ORIGINAL RESEARCH

Growth Differentiation Factor 15 Is Associated With Major Amputation and Mortality in Patients With Peripheral Artery Disease

Judith J. De Haan, MSc; Saskia Haitjema, MD, PhD; Hester M. den Ruijter, PhD; Gerard Pasterkamp, MD, PhD;* Gert J. de Borst, MD, PhD; Martin Teraa, MD, PhD; Marianne C. Verhaar, MD, PhD; Hendrik Gremmels, MD, PhD;* Saskia C. A. de Jager, PhD*

***Background*—**Peripheral artery disease (PAD) is one of the most common clinical presentations of atherosclerosis, and its prevalence is still increasing. Despite improvement of health care, morbidity and mortality risks remain high, including the risk of amputation. GDF15 (growth differentiation factor 15) is a member of the transforming growth factor family that is involved in apoptosis and inflammation; therefore, GDF15 is a potential biomarker to identify patients at high risk of adverse clinical outcomes.

***Methods and Results*—**Circulating GDF15 levels were measured using a multiplex immunoassay in patients with critical limb ischemia and PAD from 2 different patient cohorts that included patients with clinically manifest PAD: the JUVENTAS (Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation) trial (n=160, 67 major events; critical limb ischemia) and the Athero-Express Biobank (n=386, 64 major events; PAD). Kaplan–Meier curves demonstrated that high levels of GDF15 were associated with increased risk of major events, defined as major amputation (at or above the ankle joint) and all-cause mortality, in both cohorts (highest versus lowest, JUVENTAS: hazard ratio: 4.01 [95% confidence interval, 2.05–7.84; $P<0.0001$]; Athero-Express: hazard ratio: 3.27 [95% confidence interval, 1.64–6.54; $P=0.0008$]). In the JUVENTAS trial, this was more pronounced in women. Cox proportional multivariable regression models with median follow-up of 3 years, corrected for common confounders, showed hazard ratios of 1.70 (95% confidence interval, 1.18–2.69; $P=0.0053$) and 1.57 (95% confidence interval, 1.02–2.41; $P=0.041$) per 2.78-fold increase of GDF15 in JUVENTAS and Athero-Express, respectively.

***Conclusions*—**High GDF15 levels are associated with increased risk of major amputation and/or death in PAD patients. GDF15 levels could be of additive value to identify patients who are at high risk of amputation or death and could help guide treatment choices. **(*J Am Heart Assoc*. 2017;6:e006225. DOI: 10.1161/JAHA.117.006225.)**

Key Words: biomarker • cardiovascular disease risk factors • cytokine • growth differentiation factor 15 • follow-up studies • mortality • peripheral artery disease • revascularization • secondary prevention • vascular biology • women

Peripheral artery disease (PAD) is one of the most common clinical presentations of atherosclerosis.[1] In 2010, the global prevalence of PAD was 202 million, and this is ever increasing.[2] Critical limb ischemia is the most severe form of PAD, and mortality rates reach up to 20% in the first 6 months after diagnosis.[3] Despite improved health care for atherosclerosis and subsequent increased survival in PAD patients, morbidity and mortality risks in PAD patients are still high.[2,4] Patients with critical limb ischemia are at particularly high risk of major amputation. To improve patient management, it is important to identify those patients who are at exceedingly high risk of amputation and death.

GDF15 (growth differentiation factor 15) is a protein found in circulation that is a member of the transforming growth factor family. GDF15 is a promising prognostic biomarker for cardiovascular disease. GDF15 levels are a strong predictor of mortality

From the Laboratory for Experimental Cardiology (J.J.D.H., S.H., H.d.R., G.P., S.C.A.D.J.), Laboratory for Clinical Chemistry and Haematology (G.P.), Department of Vascular Surgery (G.J.d.B., M.T.), Department of Nephrology and Hypertension (M.C.V., H.G.), Laboratory of Translational Immunology (S.C.A.D.J.), University Medical Center Utrecht, the Netherlands.

Accompanying Figures S1 through S4 and Table S1 are available at http://jaha.ahajournals.org/content/6/9/e006225/DC1/embed/inline-supplementary-material-1.pdf

*Dr Gremmels and Dr De Jager contributed equally to this work.

Correspondence to: Saskia C. A. de Jager, PhD, Heidelberglaan 100, Huispostnummer G03.550, 3584 CX Utrecht, the Netherlands. E-mail: s.c.a.dejager@umcutrecht.nl

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Clinical Perspective

What Is New?

- High GDF15 (growth differentiation factor 15) levels in blood are associated with increased risk of amputation or death in patients with peripheral artery disease (PAD).
- In patients with severe PAD, GDF15 levels in blood appear to be more strongly associated with major events in women.
- Circulating GDF15 is highly correlated with hemoglobin levels.
- Circulating GDF15 levels do not correlate with specific plaque characteristics.

What Are the Clinical Implications?

- GDF15 levels in blood can predict outcome in PAD patients.
- Circulating GDF15 levels can improve prediction beyond traditional risk factors in patients with severe PAD.
- Sex-adjusted risk prediction might be needed for patients with severe PAD.

and recurrent myocardial infarction in patients with acute coronary syndrome.[5–10] Furthermore, high levels of GDF15 are associated with the risk of developing adverse left ventricular remodeling and heart failure.[11–15] In heart failure patients, both with preserved and reduced ejection fraction, high GDF15 levels identify patients at high risk of rehospitalization and death.[14]

The causal role of GDF15 in atherosclerosis has been studied in vitro and in animal models. GDF15 affects early plaque formation, progression, and plaque composition.[16,17] From a mechanistic point of view, GDF15 modulates CCR2 (C-C motif chemokine receptor 2)–mediated macrophage chemotaxis, affects macrophage apoptosis,[18] and inhibits the proliferation of endothelial cells.[19] Macrophage accumulation and endothelial dysfunction play key roles in atherosclerosis and thus are important in PAD patients.

The prognostic value of GDF15 in PAD is currently unknown. Consequently, the aim of this study was to determine the association of GDF15 levels with major events, namely, major amputation and all-cause mortality, in PAD patients. For this study, we used 2 independent cohorts: the JUVENTAS (Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation) trial[20] and the Athero-Express Biobank study.[21]

Methods

JUVENTAS Trial

Patient population

The JUVENTAS trial was a single-center (University Medical Center in Utrecht, Utrecht, the Netherlands), placebo-controlled, double-blinded, randomized trial on repeated intra-arterial injections of bone marrow–derived mononuclear cells in patients with nonrevascularizable severe PAD (Fontaine classes IIb, III, and IV). Autologous erythrocytes were used as a placebo control. Patients with severe PAD (Fontaine classes IIb, III, and IV) who were not eligible for conventional revascularization were included in this study. The trial was registered at ClinicalTrials.gov (identifier NCT00371371). No effect by the treatment on primary outcome was observed.[20]

Patient selection

Patients with a life expectancy of <1 year were excluded.[22] In total, 160 patients were enrolled in this trial between 2006 and 2012. The primary outcome was major amputation (through or above the ankle joint) or death at 6 months of follow-up. For the present study, the follow-up was extended, leading to a median follow-up duration of 3 years.

