Clinical Track

Prognostic Value of Lymphocyte G Protein-Coupled Receptor Kinase-2 Protein Levels in Patients With Heart Failure

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Rationale: Sympathetic nervous system hyperactivity is associated with poor prognosis in patients with heart failure (HF), yet routine assessment of sympathetic nervous system activation is not recommended for clinical practice. Myocardial G protein-coupled receptor kinase-2 (GRK2) is upregulated in HF patients, causing dysfunctional β-adrenergic receptor signaling. Importantly, myocardial GRK2 levels correlate with levels found in peripheral lymphocytes of HF patients.

Objective: The independent prognostic value of blood GRK2 measurements in HF patients has never been investigated; thus, the purpose of this study was to evaluate whether lymphocyte GRK2 levels predict clinical outcome in HF patients.

Methods and Results: We prospectively studied 257 HF patients with mean left ventricular ejection fraction of 31.4±8.5%. At the time of enrollment, plasma norepinephrine, serum NT-proBNP, and lymphocyte GRK2 levels, as well as clinical and instrumental variables were measured. The prognostic value of GRK2 to predict cardiovascular (CV) death and all-cause mortality was assessed using the Cox proportional hazard model including demographic, clinical, instrumental, and laboratory data. Over a mean follow-up period of 37.5±20.2 months (range, 3–60 months), there were 102 CV deaths. Age, left ventricular ejection fraction, New York Heart Association class, chronic obstructive pulmonary disease, chronic kidney disease, N-terminal-pro brain natriuretic peptide, and lymphocyte GRK2 protein levels were independent predictors of CV mortality in HF patients. GRK2 levels showed an additional prognostic and clinical value over demographic and clinical variables. The independent prognostic value of lymphocyte GRK2 levels was also confirmed for all-cause mortality.

Conclusions: Lymphocyte GRK2 protein levels can independently predict prognosis in patients with HF.

Key Words: beta-adrenergic receptors ■ G-protein-coupled receptor kinase 2 ■ heart failure ■ natriuretic peptide, brain ■ prognosis

Heart failure (HF) is a leading cause of morbidity and mortality worldwide, with a 2-year mortality rate approaching 50% in patients with New York Heart Association (NYHA) class III and IV symptoms.1,2 Sympathetic nervous system (SNS) hyperactivity is a salient characteristic of HF.3,4 Although it represents an early compensatory response aimed at enhancing cardiac contractility, sustained SNS activation exerts detrimental effects on the failing heart in the long term,3–5 and it is correlated with increased mortality in HF patients.4 Consistently, β-adrenergic receptor (βAR) blockers have been shown to improve quality of life and to reduce rehospitalization...
and mortality in HF patients. Thus, measurement of SNS activity has been suggested to help assessment of prognosis and clinical management of HF patients. However, although SNS hyperactivity measured through plasma circulating norepinephrine (NE) levels, cardiac or renal NE spillover, heart rate variability, and iodine-123-metaiodobenzylguanidine myocardial scintigraphy has prognostic value in HF patients, none of these approaches is routinely used in clinical practice or recommended by guidelines.

It has been repeatedly reported that HF-related SNS hyperactivity is responsible for enhanced cardiac G protein-coupled receptor kinase-2 (GRK2) levels, which are critically involved in the processes of cardiac βAR downregulation/desensitization, which occurs in HF. GRK2 is a serine–threonine kinase that by phosphorylating agonist-bound G protein-coupled receptors, including βARs, leads to the recruitment of arrestins and attenuates intracellular G protein-dependent signaling. We and others have reported that enhanced GRK2 activity plays a key role in the pathogenesis of HF. Moreover, preclinical studies have shown that GRK2 inhibition in HF results in improved cardiac function, reverse remodeling, restoration of HF-related βAR abnormalities, and attenuation of SNS activity and neurohormonal responses.

Of interest for this study, GRK2 expression measured in peripheral lymphocytes of HF patients correlates directly with levels of this kinase in failing myocardium and reflects the loss of hemodynamic function, the disease severity, and the degree of left ventricular (LV) dysfunction. Additionally, GRK2 is comparably and significantly reduced in both lymphocytes and myocardium of HF patients after LV unloading and exercise training. However, the prognostic value of lymphocyte GRK2 levels has never been investigated. This study aims to assess the value of lymphocyte GRK2 to predict outcome in patients with systolic HF. Our results do indeed suggest that blood GRK2 offers advantages over existing biomarkers and provide valuable data to predict HF outcomes.

