Circulating Concentrations of Follistatin-Like 1 in Healthy Individuals and Patients with Acute Coronary Syndrome as Assessed by an Immunoluminometric Sandwich Assay

Christian Widera,1 Rüdiger Horn-Wichmann,2 Tibor Kempf,1 Kerstin Bethmann,1 Beate Fiedler,1 Sarita Sharma,1 Ralf Lichtinghagen,3 Holger Leitolf,2 Boris Ivandic,4 Hugo A. Katus,4 Evangelos Giannitsis,4 and Kai C. Wollert1*

BACKGROUND: Follistatin-like 1 (FSTL1) is a 308–amino acid secreted glycoprotein. Tissue levels of FSTL1 are induced in animal models and patients with chronic inflammatory and cardiovascular disease. We hypothesized that FSTL1 can be measured in the human circulation and used as a biomarker in acute coronary syndrome (ACS).

METHODS: We developed an immunoluminometric assay (ILMA), assessed the preanalytic characteristics of FSTL1, and determined circulating FSTL1 concentrations in 120 apparently healthy individuals and 216 patients with ACS.

RESULTS: The assay had a limit of detection of 0.17 μg/L, limit of quantification of 1.02 μg/L, intraassay imprecision of ≤12.7%, and interassay imprecision of ≤15.4%. Selectivity was demonstrated with size-exclusion chromatography and lack of cross-reactivity with related proteins. The assay was not appreciably influenced by unrelated biological substances. FSTL1 in serum or whole blood was stable at room temperature for 48 h and was resistant to 4 freeze-thaw cycles. Measured FSTL1 concentrations in citrated plasma and heparin-treated plasma were 18% and 17% lower, respectively, than concentrations measured in serum. Apparently healthy individuals presented with a median FSTL1 serum concentration of 7.18 (range 1.06–18.49) μg/L. Serum FSTL1 concentrations were increased in ACS and related to the risk of all-cause mortality during follow-up.

CONCLUSIONS: The ILMA permits detection of FSTL1 in human serum and plasma. We expect that the favorable preanalytic characteristics of FSTL1 and the reference limits defined here for apparently healthy individuals will facilitate future studies of FSTL1 as a biomarker in various disease settings, including ACS.

© 2009 American Association for Clinical Chemistry

Follistatin-like 1 (FSTL1)5 is a 308–amino acid extracellular glycoprotein that was originally cloned from a mouse osteoblastic cell line as a transforming growth factor β–inducible gene (1). Rat and human homologs have been cloned from glia cells (2). FSTL1 amino acid sequences are highly conserved across these species (>92% sequence identity). Human FSTL1 contains 3 putative N-glycosylation sites, undergoes extensive posttranslational modifications, and has a M₉ of approximately 45 000–55 000 (3). FSTL1 shares a characteristic structural module, the FS domain, with follistatin (FST), follistatin-like 3 (FSTL3), and members of the SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin) protein family.

Recent studies have implicated FSTL1 in the pathophysiology of inflammation and cardiovascular disease. FSTL1 expression is induced in synovial tissue in patients with rheumatoid arthritis (4, 5). Transfection of FSTL1 into macrophages leads to upregulation of proinflammatory cytokines (6), whereas neutralization of endogenous FSTL1 ameliorates inflammation in a mouse arthritis model (5), indicating that FSTL1 may act as a proinflammatory molecule. Cardiac expression levels of FSTL1 are upregulated in rodent models of myocardial infarction or pressure overload hypertrophy (7, 8). Cardiac expression levels of FSTL1

1 Department of Cardiology and Angiology, 2 Department of Gastroenterology, Hepatology, and Endocrinology, and 3 Department of Clinical Chemistry, Hannover Medical School, Hannover, Germany; 4 Department of Medicine III, University of Heidelberg, Heidelberg, Germany.

* Address correspondence to this author at: Klinik für Kardiologie und Angiologie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Fax +49-511-532-5307; e-mail wollert.kai@mh-hannover.de.

Received April 21, 2009; accepted June 23, 2009.