Sample collection

Blood was drawn at inclusion in the trial, and serum was collected and stored at −80°C until further analysis.

Athero-Express Biobank Study

Patient population

The Athero-Express Biobank is an ongoing cohort study that includes patients undergoing carotid and iliofemoral endarterectomy in 2 Dutch hospitals (St. Antonius Hospital, Nieuwegein, and University Medical Center Utrecht). Blood and plaque specimens are collected from these patients, and follow-up is for 3 years.[21] The study was approved by the ethics boards of both hospitals, and informed consent was obtained from all patients.

Patient selection

All patients who underwent an iliac or femoral endarterectomy between 2002 and 2013 with available plasma samples were included in the current study (n=386). Standardized questionnaires and hospital patient records were used to collect clinical data. The Athero-Express Biobank includes mainly patients with intermittent claudication.

Sample collection

The sample collection protocol was described in detail previously.[21] Briefly, preoperative blood was drawn, and citrate plasma was collected and stored at −80°C until further analysis.

Immediately after surgery, the plaque was processed. The segment with the culprit lesion (smallest lumen) or the segment with the largest plaque diameter (in case of total occlusion) was chosen to be fixed in 4% formaldehyde, decalcified, and embedded in paraffin.

Histological assessment

Histological slides were evaluated using a previously validated protocol.[23,24] In short, per-patient staining was performed with CD68 (macrophages), α-actin (smooth muscle cells), picrosirius red (collagen), elastin, hematoxylin and eosin, and Mallory's phosphotungstic acid–hematoxylin.

Intraplaque hemorrhage was determined by hematoxylin and eosin and Mallory's staining. Luminal thrombosis and intraplaque hemorrhage were considered plaque thrombosis.

Collagen and calcification were semiquantitatively scored and separated into binary groups of no or minor and moderate or heavy staining. The size of the lipid core was assessed with the use of polarized light. Smooth muscle cells and macrophages were automatically quantified as percentages of plaque area (AnalySiS version 3.2; Soft Imaging GmbH).

GDF15 Multiplex Immunoassay

GDF15 levels in plasma were determined by multiplex immunoassay.

For Athero-Express samples, a Human Magnetic Luminex Assay (R&D Systems) was used, according to the manufacturer's protocol. Citrate plasma samples were diluted 1:1 with Calibrator Diluent RD6-52 (R&D systems). For JUVENTAS samples, serum measurements were performed using an in-house developed and validated multiplex immunoassay based on Luminex technology (xMAP; Luminex), using MagPlex microspheres (as described by van Balkom et al.[25]). The assay contained a standard curve based on custom-made control peptides and controls for heterophylic antibodies. Acquisition was performed on the Bio-Plex system (Bio-Rad Laboratories) with the Bio-Plex 3D reader (Luminex FlexMAP 3D) in combination with xPONENT software version 4.2 (Luminex). Data were analyzed by 5-parameter curve fitting using Bio-Plex Manager software, version 6.1.1 (Bio-Rad).

Outcomes

The primary outcome of the present study was major amputation-free survival (AFS), for which an event was defined as either amputation through or above the ankle joint or all-cause mortality.

Statistical Analysis

Continuous data are reported as mean±SD when normally distributed and as median and interquartile range (IQR) otherwise. GDF15 plasma (Athero-Express) or serum (JUVENTAS) values were log-transformed and intercept-normalized per plate median; therefore, values are reported as lnGDF15. Independent-samples Student *t* tests were used to compare means of continuous data. Survival analysis was performed using the Kaplan–Meier method or Cox proportional hazards regression. For Cox regression, hazard ratios (HRs) are presented as tertiles or unstandardized β for lnGDF15 and thus reflect the HR per ln increase in GDF15. Multivariable Cox regression was used to create simple (age and sex) and full adjusted models based on the literature and on clinical relevance (age, sex, body mass index, estimated glomerular filtration rate, diabetes mellitus, smoking, hypertension, and history of coronary artery disease). The proportional hazards assumptions for all presented Cox models were evaluated by plotting Schoenfeld residuals. In no case were these proportional hazards assumptions violated. To estimate a dose-response relationship, a restricted cubic spline (3 knots) was fit in the time-to-event analysis. To investigate the possibility of risk stratification, multivariable logistic regression models were created to predict major events at 1 year after inclusion. Receiver operating characteristic curves were presented to summarize predictive performance of different models, Net reclassification improvement was used to compare models. To analyze the plaque characteristics in the Athero-Express Biobank, χ^2 testing was used. A P value <0.05 was considered statistically significant. All analyses were performed with R software (version 3.1.1).

Results

Baseline Patient Characteristics

In the JUVENTAS trial, median age was 67 years (range: 56–76 years), and 68% of participants were male. A history of other cardiovascular diseases, such as myocardial infarction, was abundantly present (Table 1). In the Athero-Express Biobank, median age was 69 years (range: 62–75 years), and 73% of participants were male. Most patients had a history of cerebrovascular accident or coronary artery disease (Table 1).

In JUVENTAS, at last available follow-up (median follow-up time of 3 years), 67 of 160 patients (42%) experienced a major event (41 patients underwent a major amputation; 40 patients died, of which 14 underwent an amputation before death; see Table 1 for baseline characteristics). In Athero-Express, at 3 years after inclusion, 64 of 386 patients experienced a major event (13 patients underwent a major amputation; 54 patients died, of which 3 underwent an amputation before death; Table 1).

