–1.30)	0.9628
Model 1b*	228 (5.8)	0.88 (0.77–1.02)	0.0834	34 (0.9)	0.54 (0.36–0.80)	0.0025	25 (0.6)	1.07 (0.68–1.69)	0.7604	69 (1.8)	0.98 (0.76–1.27)	0.8823
Model 1c*	215 (6.0)	0.87 (0.75–1.00)	0.0537	31 (0.9)	0.59 (0.38–0.91)	0.0165	21 (0.6)	1.06 (0.65–1.74)	0.8116	64 (1.8)	0.93 (0.71–1.22)	0.5886
Model 2*	204 (6.0)	0.89 (0.77–1.04)	0.1485	31 (0.9)	0.59 (0.38–0.92)	0.0189	19 (0.6)	1.18 (0.71–1.97)	0.5250	62 (1.9)	0.91 (0.69–1.20)	0.5052
Model 3*	204 (6.0)	0.89 (0.77–1.04)	0.1440	31 (0.9)	0.59 (0.38–0.91)	0.0176	19 (0.6)	1.19 (0.71–1.98)	0.5111	62 (1.9)	0.91 (0.68–1.20)	0.5041
Model 4*	202 (6.0)	0.91 (0.78–1.06)	0.2340	31 (0.9)	0.57 (0.37–0.88)	0.0111	19 (0.6)	1.29 (0.76–2.17)	0.3476	62 (1.9)	0.91 (0.69–1.21)	0.5145
Model 5*	202 (6.0)	0.90 (0.77–1.05)	0.1825	31 (0.9)	0.57 (0.37–0.88)	0.0114	19 (0.6)	1.30 (0.77–2.20)	0.3289	62 (1.9)	0.89 (0.67–1.18)	0.4157

Model 0 includes legumain at 1 month, adjusted for baseline legumain and randomized treatment. Model 1a includes legumain at 1 month, adjusted for baseline legumain, age, sex, body mass index, diabetes mellitus, dyslipidemia, hypertension, chronic renal disease, chronic heart failure, ST elevation myocardial infarction/non ST elevation-acute coronary syndrome, smoking, type of acute coronary syndrome, aspirin at entry, randomized treatment, previous (MI/periphery artery disease/CABG/percutaneous coronary intervention/nonhemorrhagic stroke). Model 1b includes the following covariates in addition to Model 1a: unfractionated heparin, low-molecular-weight heparin, glycoprotein IIb/IIIa inhibitor, statin, diuretic, and proton pump inhibitor during hospital stay. Model 1c includes the following covariates in addition to Model 1b: hemoglobin, platelets, and white blood cells. Model 2 includes the following covariates in addition to Model 1c: C-reactive protein. Model 3 includes the following covariates in addition to Model 2: cystatin C. Model 4 includes the following covariates in addition to Model 3: N-terminal pro–B-type natriuretic peptide and troponin T. Model 5 includes the following covariates in addition to Model 4: growth differentiation factor 15. All adjustment biomarkers are at baseline, included in the models after logarithmic transformation. CABG indicates coronary artery bypass graft; HR, hazard ratio; and MI, myocardial infarction.

*Multivariable Cox regression models.

†Stroke is a subset of cardiovascular Death/Spontaneous MI/Stroke, procedural MI and non-CABG bleed are not.

‡Incidence during follow-up, (no. events / no. of subjects) × 100%

§The HR is per 50% increase of legumain at 1 month.

‖P value for the effect of legumain at 1 month.

CONCLUSIONS

Legumain was associated with outcome in patients with ACS but not in the fully adjusted model. The association between high levels of legumain at 1 month and decreased occurrence of stroke could be of interest from a mechanistic point of view, illustrating the complex and potential dual role of legumain during ACS and atherogenesis.