Previously published online at DOI: 10.1373/clinchem.2009.129411

© 2009 American Association for Clinical Chemistry

1 Nonstandard abbreviations: FSTL1, follistatin-like 1; FST, follistatin; SPARC, secreted protein acidic and rich in cysteine; ACS, acute coronary syndrome; ILMA, immunoluminometric assay; rh, recombinant human; NT-proBNP, N-terminal pro-B-type natriuretic peptide; LV, left ventricular; NSTEMI, non-ST-elevation myocardial infarction; STEMI, ST-elevation myocardial infarction; cTnT, cardiac troponin T; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; MDRD, Modification of Diet in Renal Disease.
are also increased in patients with advanced heart failure, and return to normal following mechanical unloading by left ventricular assist device implantation (8). Adenoviral gene transfer of FSTL1 reduces myocardial infarct sizes after ischemia-reperfusion injury (7) and promotes revascularization after hind-limb ischemia in mice (9), suggesting that FSTL1 may serve a protective role after tissue ischemia.

Considering that FSTL1 is a secreted protein, and that FSTL1 expression is induced in the heart in response to ischemia and hemodynamic stress, we hypothesized that FSTL1 protein is detectable in the human circulation and that circulating concentrations of FSTL1 are increased in patients presenting with acute coronary syndrome (ACS).

In the present study, we established a sandwich immunoluminometric assay (ILMA) to measure FSTL1 in human serum and plasma. We used this technique to assess the preanalytic characteristics of the analyte and to establish reference values for FSTL1 in apparently healthy individuals. Given the potential importance of FSTL1 in heart disease, we also assessed circulating FSTL1 concentrations in 216 patients with ACS and related the FSTL1 concentration to all-cause mortality during follow-up. Our assay provides a basis for further investigation of FSTL1 as a biomarker in human disease.

Materials and Methods

MATERIALS
Recombinant human (rh) FSTL1 (custom-made, expressed in NSO mouse myeloma cells), monoclonal murine antihuman FSTL1 antibody (MAB1694), polyclonal FSTL1 affinity chromatography-purified goat antihuman FSTL1 IgG antibody (AF1694), rhFSTL1 (669-FO/CF), rhFSTL3 (1288-F3/CF), and rhSPARC (941-SP) were purchased from R&D Systems.

Blood samples
To establish the FSTL1 ILMA and assess the preanalytic characteristics of FSTL1, we obtained serum, citrated plasma, and heparin-treated plasma samples from apparently healthy individuals (n = 10; 6 men 23–41 years old) and ACS patients (n = 9; 7 men 44–65 years old). All individuals provided written informed consent, and the ethics committee of Hannover Medical School approved the study.

FSTL1 sandwich ILMA
Maxisorp Startubes (Nunc) were coated overnight at 4 °C with 0.5 µg monoclonal anti-FSTL1 antibody in 0.1 mol/L sodium carbonate buffer (pH 9) and were washed 3 times with PBS (40 mmol/L sodium phosphate, 150 mmol/L sodium chloride, pH 7.4) containing 1 mL/L Tween 20. The polyclonal anti-FSTL1 antibody was labeled with a 10-fold molar excess of acridinium for 15 min at room temperature under alkaline conditions (pH 8). Serum and plasma samples (50 µL) were diluted with 150 µL assay buffer [30 g/L BSA (Sigma), 10 g/L bovine IgG, 10 mL/L goat serum, 1 mL/L sodium azide, 1 mol/L sodium chloride, 40 mmol/L sodium phosphate, pH 7.4] containing acridinium-labeled polyclonal anti-FSTL1 (0.66 µg/mL), added to the tubes, and incubated for 16 h at 4 °C. After removal of the samples, we washed the tubes 3 times with phosphate-buffered saline containing 1 mL/L Tween 20 and quantified bound fluorescence in an Autolumat Plus LB 953 luminometer (Berthold). All measurements were performed in duplicate. In each experiment, we generated a calibration curve with various concentrations of rhFSTL1 and used it to calculate FSTL1 concentrations in individual samples (an rhFSTL1 stock solution was stored in aliquots at −70 °C). A typical calibration curve is shown in Supplemental Fig. 1, which accompanies the online version of this article at www.clinchem.org/content/vol55/issue10. Pooled control samples (also stored in aliquots at −70 °C) were analyzed with each single run.

We assessed assay linearity with serial dilutions (in assay buffer) of 6 serum samples obtained from healthy individuals and patients with ACS containing different FSTL1 concentrations (5.42–29.97 µg/L) (see online Supplemental Fig. 2).