Patients Who Experienced a Major Event Had Higher GDF15 Levels

The median GDF15 level was 1793 pg/mL (IQR: 1043–2943 pg/mL) in JUVENTAS and 1574 pg/mL (IQR: 1070–2102 pg/mL) in Athero-Express. GDF15 levels were

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Table 1. Baseline Characteristics of JUVENTAS and Athero-Express

	JUVENTAS				Athero-Express			
	No Event	Event	Total		No Event	Event	Total	
Amputation Free Survival	n=93	n=67	n=160	*P* Value	n=323	n=63	n=386	*P* Value
Sex (male), n (%)	56 (60)	53 (79)	108 (68)	0.03	235 (73)	46 (73)	281 (73)	0.99
Age, y, median (IQR)	62.0 (52–72)	71.0 (61.5–79.0)	67.0 (56.0–76.0)	0.002	68.0 (62.0–74.0)	71.0 (68.0–78.0)	69 (62.2–75.0)	0.0003
BMI, kg/m^2, median (IQR)	26.3 (23.5–28.8)	25.7 (24.1–28.1)	26.17 (23.7–28.7)	0.74	26.4 (23.6–28.4)	25.3 (23.3–28.0)	26.2 (23.5–28.4)	0.43
Smoking (current), n (%)	29 (31)	13 (19)	42 (26)	0.14	124 (38)	23 (37)	147 (38)	0.81
Systolic BP, mm Hg, mean (SD)	130.0 (18.6)	132.5 (20.8)	131 (19.5)	0.42	147.0 (22.6)	150 (32.9)	147.5 (24.5)	0.54
Diastolic BP, mm Hg, mean (SD)	73.5 (9.7)	72.1 (10.2)	72.9 (9.1)	0.39	77.4 (11.7)	72.9 (17.2)	76.7 (12.8)	0.07
Creatinine, μmol/L, median (IQR)	87.0 (75.0–107.0)	105.0 (75.0–146.5)	90.0 (74.8–114.5)	0.04	85.0 (70.0–99.5)	90.0 (65.0–119.0)	85.0 (70.0–101.0)	0.03
eGFR, MDRD, mL/min/1.73 cm^2, mean (SD)	72.3 (23.8)	66.1 (32.0)	69.7 (27.6)	0.18	80.7 (25.5)	73.1 (31.5)	79.5 (26.7)	0.08
Cholesterol, mmol/L, mean (SD)	4.4 (1.1)	4.1 (1.1)	4.3 (1.1)	0.15	4.6 (1.1)	4.8 (1.4)	4.6 [1.1)	0.56
HDL, mmol/L, mean (SD)	1.3 (0.4)	1.1 (0.4)	1.2 (0.4)	0.02	1.2 (0.4)	1.1 (0.4)	1.2 (0.4)	0.32
Triglycerides, mmol/L, mean (SD)	1.6 (0.9)	1.7 (1.1)	1.7 (1.0)	0.59	1.7 (1.0)	2.1 (1.1)	1.8 (1.0)	0.07
Hemoglobin, mmol/L, mean (SD)	8.3 (1.0)	7.8 (1.0)	8.1 (1.1)	0.004	8.6 (0.9)	8.2 (1.3)	8.5 (1.0)	0.02
History of CVA, n (%)	2 (2)	10 (15)	12 (8)	0.006	17 (5)	6 (9)	23 (6)	0.27
History of CAD, n (%)	30 (32)	36 (54)	66 (41)	0.02	134 (42)	29 (46)	163 (42)	0.53
Diabetes mellitus, n (%)	30 (32)	30 (44)	60 (38)	0.15	86 (27)	20 (31)	106 (27)	0.50
ABI, median (IQR)	0.54 (0.41–0.77)	0.47 (0.27–0.64)	0.51 (0.38–0.74)	0.17	0.59 (0.45–0.70)	0.50 (0.39–0.60)	0.57 (0.44–0.70)	0.9
Fontaine class (II, III, IV)				0.004				0.18
II, n (%)	8 (9)	0 (0)	8 (5)		202 (63)	31 (49)	233 (60)	
III, n (%)	35 (38)	16 (24)	41 (27)		78 (24)	18 (29)	96 (25)	
IV, n (%)	50 (53)	51 (76)	101 (67)		43 (13)	14 (22)	57 (15)	

ABI indicates ankle brachial index; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CVA, cerebrovascular accident; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; MDRD, Modification of Diet in Renal Disease.

significantly higher in patients who experienced a major event (amputation or death) compared with patients who remained event-free, both in JUVENTAS (2346 pg/mL [IQR: 1466 to 3191 pg/mL] versus 1398 pg/mL [IQR: 890.4–2569 pg/mL], respectively; *P*=0.0002) and Athero-Express (1957 pg/mL [IQR: 1315–3395 pg/mL] versus 1458 pg/mL [IQR: 1032–1971 pg/mL], respectively; *P*=0.0003; Figure 1). GDF15 levels increased with age and Fontaine classification in both JUVENTAS and Athero-Express (Figure S1).

High GDF15 Levels Are Associated With Increased Risk of Amputation or Death

We divided the JUVENTAS and Athero-Express patients in tertiles based on GDF15 levels (low, medium, and high) for the Kaplan–Meier curves of the estimated cumulative incidence rate of major events, defined as amputation or death (Figure 2A and 2B). Unadjusted Cox proportional hazards analysis was used to compare the different tertiles. AFS was significantly lower in patients in the high versus low GDF15 tertiles, in both JUVENTAS (HR: 4.01; 95% confidence interval [CI], 2.05–7.84; *P*=0.000005) and Athero-Express (HR: 3.27, 95% CI, 1.64–6.54; *P*=0.00077; Table 2). Of note, in JUVENTAS, GDF15 proved to be more strongly associated with AFS in women (high versus low, HR: 10.1; 95% CI, 2.14–47.81) than in men (high versus low, HR: 2.69; 95% CI, 1.28–5.66; Figures S2 and S3 and Table S1). In Athero-Express, there was no differential effect of GDF15 levels on AFS between the sexes (Figures S2 and S3 and Table S1).