### Methods

#### Study Design and Population

The study was conducted at the Department of Translational Medical Sciences of the University Federico II (Naples, Italy) and at Salvatore Maugeri Foundation, Scientific Institute of Telese Terme (Telese, BN, Italy) and was approved by the Local Ethical Committee. All patients gave written informed consent. We prospectively enrolled 303 consecutive patients with HF between January 2007 and July 2010. Of these 303 subjects, 21 subjects had to be excluded because of poor quality of lymphocyte extracts and 25 patients because of lack of serum N-terminal pro-brain natriuretic peptide (NT-proBNP) values, leaving a final study population of 257 patients.

To be included in the study, patients needed to fulfill the following criteria: diagnosis of HF due to ischemic or nonischemic causes; LV ejection fraction (LVEF) ≤50%; clinical stability of symptoms for at least 1 month before inclusion; and guidelines-based optimal pharmacotherapy. Exclusion criteria were cardiac revascularization or acute myocardial infarction within 3 months before study entry, uncontrolled hypertension (>180 mmHg systolic or >110 mmHg diastolic on measurements made on at least 3 separate dates during the preceding 3 months), severe chronic kidney disease (glomerular filtration rate <30 mL/min), comorbidity conditions associated with lymphocyte activation (ie, cancer, severe chronic diseases, ongoing infection, and excessive alcohol intake), and a life expectancy of <1 year, based on noncardiac reasons. At the time of enrollment, all subjects underwent a complete clinical examination (including NYHA class assessment and echocardiography) and blood draw for serum N-terminal pro-brain natriuretic peptide (NT-proBNP), lymphocyte GRK2, and plasma NE levels. Demographic data including age, sex, HF medications, cardiovascular (CV) risk factors, and presence of comorbidities were also collected.

#### NT-proBNP Measurements

Level of NT-proBNP was determined by chemiluminescence (Elecsys 2010, Roche). The analytic range of the NT-proBNP assay extends from 1 to 25,000 pg/mL.

#### Lymphocyte GRK2 Immunoblotting

Blood samples were collected from all patients in ethylenediaminetetraacetic acid tubes. Lymphocytes were isolated by ficoll gradient using HISTOPAQUE-1077 (Sigma), frozen, and stored at −80°C until the day of the assay. Immunodetection of GRK2 was performed using detergent-solubilized lymphocyte extracts after immunoprecipitation (IP) as previously described. IPs were done using a monoclonal anti-GRK2/3 antibody (C5/1, Upstate) followed by Western blotting with a GRK2 polyclonal antibody (C-20, Santa Cruz Biotechnology). All IPs were done in protein lysates from lymphocytes of control patients that were used for GRK2-IPs. The dilution range was used 5 different dilutions of total lysates from lymphocytes of control patients that were used for GRK2-IPs. The dilution range was in an established order of magnitude (from 2000 to 200 μg). As previously reported, post-IP lysates have been blotted for residual GRK2 amounts, and typically none has been found demonstrating the quantitative nature of these experiments. Each IP was performed in duplicate. To secure the linearity between optical density and GRK2 levels a standard curve was performed. The sample containing the protein to be quantified plus a set of standards were used. As standards we used 5 different dilutions of total lysate from lymphocytes of control patients that were used for GRK2-IPs. The dilution range was in an established order of magnitude (from 2000 to 200 μg). The 80 kDa GRK2 protein was visualized using standard enhanced chemiluminescence (ECL Kit, Amersham). For an accurate quantification it was important that the light produced was in the linear range of the film. This was achieved by making several exposures of different lengths of time. Quantification of immunoreactive GRK2 was done by scanning the autoradiography film and using ImageQuant software (Molecular Dynamics). The concentration of the protein was quantified and read off a graph. To normalize data between different blots, a reference sample was run in all immunoblots and data from each individual patient were normalized to this sample. Intra- and interassay coefficient of variation were 5% and 15%, respectively.