ARTICLE INFORMATION

Affiliations

From the Research Institute for Internal Medicine, Oslo University Hospital Rikshospitalet Oslo (I.G., A.E.M., P.A., T.U., B.H.), Institute of Clinical Medicine, Faculty of Medicine University of Oslo, Norway (I.G., A.E.M., P.A., T.U., B.H.); Department of Medical Sciences, Cardiology and Uppsala Clinical Research Center (A.Å., S.K.J., L.W.), and Uppsala Clinical Research Center (T.G.L., J.L., A.S.), Uppsala University, Uppsala, Sweden; Department of Neurology, Oslo University Hospital, Rikshospitalet, Oslo, Norway (M.S., K.R.S.); Division of Cardiovascular Health and Disease, Heart, Lung and Vascular Institute, Academic Health Center, Cincinnati, OH (R.C.B.); Section of Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy University of Oslo, Norway (N.N.L., R.S., H.T.J.); AstraZeneca Research and Development, Gothenburg, Sweden (A.H.); Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, United Kingdom (R.F.S.); Department of Cardiology Stavanger University Hospital, Stavanger, Norway (F.K.); Drammen Heart Center, Drammen, Norway (F.K.); Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Oslo, Norway (P.A.); and K.G. Jebsen TREC, The Faculty of Health Sciences, The Arctic University of Tromsø, Tromsø, Norway (P.A., T.U.).

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Disclosures

Supplementary Materials

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

<u>Sampling and laboratory analysis</u>

Baseline venous blood samples were obtained within 24 hours of admission, prior to the administration of study medication. The venous blood was centrifuged and plasma and serum samples locally frozen in aliquots and stored at -70ºC in a central repository in Uppsala Biobank until analyses. Serum legumain concentrations were determined with sandwich immunoassay from R&D Systems (Duoset DY4769), Stillwater, MN. Total and differential (i.e., neutrophils, lymphocytes and monocytes) white blood cell (WBC) counts, hemoglobin (Hb) and plasma C-reactive protein (CRP) were analyzed at the UCR laboratory, Uppsala, Sweden with a spectrophotometric analysis (Architect, Abbott). Platelet count was determined using an Electronic Cell Counter by Quintiles Laboratories. Plasma high sensitivity cardiac troponin T (hs-troponin T; TnT), N-terminal pro-B-type natriuretic peptide (NT-proBNP) and cystatin C were determined with sandwich immunoassays on the Cobas® Analytics e601 Immunoanalyzer (Roche Diagnostics, Mannheim, Germany). Plasma growth/differentiation factor (GDF)-15 was measured with a pre-commercial assay (Roche Diagnostics) using a monoclonal mouse antibody for capture and a monoclonal mouse antibody fragment for detection in a sandwich assay format. We also include data on sCD40L, sP-selectin, D-dimer, IL-6 and oxidized LDL (oxLDL), analyzed as previously described[20-22]. The results of these analyses in relation to outcomes and effects of study treatment have previously been reported[20-25].

Table S1. Baseline characteristics of patients in the total PLATO population and in the current study sub-population.

Group of characteristics	Characteristic	PLATO total population N=18421	Legumain population (Biomarker substudy) N=4883
Demographics	Age yrs	62 (54-71)	62 (54-71)
	Female	5237 (28.4%)	1467 (30.0%)
	Weight, kg	80 (70-90)	80 (70-90)
	BMI, kg/m^2	27.4 (24.7-30.4)	27.7 (25.0-30.6)
Risk factor	Habitual smoker	6613 (35.9%)	1781 (36.5%)
	Hypertension	12047 (65.4%)	3228 (66.1%)
	Dyslipidemia	8593 (46.7%)	2057 (42.1%)
	Diabetes mellitus	4621 (25.1%)	1099 (22.5%)
Medical history	Angina pectoris	8277 (44.9%)	2278 (46.7%)
	Myocardial infarction	3784 (20.5%)	965 (19.8%)
	Congestive heart failure	1041 (5.7%)	284 (5.8%)
	PCI	2456 (13.3%)	607 (12.4%)
	CABG	1092 (5.9%)	242 (5.0%)
	TIA	495 (2.7%)	113 (2.3%)
	Non-hemorrhagic stroke	710 (3.9%)	165 (3.4%)
	Peripheral arterial disease	1128 (6.1%)	335 (6.9%)
	Chronic renal disease	775 (4.2%)	172 (3.5%)
Type of ACS	ST-elevation MI	7471 (40.6%)	2185 (44.7%)
Risk scores	TIMI risk score	4.0 (2.0-5.0)	4.0 (2.0-5.0)
	GRACE risk score	133 (117-152)	133 (117-151)
Anti-thrombotic treatment in hospital	Aspirin	17906 (97.2%)	4801 (98.3%)
	Unfractioned heparin	10661 (57.9%)	2659 (54.5%)
	LMW heparin	9581 (52.0%)	2642 (54.1%)
	Fondaparinux	505 (2.7%)	73 (1.5%)
	Bivalirudin	371 (2.0%)	75 (1.5%)
	GPIIb/IIIa inhibitor	5003 (27.2%)	1271 (26.0%)
Other medication in hospital	Beta-blocker	15810 (85.8%)	4254 (87.1%)
	ACE-inhibitor and/or ARB	15351 (83.3%)	4237 (86.8%)
	Cholesterol lowering drug (statin)	17319 (94.0%)	4563 (93.4%)
	Ca-inhibitor	3984 (21.6%)	1028 (21.1%)
	Diuretic	6906 (37.5%)	1847 (37.8%)
	Proton pump inhibitor	8490 (46.1%)	2126 (43.5%)
Biomarkers	Troponin T, ng/L	191.0 (45.4-654.0)	159.0 (39.8-549.5)
	NT-proBNP, pmol/L	460.0 (149.0-1300)	420.0 (135.0-1150)
	Cystatin C, mg/L	0.83 (0.68-1.01)	0.82 (0.67-1.00)
	GDF-15, ng/mL	1550 (1145-2219)	1535 (1146-2152)
	CRP, mg/L	3.7 (1.6-9.5)	3.5 (1.6-8.8)
	IL-6, ng/mL	3.4 (1.9-7.3)	3.4 (1.9-7.3)