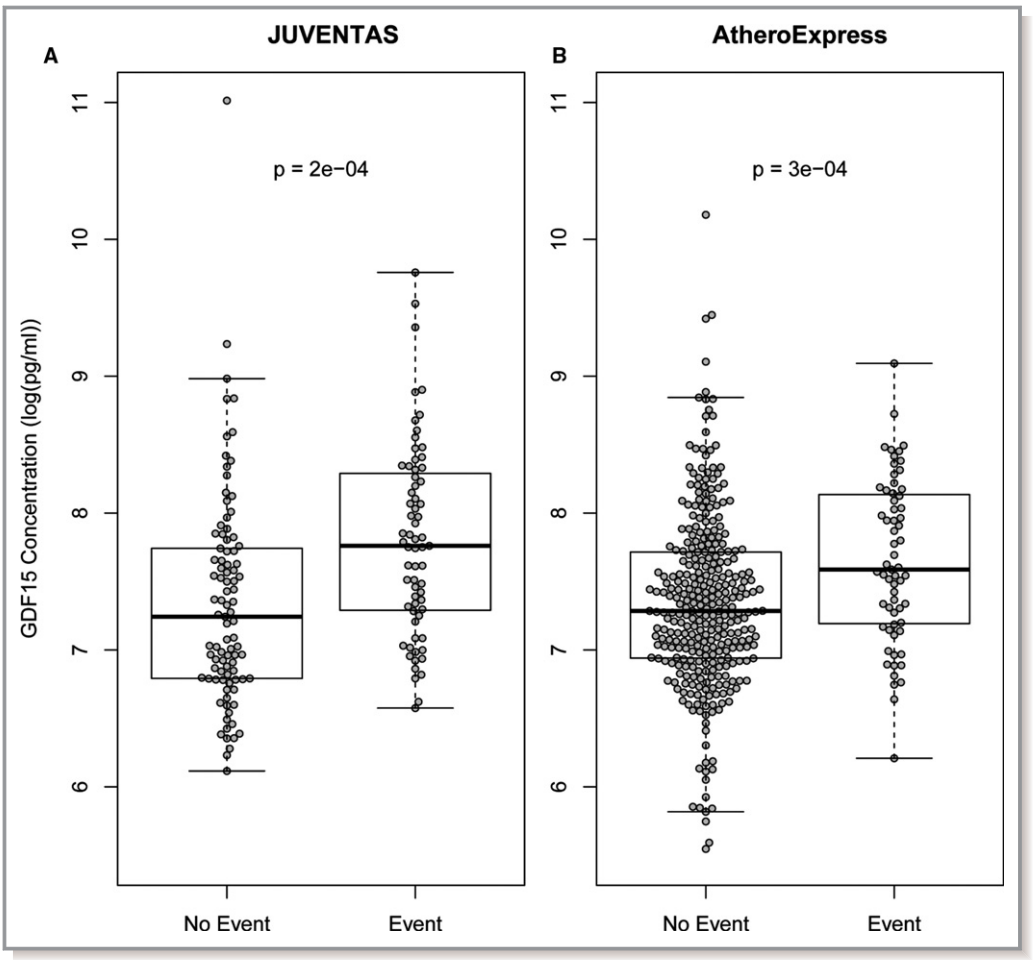

Figure 1. Box plots of log-transformed and intercept-normalized GDF15 levels in patients with no event or with an event in the JUVENTAS and Athero-Express cohorts. A, Median GDF15 levels in the JUVENTAS trial were 2346 pg/mL (IQR: 1466–3191 pg/mL) for patients with an event and 1398 pg/mL (IQR: 890.4–2569 pg/mL) for patients without an event (*P*=0.0002). B, Median GDF15 levels in the Athero-Express Biobank were 1957 pg/mL (IQR: 1315–3395 pg/mL) for patients with an event and 1458 pg/mL (IQR: 1032–1971 pg/mL) for patients without an event (*P*=0.0003). IQR indicates interquartile range; GDF15, growth differentiation factor 15; JUVENTAS, Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation Trial.

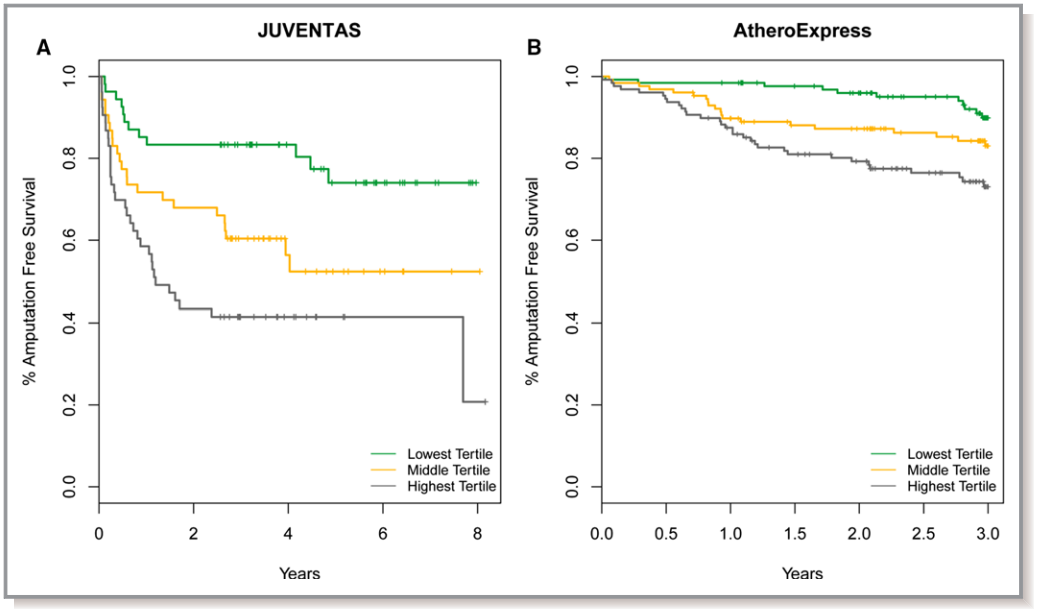

Figure 2. Kaplan–Meier curves representing the estimated cumulative incidence rates of major amputation or death in patients with low, medium, and high GDF15 levels. A, In JUVENTAS, patients in the middle tertile showed an HR of 2.41 (95% CI, 1.20–4.85; *P*=0.014) compared with the lowest tertile, and patients in the highest tertile showed an HR of 4.01 (95% CI, 2.05–7.84; *P*=0.000048) compared with the lowest tertile. B, In Athero-Express, patients in the middle tertile showed an HR of 2.19 (95% CI, 1.07–4.49; *P*=0.033) compared with the lowest tertile, and the highest tertile showed an HR of 3.27 (95% CI, 1.64–6.54; *P*=0.00077) compared with the lowest tertile. CI indicates confidence interval; HR, hazard ratio; GDF15, growth differentiation factor 15; JUVENTAS, Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation Trial.

Circulating GDF15 levels are highly correlated with the traditional risk factors, including age and eGFR (estimated glomerular filtration rate). Interestingly, GDF15 was also strongly correlated with blood hemoglobin levels (Figure S1). The unadjusted univariate HR for continuous GDF15 levels for the risk of death or major amputation was 1.62 (95% CI, 1.27–2.06; *P*=0.00012) in JUVENTAS and 1.95 (95% CI, 1.38–2.76; *P*=0.00016) in Athero-Express (Table 3). The multivariate analysis, corrected for age, sex, body mass index, eGFR, diabetes mellitus, smoking, hypertension, and history of coronary artery disease showed an HR for continuous GDF15 of 1.78 (95% CI, 1.18–2.69; *P*=0.0053) for JUVENTAS and 1.57 (95% CI, 1.02–2.41; *P*=0.041) for Athero-Express. After adjustment of the full model for hemoglobin, HRs were marginally lower in both cohorts (1.64 versus 1.70 in JUVENTAS and 1.39 versus 1.57 in Athero-Express; Table 2).