Assays of pools of 5 serum samples with low FSTL1 concentrations (8.23–13.53 µg/L) with 5 serum samples with higher concentrations (16.34–22.58 µg/L) in 5 different combinations yielded measured concentrations with means within the range of the expected concentrations (89%–107%).

Size-exclusion chromatography
We equilibrated Sephadex G-100 with Tris buffer (20 mmol/L Tris base, 150 mmol/L sodium chloride, 1 mL/L sodium azide, pH 7.0) for 2 h at 80 °C, filled a 408-mL column (Pharmacia) with the swollen dextran beads, and equilibrated the column with this Tris buffer containing 5 g/L BSA. We loaded a 1-mL serum sample from a patient with ACS (FSTL1 concentration 21.27 µg/L) onto the column and collected the eluate in 48 60-drop fractions. We then determined the FSTL1 concentration in each fraction by ILMA. We used blue dextran (Mw, 2 000 000; Pharmacia) to determine the column’s void volume and BSA (Mw, 66 000; Sigma) and parathyroid hormone (Mw, 4000; Bachem) as molecular mass markers. In an additional experiment, a 1-mL serum sample from a healthy individual (FSTL1 concentration 11.44 µg/L) was spiked with 360 ng rhFSTL1 and subjected to Sephadex column chromatography.
FSTL1 in Healthy Individuals and ACS

APPARENTLY HEALTHY COHORT
We obtained serum samples from 120 apparently healthy individuals (60 men) who were recruited in Heidelberg, Germany. The median age of this population was 43 (range 21–69) years. Individuals taking regular medication, individuals with chronic drug or alcohol abuse, and individuals with established cardiovascular disease or other chronic disease or acute illness were excluded. All participants were required to have nonpathologic serum creatinine, aspartate aminotransferase, alanine aminotransferase, thyroid-stimulating hormone, and hemoglobin concentrations, normal leukocyte and platelet counts, normal resting 12-lead electrocardiogram, normal physical examination, normal oral glucose tolerance test, and serum N-terminal pro–B-type natriuretic peptide (NT-proBNP) concentration <125 ng/L. In addition, all individuals underwent cardiac magnetic resonance imaging with dobutamine or adenosine stress to exclude individuals with left ventricular (LV) hypertrophy, LV dilation, LV systolic dysfunction, valvular heart disease, or stress-induced LV wall motion abnormalities or perfusion defects. All participants provided written informed consent, and the ethics committee of the University of Heidelberg approved the study.

ACS POPULATION
We also obtained serum samples from 216 consecutive patients (180 men) with ACS who were recruited at Hannover Medical School between August 2007 and September 2008. The median age of the patients was 67 (range 36–87) years. All patients were required to present with 1 or more episodes of angina at rest lasting ≥15 min. Sixty-two of these patients were diagnosed with unstable angina, 68 with non–ST-elevation myocardial infarction (NSTEMI), and 86 with ST-elevation myocardial infarction (STEMI). To be included in the present analysis, patients with unstable angina were required to have at least 1 angiographically documented stenosis ≥70% in a major coronary artery. Myocardial infarction was diagnosed based on the electrocardiogram and repeated cardiac troponin T (cTnT) measurements, using a cTnT decision threshold for the diagnosis of myocardial infarction of 0.03 μg/L, the lowest concentration assuring an analytical imprecision of <10% (10). Serum samples were obtained on admission and stored at −70 °C. We obtained information on survival status for all patients by telephone contact with the patient, spouse, or general practitioner after 6 months. Information on survival status at 12 months was available from 89 patients. All patients provided written informed consent, and the ethics committee of Hannover Medical School approved the study.

LABORATORY ANALYSES
We measured cTnT on an Elecsys instrument (Roche Diagnostics) and NT-proBNP on a Cobas e411 analyzer (Roche Diagnostics). We measured highsensitivity C-reactive protein (CRP) was measured with an immunoturbidimetric assay (Hitachi 917; Roche Diagnostics); samples with CRP concentrations >20 mg/L were reanalyzed with a Hitachi Modular (Roche Diagnostics). We calculated estimated glomerular filtration rate (eGFR) using the 4-variable Modification of Diet in Renal Disease (MDRD) Study equation as described (11).