#### Assessment of Outcomes and Follow-Up

The primary end point of the study was CV death, and all-cause mortality was a secondary end point. Causes of cardiac death were established after a review of hospital records, death certificates, and interviews with family members and family doctors. The patients either visited the outpatient unit of the reference hospitals or were contacted by telephone to determine their survival status. All causes of
death were adjudicated by 2 physicians with disagreements resolved by referring to a third physician (Online Table I). Follow-up period was terminated at the end of the study period (on July 30, 2012) or in the case of death.

### Statistical Analysis

Continuous variables are expressed as mean±standard deviation (SD) and compared by the use of Student’s t test (normally distributed) or as median-interquartile range value and compared by the use of Mann–Whitney U test (not normally distributed), as appropriate. Normality of data distribution was evaluated using the Kolmogorov–Smirnov test. Not normally distributed continuous variables were natural log transformed (ln NE, ln GRK2, and ln NT-proBNP). Categorical variables are expressed as proportion and compared by use of χ² test. Pearson correlation coefficient is calculated to assess correlation between data. The Cox proportional hazard analysis was used to identify the factors associated with CV and all-cause mortality. Using parsimonious criteria and taking into account the study sample size, 19 potentially prognostic independent variables have been selected (at least 10 patients were available for each prognostic factor tested). These factors were representative of several characteristics: basic demographic data (age and sex), presence of comorbidities (chronic kidney disease, defined as glomerular filtration rate ≤50 mL/min, diabetes mellitus, hypertension, and chronic obstructive pulmonary disease), resting systolic blood pressure and heart rate, presence of left bundle branch block, presence of low serum sodium (≤130 mEq/L), presence of low serum cholesterol (≤130 mg/dL), presence of hyperuricemia (≥6.7 mg/dL), medications (angiotensin-converting enzyme inhibitors/angiotensin receptor blockers and β-blockers), functional classification by NYHA class, LVEF, circulating NE, serum NT-proBNP, 6.86±0.8, 7.13±0.9, and 7.06±1.0, respectively). To overcome this confounding effects, we performed a multivariate Cox analysis and the resulting adjusted values are reported using variables included in the final Cox models were related to clinically meaningful variations (10 years period for age and 5% unit for LVEF) or to the interquartile range (75°–25° percentile difference) in case of NT-proBNP and lymphocyte GRK2 levels (because these factors lack a range with a definite clinical correlate).

Directly adjusted survival plots obtained averaging the Cox survival curves estimated using the covariate values of each subject are used to compare the survivals at specific factors’ values to the overall Kaplan–Meier.45 Data were analyzed by stata version 13.0 (StataCorp LP, College Station, Texas). Statistical significance was accepted at P<0.05.

### Results

#### Patient Characteristics

The final study group consisted of 257 patients (71.6% male) with mean age of 70.5±10.7, mean LVEF of 31.4±8.5%, and mean NT-proBNP of 1310±852 pg/dL. Only NYHA class II and III were present in the studied population (NYHA class II frequency of 18.3%). Demographic data of the overall study population and differences in the outcome groups are reported in Table 1. To note, after patient stratification according to lymphocyte GRK2 median value (1.31 Densitometric Unit [DU]), β-blockers use and doses (low, medium, high) were equally distributed among patients with high and low lymphocyte GRK2 levels (Online Table II). Moreover, there were no differences in lymphocyte GRK2 levels between patients assuming different doses or patients not taking β-blockers (ANOVA P=0.46; Online Figure I).There were no differences in sex and medication usage between survivors and nonsurvivors. Subjects who died were more likely to be older; diabetic and to have NYHA functional class III; worse kidney function; lower LVEF; and higher levels of NT-proBNP, NE, and lymphocyte GRK2 protein levels. Mean lymphocyte GRK2 protein levels of HF patients was 1.42±0.71 DU, significantly higher (>2.5-fold; P<0.01) compared with that of 37 healthy subjects (0.51±0.13 DU). Interestingly, lymphocyte GRK2 showed a significant correlation with age, LVEF, NE, and NT-proBNP levels. NT-proBNP presented a similar correlation profile (Table 2).