Continuous variables are expressed median (interquartile range). Categorical variables are expressed as frequency (%). p-values from the Chi-square test (categorical variables) or Kruskal-Wallis test (continuous variables). BMI, body mass index; PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft; TIA, transient ischemic attack; MI, myocardial infarction; TIMI, thrombolysis in myocardial infarction; GRACE, global registry of acute coronary events; LMW, low-molecular-weight; ACE, angiotensin-converting enzyme; ARB, angiotensin-receptor blockers; NT-proBNP, N-terminal pro−B-type natriuretic peptide, GDF-15, growth differentiation factor 15; CRP; C-reactive protein.

Table S2. Multivariable effect of baseline characteristics and biomarkers on legumain levels at baseline.

Background characteristic	Relative change	95% C.I.	*p* value
Age, 10 year increase	0.9359	(0.9169 ; 0.9552)	<.0001
STEMI	0.9146	(0.8799 ; 0.9506)	<.0001
GPIIb/IIIa inhibitor	0.9199	(0.8834 ; 0.9579)	<.0001
GDF-15, 10% increase	1.0108	(1.0068 ; 1.0147)	<.0001
Platelet count, 10% increase	1.0287	(1.0220 ; 1.0354)	<.0001
hsCRP, 10% increase	1.0029	(1.0014 ; 1.0044)	0.0002
IL-6, 10% increase	1.0047	(1.0022 ; 1.0072)	0.0002
Hb, 10% increase	1.0316	(1.0151 ; 1.0483)	0.0002
WBC, 10% increase	0.9891	(0.9833 ; 0.9950)	0.0003
Chronic renal disease	0.8451	(0.7669 ; 0.9312)	0.0007
NT-proBNP, 10% increase	1.0026	(1.0010 ; 1.0042)	0.0014
sCD40L, 10% increase	1.0023	(1.0008 ; 1.0037)	0.0025
Dyslipidemia	0.9483	(0.9147 ; 0.9832)	0.0040
Female sex	0.9474	(0.9084 ; 0.9882)	0.0120
Unfractioned heparin	0.9560	(0.9216 ; 0.9915)	0.0157
Proton pump inhibitor	0.9591	(0.9264 ; 0.9929)	0.0182
Diabetes	1.0496	(1.0059 ; 1.0952)	0.0258
LMW heparin	1.0431	(1.0048 ; 1.0828)	0.0269
PAD	1.0736	(1.0022 ; 1.1500)	0.0431
Cholesterol lowering (statin)	0.9254	(0.8556 ; 1.0008)	0.0523
Myocardial infarction	1.0499	(0.9989 ; 1.1035)	0.0551
Diuretic	1.0352	(0.9967 ; 1.0752)	0.0736
Aspirin at admission	0.9211	(0.8341 ; 1.0172)	0.1044
Hypertension	1.0287	(0.9901 ; 1.0689)	0.1465
CABG	1.0573	(0.9728 ; 1.1490)	0.1896
sP-selectin, 10% increase	1.0022	(0.9988 ; 1.0056)	0.2117
CHF	1.0492	(0.9700 ; 1.1348)	0.2305
Habitual smoker	1.0189	(0.9790 ; 1.0604)	0.3589
Troponin (hs-cTn), 10% increase	0.9995	(0.9984 ; 1.0006)	0.3970
Ox-LDL, 10% increase	0.9984	(0.9940 ; 1.0028)	0.4747
BMI, ≥ 30 kg/m2	1.0079	(0.9700 ; 1.0472)	0.6887
Cystatin C, 10% increase	0.9993	(0.9935 ; 1.0051)	0.8003
Non-haemorrhagic stroke	1.0102	(0.9236 ; 1.1048)	0.8250
D-dimer, 10% increase	0.9999	(0.9974 ; 1.0024)	0.9410
PCI	0.9993	(0.9405 ; 1.0618)	0.9826