GDF15 Levels Alone Can Predict Outcome in Both Cohorts and Can Even Improve Prediction Beyond Traditional Risk Factors in JUVENTAS

GDF15 levels alone performed equally as well in predicting outcome as 9 traditional risk factors combined, as shown by the C-statistics for the receiver operative characteristic curves in Athero-Express (66.1% AUC; CI, 57.1–75.2, versus 67.6% AUC; CI, 57.2–78.1; fixed end point at 1 year, eg, as in PREVENT III[26]; Figure S4). In JUVENTAS, GDF15 even has additive value beyond the 9 traditional risk factors (74.4% AUC; CI, 66.3–82.6, versus 70.8% AUC; CI, 62.4–79.2; Figure S4).

Table 2. Cox Proportional Hazards Ratios Between Low and Middle and Low And High Tertiles in JUVENTAS and Athero-Express

	JUVENTAS			Athero-Express		
Model	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Middle tertile	2.41	1.20–4.85	0.014	2.19	1.07–4.49	0.033
Highest tertile	4.01	2.05–7.84	0.000005	3.27	1.64–6.54	0.00077

CI indicates confidence interval; HR, hazard ratio; JUVENTAS, Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation Trial.

Table 3. Cox Proportional Multivariable Hazards Regression Models for Major Events in JUVENTAS and Athero-Express

	JUVENTAS			Athero-Express		
Model	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Unadjusted	1.62	1.27–2.06	0.00012	1.95	1.38–2.76	0.00016
Age plus sex	1.46	1.1–1.95	0.0093	1.73	1.19–2.51	0.0044
Full*	1.70	1.14–2.52	0.0053	1.57	1.02–2.41	0.041
Full plus hemoglobin	1.64	1.07–2.52	0.023	1.39	0.89–2.18	0.15

CI indicates confidence interval; HR, hazard ratio; JUVENTAS, Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-Arterial Supplementation Trial.
*Full model is corrected for age, sex, body mass index, estimated glomerular filtration rate, diabetes mellitus, smoking, hypertension, and coronary artery disease history.

GDF15 Levels Do Not Correlate With Plaque Characteristics

In Athero-Express, plaque specimens were studied to characterize plaque phenotype. We did not observe differences in plaque phenotype in patients who had different GDF15 levels (low, medium, high) and who experienced an event or remained event-free (Table 4).

Dose-Response Model Illustrates That Increased Levels of GDF15 Exponentially Increase Relative Hazard

We attempted to create an unbiased estimate of the relationship between GDF15 levels and the risk of a major event for PAD patients. We used the pooled data from both cohorts and observed an exponential increase in the relative hazard of amputation or death with increased GDF15 levels (Figure 3). Consequently, with higher GDF15 levels, there is an exponentially higher risk of an event.

Discussion

In this study, we showed that high levels of GDF15 are associated with major amputation and all-cause mortality in patients with PAD. This was true for patients with severe non-revascularizable PAD and critical limb ischemia (JUVENTAS) and for those with milder PAD (Athero-Express). Patients in the highest tertile of GDF15 levels had >3 times higher risk of major amputation or death compared with the lowest tertile in both cohorts. In JUVENTAS, high GDF15 levels were more predictive in women than in men. We did not observe this sex difference in Athero-Express. We could not find any difference in estimated glomerular filtration rate, Fontaine classification, hemoglobin, history of stroke, history of coronary artery

Table 4. Plaque Characteristics in Athero-Express

		GDF15, n (%)					
		Low		Medium		High	
		Event	No Event	Event	No Event	Event	No Event
Calcification *P*=0.17	No/minor	38 (18)	4 (9)	27 (13)	6 (13)	28 (13)	11 (24)
	Moderate/heavy	37 (17)	4 (9)	45 (21)	11 (24)	40 (18)	9 (20)
Collagen *P*=0.22	No/minor	11 (5)	1 (2)	12 (6)	3 (7)	16 (8)	6 (13)
	Moderate/heavy	64 (30)	7 (16)	57 (27)	14 (31)	53 (25)	14 (31)
Smooth muscle cells *P*=0.26	No/minor	18 (9)	2 (4)	22 (10)	7 (16)	17 (8)	4 (9)
	Moderate/heavy	56 (26)	6 (13)	49 (23)	10 (22)	51 (24)	16 (36)
Macrophages *P*=0.05	No/minor	65 (31)	8 (18)	56 (26)	17 (38)	57 (27)	15 (33)
	Moderate/heavy	9 (4)	0 (0)	15 (7)	0 (0)	10 (5)	5 (11)
Intraplaque fat *P*=0.42	<10%	62 (29)	7 (16)	59 (27)	13 (29)	51 (24)	16 (36)
	>10%	14 (7)	1 (2)	13 (6)	4 (9)	17 (8)	4 (9)
Intraplaque hemorrhage *P*=0.44	No	36 (17)	5 (11)	39 (18)	9 (20)	38 (18)	9 (20)
	Yes	39 (18)	3 (7)	33 (15)	8 (18)	31 (14)	11 (24)

Percentages are given per event or no event. GDF15 indicates growth differentiation factor 15.

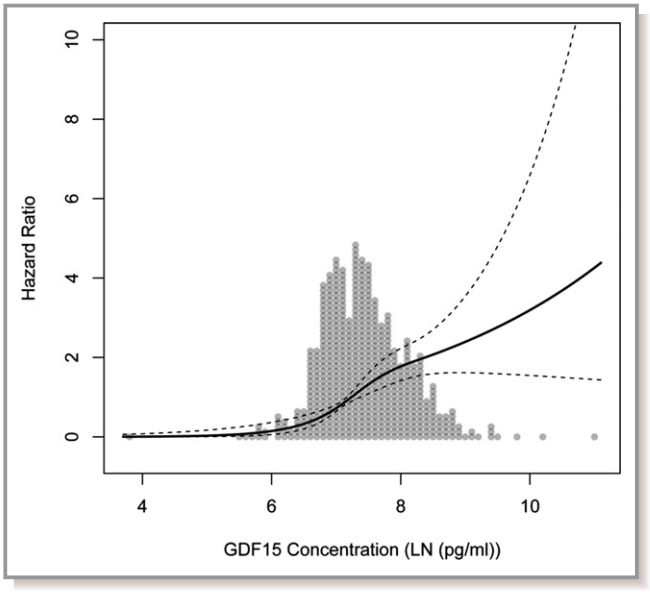

Figure 3. Dose-response curve of the hazard ratio for amputation or death to log-transformed (LN) and intercept-normalized GDF15 (growth differentiation factor 15) levels based on both JUVENTAS and Athero-Express data. An increase in GDF15 levels led to an exponential increase in HR. GDF15 indicates growth differentiation factor 15; HR, hazard ratio.

disease, or age that could explain this sex discrepancy between JUVENTAS and Athero-Express.