#### Analysis of Outcomes

Over a mean follow-up of 37.5±20.2 months (range: 3–60 months) CV and all-cause deaths were 102 and 131, respectively (final cumulative year mortality rate of 47.3±3.9% and 61.5±3.8%, with 60.3% and 49.0% censoring for CV and all-cause deaths, respectively). Specific causes of deaths are reported in Online Table I. Table 3 shows the rate of CV and all-cause deaths for quartiles of age, LVEF, lymphocyte GRK2, and NT-proBNP levels. Both CV and all-cause death rates show a significant constant increase along the quartiles of these 4 factors. Of note, the fourth GRK2 quartiles show a slight decrease in death rate compared with the third quartile.

The stability attained in each final model in which a given prognostic factor was included as significant was measured by the number of times that the given variable was included as significant in a large (5000) number of bootstrap replications, applying the same multivariable fractional polynomials selection procedure (bootstrap inclusion frequency). The stability of the relationship between each variable and the survival was measured by the frequency (in the bootstrap subset) of a significant linear versus nonlinear association.

To have a comprehensive view of the relative weight of each factor on the survival curve, the HR of the significant continuous
Cox proportional hazard assumption was verified and hold for all variables in all final models. The stability of the results was documented by the high frequency of significant inclusion (above 90% for both NT-proBNP and GRK2) >5000 bootstrap study sample replications (Table 4), with the linear relationships being the most frequent functional form.  

CV Death

Multivariate Cox proportional hazards regression analysis (Table 4 (cardiovascular death)) revealed that age, LVEF, NYHA class, chronic kidney disease, chronic obstructive pulmonary disease, serum NT-proBNP, and lymphocyte GRK2 protein levels were all independent and significant factors associated with CV mortality (full model). The model shows a global $R^2$ of 0.48 and a Haller’s $C=0.79\pm0.02$, denoting that a good fraction of the outcome variability is explained and, hence, denoting an adequate discrimination. Other potential prognostic factors in HF, such as heart rate, systolic blood pressure, low serum cholesterol, low serum sodium, hyperuricemia, and presence of left bundle branch block, did not show a significant association with CV death. However, low serum sodium presence was associated with a 69% greater hazard of CV death, just above the significant threshold ($P=0.06$).

Good agreement between observed and Cox estimated death rate in 5 risk groups was acknowledged by a nonsignificant Gronnesby and Borgan calibration test ($P=0.70$). NYHA functional class III, NT-proBNP and blood GRK2 showed the greatest impact on cardiac mortality, as documented by their HR (>2.0). Interestingly, the partial $R^2$ contributions was 27.5%, 18.9%, and 8.7% for NT-proBNP, GRK2, and NYHA class, respectively, indicating that the 2 circulating biomarkers (NT-proBNP first) had the strongest contribution to the variation of the prognostic index (the linear combination of all factors in the Cox model). For the NYHA class, the apparent inconsistency between the HR and the $R^2$ contribution could be explained by its skewed distribution (81.7% prevalence of the NYHA class III), considered that the actual distribution of a factor is one of the contributing determinants of the partial $R^2$ other than its intrinsic weight (ie, HR).

<table>
<thead>
<tr>
<th>Table 1. Characteristic of Patients in the Overall Study Population and in Patients Stratified in Survivors and Nonsurvivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n=257)</td>
</tr>
<tr>
<td>Age, yr</td>
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<tr>
<td>Sex, % male (n)</td>
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<tr>
<td>LVEF, %</td>
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<td>NYHA class, % (n)</td>
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<tr>
<td>SBP (mmHg)</td>
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<td>Resting HR (bpm)</td>
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<td>LBBB, % (n)</td>
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<tr>
<td>Low serum sodium (&lt;130 mEq/L), % (n)</td>
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<tr>
<td>Low serum cholesterol (&lt;130 mg/dL), % (n)</td>
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<tr>
<td>Hyperuricemia (&gt;9.5 mg/dL), % (n)</td>
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<tr>
<td>Comorbidity</td>
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<tr>
<td>Hypertension, % (n)</td>
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<tr>
<td>Diabetes mellitus, % (n)</td>
</tr>
<tr>
<td>COPD, % (n)</td>
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<tr>
<td>GFR &lt;50 mL/min, % (n)</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>ACE I/ARBs, % (n)</td>
</tr>
<tr>
<td>β-Blockers, % (n)</td>
</tr>
<tr>
<td>Biochemical determinations</td>
</tr>
<tr>
<td>Serum NT-proBNP,* pg/dL</td>
</tr>
<tr>
<td>Plasma norepinephrine,* pg/dL</td>
</tr>
<tr>
<td>Lymphocyte GRK2,* DU</td>
</tr>
</tbody>
</table>