Linear model for log-transformed legumain. The relative increase is the adjusted geometric mean ratio between subgroups or for the stated change in continuous variables. STEMI, ST-segment elevation myocardial infarction; GPIIb/IIIa, glycoprotein GPIIb/IIIa; GDF-15, growth differentiation factor 15; CRP; C-reactive protein; Hb, hemoglobin; WBC, white blood cells; NT-proBNP, N-terminal pro−B-type natriuretic peptide, LMW, low-

molecular-weight; PAD, peripheral artery disease; CABG, coronary artery bypass graft; CHF, chronic heart failure, Ox-LDL, oxidized low density lipoprotein, BMI, body mass index; PCI, percutaneous coronary intervention.

Figure S1. Restricted cubic-spline of the interaction between baseline legumain levels and the primary outcome (composite of cardiovascular death (CV), spontaneous myocardial infarction, and stroke) of the two treatment groups; clopidogrel or ticagrelor.
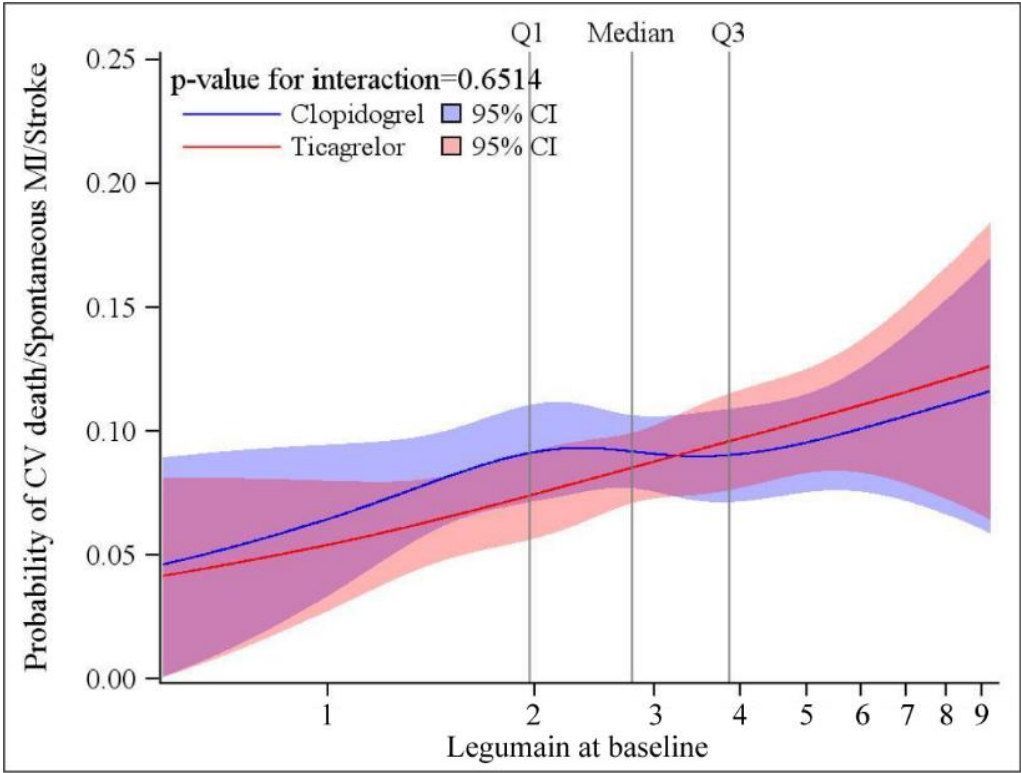
Figure S2. Cubic spline curves for legumain levels at one month against the primary and secondary outcomes.