GDF15 colocalizes with macrophages in carotid artery plaques,[16,18] and oxidized low-density lipoprotein has been shown to increase GDF15 production by human macrophages ex vivo. In addition, atherosclerotic mouse models show a causal role of GDF15 in the chemotaxis of macrophages to the plaque specifically during the early phase of atherosclerosis.[16] In our Athero-Express plaques of PAD patients, we did not observe a difference in the amount of macrophages; however, we were looking at an advanced stage of atherosclerosis. Furthermore, GDF15 was shown to promote cholesterol efflux in an in vitro human foam cell model[27]; though, we did not observe differences in GDF15 levels in relation to fat content in the plaque. During plaque progression and in the more advanced stages of atherosclerosis, data on GDF15 are conflicting because GDF15 has been shown to be protective,[28,29] whereas it was also shown that the absence of GDF15 can positively affect plaque stability.[16] Mechanistic studies are needed to examine the role of GDF15 in plaque initiation and progression in PAD patients.

GDF15 has been reported to be involved in apoptosis in various disease models. In cultured adult rat cardiomyocytes, GDF15 seems to have an anti-apoptotic effect.[30] Also in cardiac ischemia/reperfusion injury in mice, GDF15 functions as an anti-apoptotic cytokine for cardiomyocytes.[31] In contrast, GDF15 appears to have a pro-apoptotic effect in numerous cancer types, such as glioblastoma,[32] prostate cancer,[33] and colorectal cancer.[34] In a mouse model of atherosclerosis, GDF15 showed to be involved in apoptosis of mononuclear cells in the atherosclerotic plaque.[16] Whether GDF15 plays a role in apoptosis of mononuclear cells in the plaques of PAD patients, and thus the cellular composition of the plaque and its characteristics, is currently unknown. We did not observe a relationship between circulating GDF15 levels and the number of macrophages in the plaques of the Athero-Express cohort.

Levels of GDF15 were highly correlated with disease severity (Fontaine classification), and levels were higher in the JUVENTAS cohort compared with the Athero-Express cohort; this could be a reflection of the severity of tissue hypoxia. It is known that in vitro hypoxia can induce GDF15 expression in human umbilical vein endothelial cells[35] and in human glioblastoma cells.[36] Increased levels of GDF15 can promote angiogenesis[35,37]; therefore, GDF15 might be an important activator of local angiogenesis. In contrast, it also has been shown that GDF15 can inhibit CCN2-induced angiogenesis[38] and the proliferation of endothelial cells,[19] highlighting that additional factors and conditions are involved in the regulation of angiogenesis and that GDF15 does not simply reflect the angiogenic activity. GDF15 levels rather seem to reflect a cumulative risk factor burden and precisely follow established risk factors for events in PAD, such as age,[1] renal damage,[39] and diabetes mellitus.[40]

Interestingly, we observed that GDF15 levels are strongly negatively correlated with blood hemoglobin. A low blood hematocrit, the fractional hemoglobin volume of blood, was previously shown to be an independent predictor of AFS in the PREVENT III prediction model.[26] At present, no mechanistic explanation has been offered with regard to this association. GDF15 has been linked to anemia in various pathogenic conditions, such as chronic heart failure,[13,41] thalassemia,[42] and pyruvate kinase deficiency,[43] and in kidney[44] or heart[41] allograft recipients. It has been described that an insufficient erythropoiesis can lead to an increase in GDF15 levels. GDF15, however, is also involved in the inhibition of hepcidin (a key regulator of the entry of iron into the blood stream) and thus iron uptake. Whether high GDF15 levels are the cause or consequence of a decrease in hemoglobin remains to be elucidated. The fact that the pathways of GDF15 and erythropoiesis are related might explain that both are related to prognosis in PAD.

The prognostic value of GDF15 has been reported extensively for various cardiovascular diseases[5–15]; however, the current study is the first to report the role of GDF15 in the prediction of major amputation or death in patients with PAD. The dose-response curve shows that risk of amputation or death increases exponentially with increased levels of GDF15. Furthermore, C-statistics of the receiver operating

characteristic curves showed that GDF15 could be of additive value as a classification parameter.

GDF15 levels could be used in the clinic to predict major events and thus may be useful in improving risk stratification of PAD patients. Patients with high GDF15 levels should be monitored more closely. Furthermore, GDF15 levels could be helpful in deciding whether an affected limb should be amputated.

Conclusion

We showed for the first time, to our knowledge, that GDF15 levels predict major amputation or death in PAD patients. Circulating GDF15 levels could be implemented in clinical practice as a biomarker to identify patients with PAD and critical limb ischemia who are at high risk of major events and to guide treatment choices.

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Disclosures

None.

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Supplemental Material

Figure S1. A) Correlation between lnGDF15 and Hb, age and eGDR based on pooled data of JUVENTAS and AtheroExpress. **B**) Boxplots of lnGDF15 levels in males and females that smoke, have DM, hypertension, history of CAD, history of stroke or for Fontaine classification on pooled data of JUVENTAS and AtheroExpress