Normally distributed variables are expressed as mean±SD, binary data as percentage. $P$ value refers to the survivors/nonsurvivors comparisons. ACE I indicates angiotensin-converting-enzyme inhibitor; ARBs, angiotensin receptor blockers; COPD, chronic obstructive pulmonary disease; GFR, glomerular filtration rate; GRK2, G coupled-receptor kinase; HR, hazard ratio; LBBB, left bundle branch block; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; and SBP, systolic blood pressure.

*Not normally distributed variables are expressed as mean±SD (median/interquartile range value).
The independent prognostic value of NT-proBNP and lymphocyte GRK2 on CV survival is graphically represented in Figures 1A and 1B. These curves were generated from the Cox parameters and showed the survival of the population at the same percentile value of the 2 markers compared with the overall Kaplan–Meier. The overall trend of these survival curves was comparable, depicting the similar impact of NT-proBNP and GRK2 on cardiac survival.

Figure 2 shows the clinical net benefit profiles of the full model and of the 2 partial models obtained excluding alternatively NT-proBNP or GRK2. The full model shows a clinical net benefit higher than the 2 partial models that, in turn, show practically overlapping net benefit profiles. Consistent results have been obtained with net reclassification improvement analysis as reported in Table 5.

**All-Cause Death**

Multivariate Cox model, shown in Table 2 (all-cause death), indicated that age, LVEF, chronic kidney disease, chronic obstructive pulmonary disease, serum NT-proBNP, and lymphocyte GRK2 protein levels were significantly associated with all-cause mortality, showing a global $R^2$ of 0.45, a Haller’s C=0.77±0.02 and a good calibration (nonsignificant Gronnesby and Borgan calibration test, $P=0.98$). Differently from cardiac mortality, NYHA class did not result an independent predictor of all-cause mortality. As for CV mortality, heart rate, systolic blood pressure, low serum cholesterol, hyperuricemia, and presence of left bundle branch block did not show a significant association with all-cause death. Similar to the results obtained for cardiac death, NT-proBNP and GRK2 were the strongest factors with an equivalent impact on survival (HR, 2.1 and 1.94 and partial $R^2$ contributions, 29.6% and 21.7%, respectively).

**Discussion**

The findings of this study demonstrate, for the first time, that peripheral lymphocyte levels of GRK2 can independently predict mortality in patients with HF. The prognostic power of GRK2 measurement seems incremental to that provided by clinical, functional, and biohumoral parameters commonly used for risk stratification of HF patients, with an impact on both cardiac and all-cause mortality similar to that exerted by the NT-proBNP in our studied population.

**GRK2 Signaling in HF**

Increased cardiac sympathetic activity is associated with progressive myocardial remodeling, decline in LV contractility, worsening symptoms, and increased mortality in patients with chronic HF. Thus, SNS hyperactivity has been clinically investigated with the aim to improve risk stratification, and, potentially, management of HF patients. Importantly, increased NE concentration induces downregulation and desensitization of myocardial βARs via GRK activity, and cardiac GRK2 up-regulation has been recognized as 1 of the major responsible mechanisms of catecholamine-dependent βAR dysregulation and HF progression. Notably, increased lymphocyte and cardiac GRK2 levels have been detected in HF patients with lower LVEF and more severe symptoms. Moreover, mechanical unloading of failing human hearts is associated with decreased peripheral and cardiac GRK2 levels. Despite this evidence, no clinical studies have assessed the prognostic power of peripheral GRK2 measurements although this information would be relevant to establish the clinical value of this novel biomarker in HF.
GRK2 as a Marker of Prognosis in HF

In this study, lymphocyte GRK2 protein levels were independently associated, at multivariate analysis, with CV mortality and all-cause mortality. Lower lymphocyte GRK2 levels identified patients at low-risk mortality. In particular, patients with lymphocyte GRK2 protein levels at 25th percentile showed a lower incidence rate of CV death and all-cause mortality compared with patients with lymphocyte GRK2 protein levels at 75th percentile (Figure 2B). Notably, the prognostic information provided by GRK2 values proved to be additional and comparable with that obtained by the most common prognostic parameters used for risk stratification of HF patients, ie, LVEF and NT-proBNP. Moreover, assessment of lymphocyte GRK2 might have a role in the clinical practice, as demonstrated by the improvement of the clinical net benefit derived from its introduction in the decision curve analysis (Figure 2) and by net reclassification improvement analysis (Table 5). Even in this condition, NT-proBNP and GRK2 contribution resulted to be equivalent.