STATISTICAL ANALYSES
Data are presented as mean (SD) or median with 25th and 75th percentiles. We used the CV, calculated as the (SD/mean) × 100%, as a measure of assay imprecision. We used the Mann–Whitney U-test to analyze differences between the medians of 2 groups, Student’s t-test to analyze differences between the means of 2 groups, linear regression to identify variables associated with FSTL1, and multiple linear regression analysis to identify variables that were independently associated with FSTL1. Variables that were not normally distributed were transformed to their natural logarithm (ln) for the regression analyses. We used the Kolmogorov–Smirnov test to assess normal distribution of variables, the Kaplan–Meier method to illustrate patient survival during follow-up in relation to baseline concentrations of FSTL1, and the log-rank test for statistical assessment. We used simple Cox regression analysis to further characterize the relation of FSTL1 to mortality and multiple Cox regression analysis to adjust for age and sex. All analyses were performed with StatView 5.0.1 (SAS Institute Inc.).

Results
TECHNICAL CHARACTERISTICS OF THE FSTL1 IIMA
Intraassay imprecision, determined by measuring 14 serum samples in 8–15 parallel measurements, ranged from 4.6% to 12.7% for samples containing 2.93–19.87 μg/L FSTL1. Total (interassay) imprecision was determined by measuring 19 serum samples in 8–20 assay runs on different days, by 2 different operators, and with different lots of tubes, tracer, and calibrator. The interassay imprecision ranged from 4.3% to 15.4% for samples containing 1.62–27.24 μg/L FSTL1. The limit of detection, calculated as the mean + 3SD for 10 replicate measurements of the zero standard (calibrator free of analyte), was 0.17 μg/L. The limit of quantification, defined as the lowest concentration that can be measured with a precision and deviation from target of <20%, was 1.02 μg/L (the limit of quantification was determined in serum samples diluted with assay buffer).
After adding 10 µg/L rhFSTL1 to 4 serum samples containing 9.41–14.14 µg/L FSTL1, recoveries of rhFSTL1 ranged from 83% to 95%.

Size-exclusion chromatographic results for a serum sample obtained from a patient with ACS revealed a single peak corresponding in Mr (approximately 50 000) to the mature, glycosylated human FSTL1 protein (Fig. 1). A virtually identical peak was obtained with another serum sample that had been spiked with rhFSTL1 (data not shown). To test for potential cross-reactivity with FSTL1-related proteins, we added increasing amounts of FST, FSTL3, or SPARC to 6 serum samples obtained from apparently healthy individuals and patients with ACS. We observed no cross-reactivity for the 3 proteins up to a tested concentration of 100 µg/L.

To assess whether unrelated biological substances interfere with the FSTL1 ILMA, we added several potentially interfering substances to 6 serum samples obtained from apparently healthy individuals and patients with ACS (FSTL1 concentration range 7.12–32.07 µg/L), as recommended by the Clinical Laboratory Standards Institute. The assay was not appreciably influenced by albumin (tested concentration 40 g/L), bilirubin (320 µmol/L), or hemoglobin (3.2 g/L). The FSTL1 concentrations obtained for samples with and without added interfering substances differed by <24% in all cases (P > 0.10).

PREANALYTIC PERFORMANCE OF FSTL1
We analyzed FSTL1 concentrations in parallel in serum, citrated plasma, EDTA-treated plasma, and heparin-treated plasma samples obtained from 4 healthy individuals and 6 patients with ACS containing different FSTL1 concentrations (range in serum 7.17–32.07 µg/L). After correction for sample dilution by the added anticoagulants, the mean (SD) FSTL1 concentrations obtained for citrated plasma [82% (19%); n = 10; P = 0.002] and heparin-treated plasma [83% (8%); n = 7; P = 0.020] were significantly lower than the values measured in serum (100%). FSTL1 concentrations measured in EDTA-treated plasma were not significantly different from the values measured in serum [97% (12%); n = 10; P = 0.54].

Four cycles of freezing (20 h at −70 °C) and thawing (4 h at room temperature) induced no discernible loss of FSTL1 immunoreactivity (88%–103% vs 100% at baseline) in samples of serum or plasma (containing citrate, EDTA, or heparin) from 9 healthy individuals (FSTL1 concentration range in serum 6.02–10.80 µg/L). Storage of these 9 serum samples for 48 h at room temperature or at 4 °C did not produce a discernible loss of FSTL1 immunoreactivity (87–123% vs 100% at baseline). Moreover, no discernible loss of FSTL1 immunoreactivity was observed in 5 samples of whole blood (FSTL1 concentration range in serum 10.32–14.76 µg/L) stored for 48 h at room temperature or 4 °C (86%–103% vs 100% at baseline).