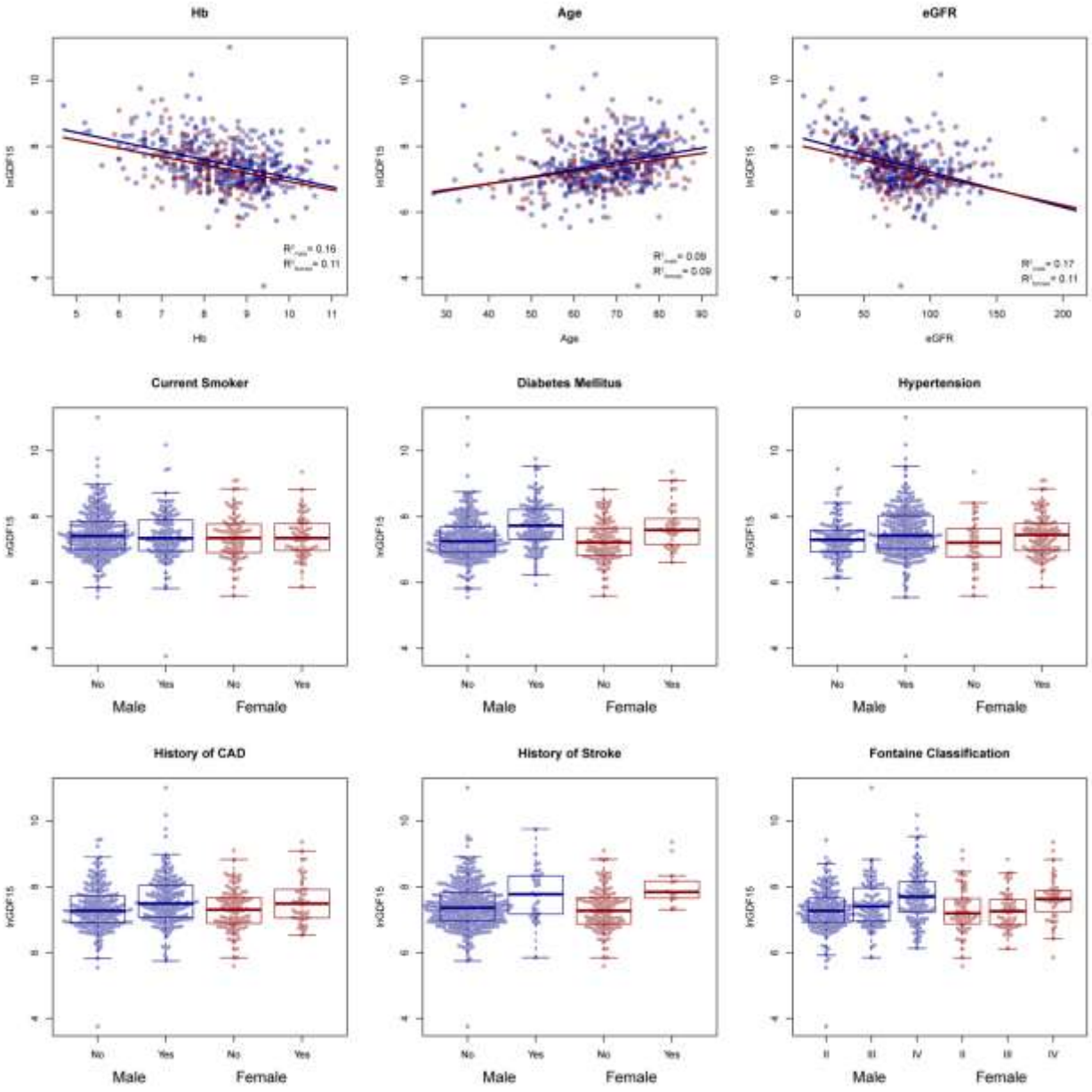

Figure S2. Boxplots of lnGDF15 levels in males and females with no event or with an event in the JUVENTAS and in the AtheroExpress.

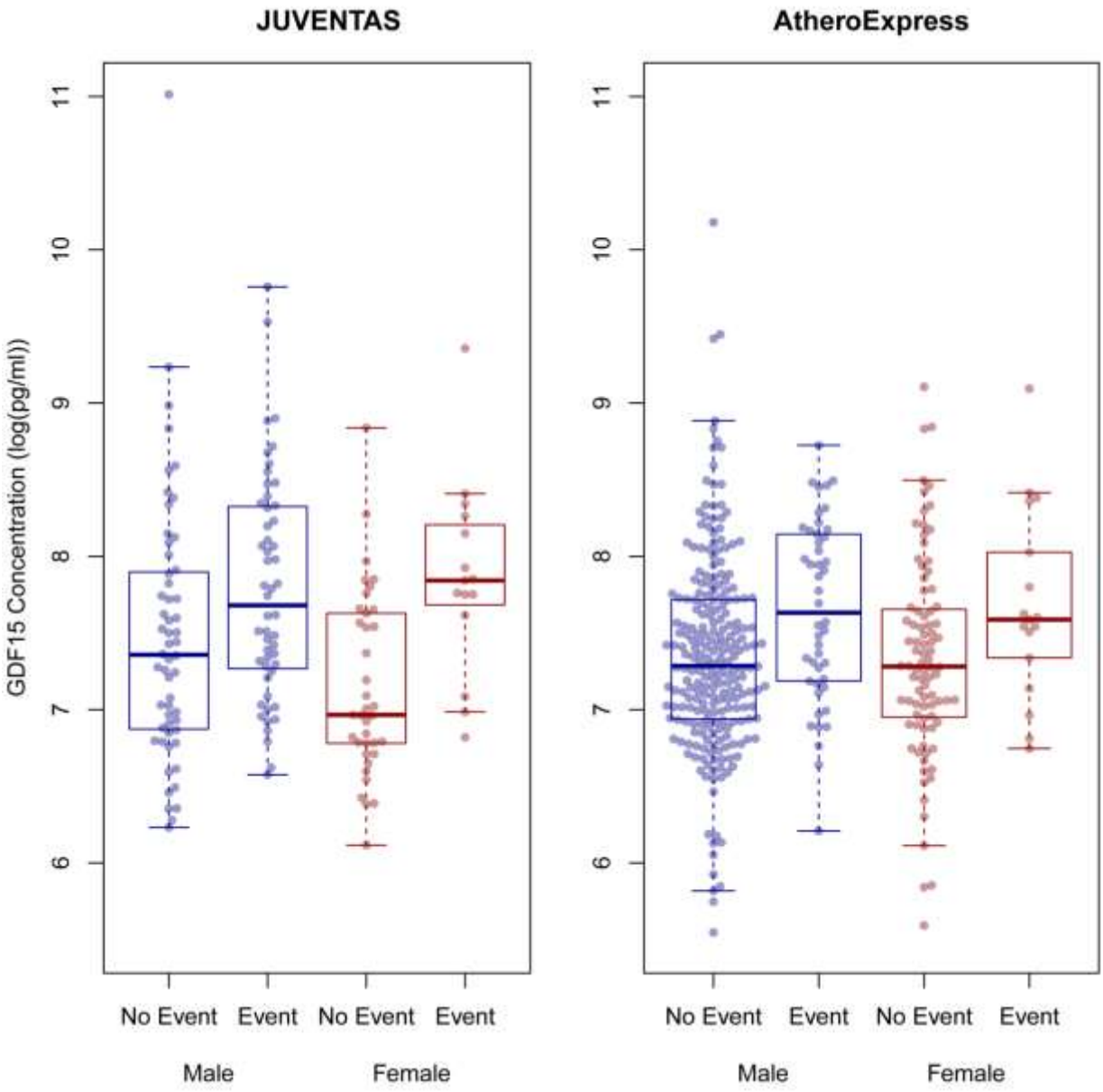

Figure S3. Kaplan-Meier curves representing the estimated cumulative incidence rates of major amputation or death in patients with low, medium or high GDF15 levels, stratified by sex in A) JUVENTAS and B) AtheroExpress