These findings are consistent with those reported in the AdreView Myocardial Imaging for Risk Evaluation in Heart Failure (ADMIRE-HF) trial10 that assessed the prognostic value of 123I-MIBG cardiac imaging for risk stratification of patients with severe systolic HF. In that study, enrolling 961 patients with mean LVEF of 27.1%, patients with 123I-MIBG heart to mediastinum ratio below the median (<1.60), reflecting impaired cardiac innervations, showed a 6.22-fold increase in the occurrence of cardiac death, with a prognostic value additional to that of LVEF and NT-proBNP, as reported in this study.10 As 123I-MIBG ratio parallels the status of βAR density in patients with HF,23,46 altogether the findings from the ADMIRE trial and the current findings similarly indicate that indexes of cardiac adrenergic derangement provide independent prognostic information on top of commonly used prognostic parameters in HF patients. Therefore, GRK2 levels in white blood cells have the potential to add, over the currently available biomarkers, important information on cardiac βAR signaling and function, whose status is progressively impaired in HF patients with relevant pathophysiological consequences.

Of note, despite a significant albeit weak ($R^2=5.7\%$; $P=0.001$) correlation between lymphocyte GRK2 protein levels and NT-proBNP, the prognostic information provided by GRK2 values proved to be additional and comparable with that obtained by the most common prognostic parameters used for risk stratification of HF patients. This is consistent with the findings from the ADMIRE-HF trial and the current findings similarly indicate that indexes of cardiac adrenergic derangement provide independent prognostic information on top of commonly used prognostic parameters in HF patients. Therefore, GRK2 levels in white blood cells have the potential to add, over the currently available biomarkers, important information on cardiac βAR signaling and function, whose status is progressively impaired in HF patients with relevant pathophysiological consequences.