FSTL1 CONCENTRATIONS IN APPARENTLY HEALTHY INDIVIDUALS
We used the ILMA to measure serum FSTL1 concentrations in a cohort of 120 apparently healthy individuals consisting of 60 men and 60 women with a median age of 43 (range 21–69) years. FSTL1 was detected in all samples. The median FSTL1 concentration was 7.18 µg/L (25th–75th percentiles 5.77–8.39 µg/L; range 1.06–18.49 µg/L) (Fig. 2). Men presented with slightly higher FSTL1 concentrations than women [median (25th–75th percentiles) 7.62 (6.43–8.56) µg/L vs 6.74 (5.40–7.94) µg/L; P = 0.033]. FSTL1 concentrations

---

**Fig. 1.** Size-exclusion chromatogram of a serum sample analyzed for FSTL1 concentration. Numbers in parentheses indicate molecular masses. V₀ indicates void volume.

---

**Fig. 2.** Distribution of serum FSTL1 concentrations in 120 apparently healthy individuals and 216 patients with ACS.
decreased slightly with age ($R = -0.19; P = 0.037$) (Fig. 3).

**FSTL1 Concentrations in Patients with ACS**

Next, we determined serum FSTL1 concentrations on admission in a cohort of 216 patients with ACS, consisting of 180 men (83.3%) and 36 women (16.7%) with a median age of 67 (range 36–87) years. The patient cohort was older than the healthy control group ($P < 0.001$) and included more men ($P = 0.014$).

FSTL1 was detected in all patients. The median FSTL1 concentration was 13.50 µg/L (25th–75th percentiles 11.96–15.60 µg/L; range 8.82–30.46 µg/L). FSTL1 concentrations were significantly higher in ACS patients than in healthy controls ($P < 0.001$) (Fig. 2). A considerable overlap in the FSTL1 concentrations was observed between patients diagnosed with unstable angina [median (25th–75th percentiles) 13.48 (12.21–14.82) µg/L], NSTEMI [14.44 (12.63–16.16) µg/L], and STEMI [13.02 (11.41–15.24) µg/L]. The FSTL1 concentration in patients with cTnT concentrations ≤0.01 µg/L on admission and throughout the hospital stay was 13.24 (11.97–14.46) µg/L.

FSTL1 concentrations were significantly higher in patients with diabetes, Killip class >1, and cTnT concentration ≥0.03 µg/L on admission (Table 1). FSTL1 concentrations were also related to the concentrations of NT-proBNP and CRP; moreover, FSTL1 was inversely related to eGFR (Table 1). In a multiple linear regression analysis that used ln(FSTL1) as the dependent variable, and that included all variables shown in Table 1 except for LDL cholesterol (available in only 71 patients), younger age ($P < 0.001$), male sex ($P = 0.014$), diabetes ($P < 0.001$), the concentrations of NT-proBNP ($P < 0.001$) and CRP ($P < 0.001$), and reduced eGFR ($P < 0.001$) were independently associated with FSTL1 (Table 2). The $R^2$ value of this model was 0.33.

![Fig. 3. Correlation between FSTL1 and age in 120 apparently healthy individuals.](image-url)