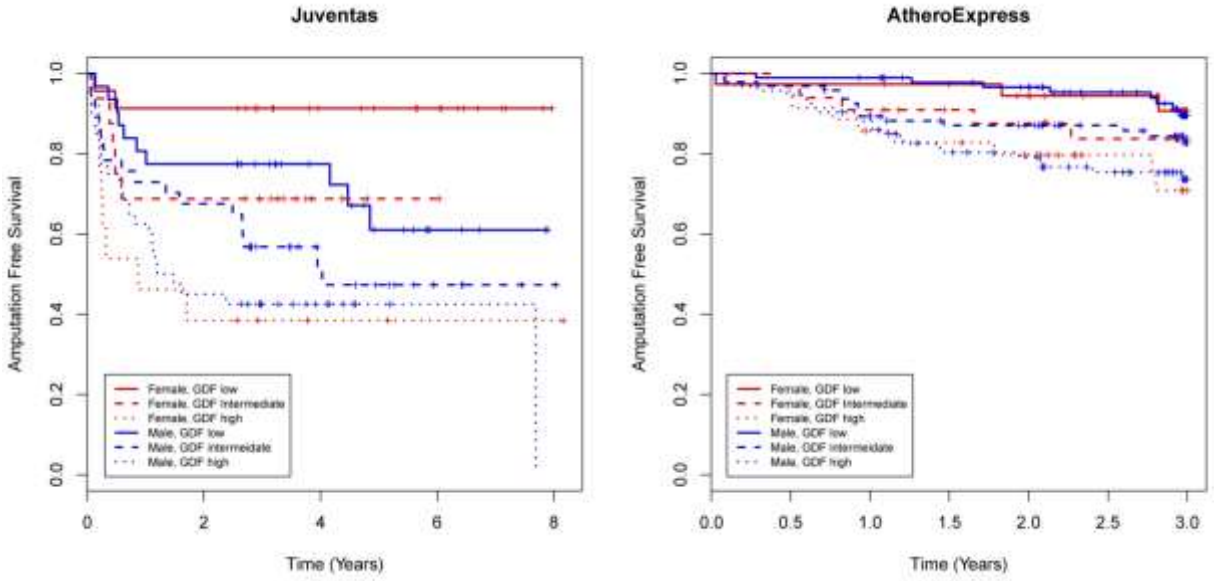

Figure S4. ROC curves showing additive value of GDF15 in JUVENTAS as classification parameter. In Juventas, GDF15 showed an added value as classification parameter upon the full model + hb with a C- statistic of 74.4% vs 70.8% (with vs without GDF). NRI is 0.3459 [0.0055 - 0.6863]; p-value: 0.04642; the IDI is 0.0327 [0.0059 - 0.0596] ; p-value: 0.01683. In Atheroexpress GDF15 did not show an improved classification with a C-statistic of 69.1% vs 67.6% (with vs without GDF). NRI is 0.1593 [-0.2187 - 0.5374] ; p-value: 0.40876, and the IDI is 8e-04 [-2e-04 - 0.0018] ; p-value: 0.11431.

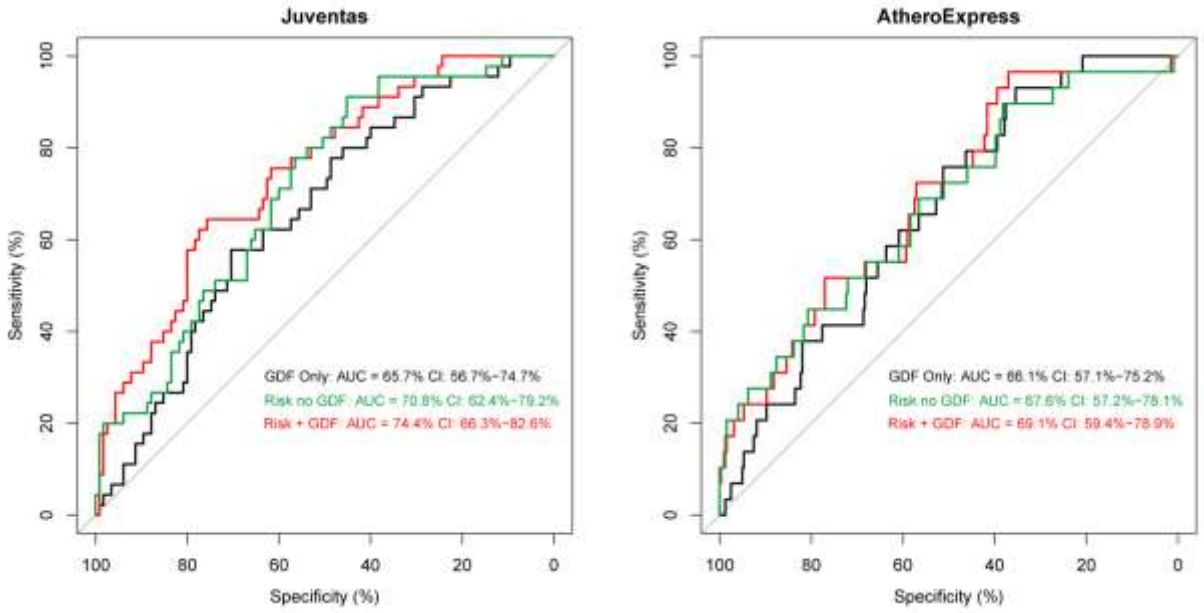

Table S1. Cox proportional hazard regression models for major events in JUVENTAS and AtheroExpress stratified by sex.

	JUVENTAS			AtheroExpress		
	HR	95% CI	P-val	HR	95% CI	P-val
Male	2.69	1.28 - 5.66	0.009	3.26	1.46 - 7.30	0.004
Female	10.1	2.14 - 47.81	0.004	3.66	0.99 - 13.53	0.052

HR showed for high versus low GDF15 levels.

HR: Hazard Ratio; CI: Confidence Interval