### Table 4. Cox Proportional Hazard Models for Cardiac Death and All-Cause Mortality

<table>
<thead>
<tr>
<th></th>
<th>Hazard Ratio</th>
<th>$P$</th>
<th>Percent Fraction of Global R2 (%)</th>
<th>Bootstrap Inclusion Frequency (%)</th>
<th>Linearity Stability (%)</th>
<th>Hazard Ratio</th>
<th>$P$</th>
<th>Percent Fraction of Global R2 (%)</th>
<th>Bootstrap Inclusion Frequency (%)</th>
<th>Linearity Stability (%)</th>
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</thead>
<tbody>
<tr>
<td>Age (10 yr)</td>
<td>1.51*</td>
<td>0.001*</td>
<td>17.5*</td>
<td>78.6*</td>
<td>76.30*</td>
<td>1.41*</td>
<td>0.002*</td>
<td>15.9*</td>
<td>80.0*</td>
<td>82.00*</td>
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<tr>
<td>Sex</td>
<td>0.96</td>
<td>0.86</td>
<td>NA</td>
<td>13.5</td>
<td>NA</td>
<td>1.09</td>
<td>0.67</td>
<td>NA</td>
<td>7.3</td>
<td>NA</td>
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<td>LVEF (5% units)</td>
<td>0.86*</td>
<td>0.03*</td>
<td>13.2*</td>
<td>62.2*</td>
<td>87.60*</td>
<td>0.83*</td>
<td>0.003*</td>
<td>19.3*</td>
<td>76.1*</td>
<td>91.30*</td>
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<td>NYHA</td>
<td>2.45*</td>
<td>0.02*</td>
<td>8.7*</td>
<td>62.7*</td>
<td>NA*</td>
<td>1.56</td>
<td>0.12</td>
<td>NA</td>
<td>40.2</td>
<td>NA</td>
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<tr>
<td>In NT-proBNP (IQ units)</td>
<td>2.23*</td>
<td>≤0.0001*</td>
<td>27.5*</td>
<td>95.7*</td>
<td>97.7†</td>
<td>2.05*</td>
<td>≤0.0001*</td>
<td>29.6*</td>
<td>96.2*</td>
<td>99.2†</td>
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<tr>
<td>In GRK2 (IQ units)</td>
<td>2.01*</td>
<td>≤0.0001*</td>
<td>18.9*</td>
<td>89.1*</td>
<td>85.8†</td>
<td>1.94*</td>
<td>≤0.0001*</td>
<td>21.7*</td>
<td>98.1*</td>
<td>75.9†</td>
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<td>Norepinephrine</td>
<td>0.90</td>
<td>0.53</td>
<td>NA</td>
<td>53.5</td>
<td>NA</td>
<td>0.99</td>
<td>0.40</td>
<td>NA</td>
<td>26.4</td>
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<td>NA</td>
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<td>0.80</td>
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<td>35.6</td>
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<td>Low serum cholesterol</td>
<td>1.41</td>
<td>0.32</td>
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<td>16.4</td>
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<td>1.12</td>
<td>0.94</td>
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<td>11.3</td>
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<td>19.3</td>
<td>NA</td>
<td>0.78</td>
<td>0.56</td>
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<td>20.1</td>
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<td>Comorbidity</td>
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<td></td>
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<td>Diabetes mellitus</td>
<td>1.16</td>
<td>0.46</td>
<td>NA</td>
<td>24.3</td>
<td>NA</td>
<td>1.10</td>
<td>0.59</td>
<td>NA</td>
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<td>Hypertension</td>
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<td>28.8</td>
<td>NA</td>
<td>0.86</td>
<td>0.48</td>
<td>NA</td>
<td>40.5</td>
<td>NA</td>
</tr>
<tr>
<td>COPD</td>
<td>1.81*</td>
<td>0.008*</td>
<td>3.0*</td>
<td>73.7*</td>
<td>NA*</td>
<td>1.94*</td>
<td>0.001*</td>
<td>4.8*</td>
<td>84.0*</td>
<td>NA*</td>
</tr>
<tr>
<td>CKD</td>
<td>1.86*</td>
<td>0.003*</td>
<td>11.4*</td>
<td>70.3*</td>
<td>NA*</td>
<td>1.54*</td>
<td>0.02*</td>
<td>8.8*</td>
<td>60.7*</td>
<td>NA*</td>
</tr>
<tr>
<td>Drugs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE I/ARBs</td>
<td>1.61</td>
<td>0.14</td>
<td>NA</td>
<td>55.6</td>
<td>NA</td>
<td>1.15</td>
<td>0.56</td>
<td>NA</td>
<td>17.9</td>
<td>NA</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>0.87</td>
<td>0.55</td>
<td>NA</td>
<td>16.8</td>
<td>NA</td>
<td>0.97</td>
<td>0.88</td>
<td>NA</td>
<td>9.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

ACE I indicates angiotensin-converting-enzyme inhibitor; ARBs, angiotensin receptor blockers; CKD, chronic kidney disease (glomerular filtration rate ≤50 mL/min); COPD, chronic obstructive pulmonary disease; GRK2, G protein-coupled receptor kinase-2; IQ, interquartile (25°–75° percentile); LBBB, left bundle branch block; LVEF, left ventricular ejection fraction; NA, not applicable; NT-proBNP, N-terminal pro-brain natriuretic peptide; and NYHA, New York Heart Association.

*Statistically significant factors.

†Linearity stability for NT-proBNP and GRK2 refers to the stability of the logarithm form of these factors.
levels and circulating plasma NE concentrations, NE did not independently predict CV death or all-cause mortality at multivariate analysis. This result might have at least 2 plausible explanations: (1) the low reproducibility and sensitivity of plasma NE values, whereas GRK2 protein levels, more closely reflecting sustained hyperactivation of βAR by catecholamines, may represent a more stable surrogate of SNS hyperactivity than circulating NE concentration; (2) lymphocyte GRK2 exerts a relevant effect on the mortality that captures the NE association with outcome and actually fully overcomes its prognostic information.