**Table 1. Baseline characteristics of the ACS patients according to FSTL1 level.**

<table>
<thead>
<tr>
<th>FSTL1 concentration, µg/L</th>
<th>First quartile (7.27–11.9)</th>
<th>Second quartile (11.96–13.49)</th>
<th>Third quartile (13.50–15.55)</th>
<th>Fourth quartile (15.65–30.46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>54</td>
<td>54</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>Age, years</td>
<td>67 (56–72)</td>
<td>64 (57–70)</td>
<td>69 (59–78)</td>
<td>69 (58–76)</td>
</tr>
<tr>
<td>Male sex</td>
<td>43 (80)</td>
<td>46 (85)</td>
<td>43 (80)</td>
<td>48 (89)</td>
</tr>
<tr>
<td>Smokingb</td>
<td>42 (78)</td>
<td>40 (74)</td>
<td>40 (74)</td>
<td>37 (69)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35 (65)</td>
<td>38 (70)</td>
<td>34 (63)</td>
<td>33 (61)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4 (7)</td>
<td>8 (15)</td>
<td>16 (30)</td>
<td>16 (30)</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dLc</td>
<td>135 (104–164)</td>
<td>124 (96–169)</td>
<td>114 (105–169)</td>
<td>134 (107–148)</td>
</tr>
<tr>
<td>Previous myocardal infarction</td>
<td>14 (26)</td>
<td>17 (31)</td>
<td>10 (19)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Killip class &gt;1</td>
<td>1 (2)</td>
<td>3 (6)</td>
<td>2 (4)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>cTnT ≥0.03 µg/L</td>
<td>22 (41)</td>
<td>27 (50)</td>
<td>21 (39)</td>
<td>39 (72)</td>
</tr>
<tr>
<td>NT-proBNP, ng/L</td>
<td>261 (79–652)</td>
<td>236 (101–862)</td>
<td>427 (134–1079)</td>
<td>1619 (300–6838)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.7 (0.9–3.9)</td>
<td>3.5 (1.9–7.3)</td>
<td>2.0 (1.2–5.0)</td>
<td>7.0 (2.6–30.0)</td>
</tr>
<tr>
<td>eGFR, mL/min</td>
<td>72 (62–82)</td>
<td>66 (55–82)</td>
<td>66 (56–79)</td>
<td>51 (34–70)</td>
</tr>
</tbody>
</table>

* Data are n (%) or median (25th–75th percentile). Linear regressions were used to evaluate relations of FSTL1 to baseline characteristics and calculate $P$ values (FSTL1, NT-proBNP, and CRP were not normally distributed and were entered as ln-transformed variables).

b Includes current and previous smoking.

c Data on LDL cholesterol were available from 71 patients.
Fifteen patients died during follow-up. The risk of death was significantly higher in patients presenting with a FSTL1 concentration above the median than in patients with a FSTL1 concentration below the median ($P = 0.019$) (Fig. 4). An increase of 1 SD of ln(FSTL1) was associated with a mortality hazard ratio of 2.1 (95% CI 1.4–3.1; $P = 0.001$). After correction for age and sex, the hazard ratio was 2.1 (95% CI 1.3–3.3; $P = 0.002$).

**Discussion**

We have developed a sandwich ILMA for the measurement of human FSTL1, assessed the preanalytic characteristics of FSTL1, evaluated circulating FSTL1 concentrations in a carefully selected cohort of apparently healthy individuals, and demonstrated that the circulating FSTL1 concentrations are increased and related to all-cause mortality in patients with ACS. To the best of our knowledge, this study is the first to measure FSTL1 in the human circulation.

The FSTL1 ILMA has a limit of detection of 0.17 μg/L and a limit of quantification of 1.02 μg/L. No individual in the present study presented with a FSTL1 concentration below this concentration. The assay has an adequate intraassay and interassay imprecision. The assay is not appreciably influenced by unrelated biological substances, such as albumin, bilirubin, and hemoglobin, and shows no cross-reactivity with human FST, FSTL3, or SPARC. Size-exclusion chromatography of a serum sample from an ACS patient and a serum sample from an apparently healthy individual that had been spiked with an excess amount of rhFSTL1 produced a single peak consistent with the $M_r$ of glycosylated human FSTL1 (3).

Linearity studies indicated a curvilinear relationship between dilution and measured concentration. We suggest that there may be 1 or more fragments of FSTL1 that saturate part of the detection antibody. This will then lead to falsely low concentrations in original serum that will be diluted out using assay buffer.

The FSTL1 protein was stable in serum and whole blood at room temperature for at least 48 h and was resistant to 4 freeze-thaw cycles. The measured FSTL1 concentrations in citrated plasma and heparin-treated plasma were somewhat lower than concentrations measured in serum or EDTA-treated plasma.

We observed a weak inverse correlation between serum FSTL1 concentrations and age in apparently healthy individuals. FSTL1 concentrations were 13.1% higher in apparently healthy men than in women. Considering the substantial overlap in FSTL1 concentrations between younger and older individuals, and between males and females, definition of separate reference intervals for these subgroups may not be justified, and we propose to use 12.0 μg/L, the rounded mean + 2SD, as the upper limit of the reference interval in apparently healthy individuals [serum FSTL1 concentrations in the apparently healthy cohort were normally distributed; 3 of these 120 individuals (2.5%) had FSTL1 concentrations >12.0 μg/L].

Serum FSTL1 concentrations were increased significantly in patients with ACS, 74.1% of whom presented with a FSTL1 concentration >12.0 μg/L. Although the ACS patients were older and included more men than the control group of apparently healthy individuals, these differences cannot explain the 1.88-fold higher median FSTL1 concentration in ACS, considering that FSTL1 concentrations decreased with age.

**Table 2. Independent association of FSTL1 with baseline variables in ACS.**

<table>
<thead>
<tr>
<th>B</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per 10 years)</td>
<td>-0.039</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.079</td>
</tr>
<tr>
<td>Smokingb</td>
<td>-0.010</td>
</tr>
<tr>
<td>Hypertension</td>
<td>-0.004</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.105</td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>-0.030</td>
</tr>
<tr>
<td>Killip class &gt;1</td>
<td>0.062</td>
</tr>
<tr>
<td>cTnT ($\geq 0.03$ vs &lt;0.03 μg/L)</td>
<td>-0.033</td>
</tr>
<tr>
<td>NT-proBNP (per 1 SD in ln scale)</td>
<td>0.075</td>
</tr>
<tr>
<td>CRP (per 1 SD in ln scale)</td>
<td>0.050</td>
</tr>
<tr>
<td>eGFR (per 1 mL/min)</td>
<td>-0.002</td>
</tr>
</tbody>
</table>

*a Multiple linear regression analysis—association with ln(FSTL1). B, unstandardized B coefficient.

*b Includes current and previous smoking.

**Fig. 4. Kaplan–Meier survival curves in 216 patients with ACS stratified according to serum FSTL1 concentration on admission.**
and were only slightly increased in men in the control group. By multiple linear regression analysis, we identified several clinical (younger age, male sex, diabetes, low eGFR) and biochemical (NT-proBNP, CRP) variables that were independently predictive of increased FSTL1 concentration in patients with ACS. The independent relation to NT-proBNP indicates that FSTL1 may reflect, to some extent, cardiac pathologies. Supporting this hypothesis, FSTL1 is induced in the heart in rodent models of pressure overload and myocardial infarction (7, 8) and in patients with advanced heart failure (8). We also observed an independent relation of FSTL1 to CRP, which is in agreement with its proposed role as an inflammatory mediator (4–6). The independent relation of FSTL1 to eGFR suggests that FSTL1 is cleared from the circulation at least in part through the kidneys and/or that FSTL1 synthesis increases in renal disease. These clinical and biochemical characteristics explained only some of the variability of the FSTL1 concentrations in ACS ($R^2 = 0.33$), indicating that FSTL1 concentrations are influenced by additional, as yet unknown, factors.

Circulating FSTL1 concentrations were increased to a similar degree in patients diagnosed with unstable angina, NSTEMI, and STEMI, indicating that FSTL1 may not be related to the extent of myocardial necrosis during an episode of ACS. In line with this conclusion, no independent relation of FSTL1 to admission cTnT concentrations was observed. However, more studies are needed to explore the relation of FSTL1 to biomarkers of myocardial necrosis at serial time points after hospital admission in patients with ACS.

Notably, FSTL1 concentrations were related to the risk of all-cause mortality during follow-up, also after correction for age and sex. Because of the small sample size, we were unable to correct for additional confounders of the relationship between FSTL1 and mortality. Studies of larger patient populations will be necessary to determine whether FSTL1 adds to other established clinical risk predictors in ACS. Our assay uses only commercially available reagents, which should enable other groups to establish the test and measure FSTL1 in ACS and other disease settings. The favorable preanalytic characteristics of FSTL1 will be advantageous in these studies.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors’ Disclosures of Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

**Employment or Leadership:** None declared.

**Consultant or Advisory Role:** None declared.

**Stock Ownership:** None declared.

**Honoraria:** None declared.

**Research Funding:** K.C. Wollert was supported by the German Research Foundation (SFB 566).

**Expert Testimony:** None declared.

**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

**Acknowledgments:** We gratefully acknowledge Ivonne Marquardt for expert technical assistance. Reagents for measuring NT-proBNP and hsCRP were kindly provided by Roche Diagnostics.

**References**