Finally, in our study, variables with an established relevant effect on survival, such as LVEF, age, NT-proBNP, and NYHA class, resulted to be associated with outcomes, along with lymphocyte GRK2 levels. Regarding the lack of significance for factors, such as the presence of diabetes mellitus, β-blocker use, heart rate, systolic blood pressure, low serum cholesterol, hyperuricemia, and presence of left bundle branch block, we can only speculate that it might be ascribed to the capture of a weak effect by the different and more prognosis-impacting factors present in the model.

Study Limitations

This study reports a 2 center experience in a relatively small group of patients and, therefore, deserves further confirmation in a multicenter study enrolling larger number of patients, thus allowing an external validation of the present results both from a prognostic and clinical utility point of view. Our study population was at particularly high CV risk, cautioning to extrapolation of the current findings to other categories of HF patients. Thus, our results are certainly preliminary and must be confirmed in less severe HF populations. In addition, the definitive clinical relevance of our findings can only be assessed in future studies testing whether improvement of the sympathetic innervation apparatus evaluated through monitoring of GRK2 levels is associated with changes in outcomes of HF patients.

We did not verify the relationship between lymphocyte GRK2 and other measures of SNS activity, such as heart rate variability and cardiac I-MIBG. Although this might represent a study limitation for a study addressing the predictive power of a new biomarker reflecting SNS activity in HF, it is important to underline that: (1) there is still debate about the value of low frequency power of heart rate variability as a valuable measure

Figure 1. Cardiovascular death free survival curves at different percentiles (5°, 25°, 50° (median), 75°, and 95°) of ln NT-proBNP (A) and ln GRK2 (B). The curves were obtained from the full Cox model, fixing the ln NT-proBNP in A and the ln GRK2 in B at the specific percentile, and adjusting for the other covariates at the values observed in the study population (directly adjusted curves). In A, the adjusting covariates include also the ln GRK2, whereas in B, ln NT-proBNP was included. GRK2 indicates G coupled-receptor kinase; HR, hazard ratio; IQR, interquartile range (25°–75° percentile); KM, Kaplan–Meier; NT-proBNP, N-terminal-pro brain natriuretic peptide; and pct, percentile.

Figure 2. Decision curve analysis for 60 months cardiac survival. The treat none and treat all curves are compared with the net benefit curves of 3 Cox models: full model (continuous black line), partial model—no N-terminal-pro brain natriuretic peptide (NT-proBNP; continuous gray line), and partial model—no G coupled-receptor kinase (GRK2; dashed black line). The full model profile is higher than the 2 partial model profiles across the critical range of the survival threshold probabilities (40–70%). The 2 partial models profiles overlap across the threshold span. All 3 models show curves well above that of the treat none and treat all.
of cardiac sympathetic tone, (2) cardiac 123I-MIBG was not available for a key fraction of our study population because this study has been planned before the demonstration of the clinical usefulness of this cardiac imaging technique.

Conclusions
Measurement of GRK2 protein levels in circulating lymphocytes provides additional and independent prognostic information on all-cause and CV mortality in HF patients, over and above commonly used prognostic markers. Of noted importance, GRK2, differently from other biomarkers, is strictly related to cardiac adrenergic receptor function, whose dysregulation is a key point of HF pathophysiology and is the target of the most relevant therapy in this syndrome. Future important steps have to assess the clinical value of GRK2 as a new HF biomarker and for its potential introduction in the clinical management of HF patients with ad hoc planned studies, and, finally, development of a more high-throughput GRK2 quantification assay for future potential clinical use.

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Disclosures
None.

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19. McMurray JJ, Adamopoulos S, Anker SD, et al; Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology; ESC Committee for Practice Guidelines. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur J Heart Fail. 2012;14:803–869. doi: 10.1093/eurjhf/hfs105.
23. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. Decreased catecholamine measurement of GRK2 protein levels in circulating lymphocytes; (2) cardiac 123I-MIBG was not available for a key fraction of our study population because this study has been planned before the demonstration of the clinical usefulness of this cardiac imaging technique.

**Table 5. Net Reclassification Improvement of Clinical Model Relative to NT-proBNP or Lymphocyte GRK2 Inclusion**

<table>
<thead>
<tr>
<th></th>
<th>NT-proBNP (%)</th>
<th>Lymphocyte GRK2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P_up in deceased</strong></td>
<td>69.60</td>
<td>67.70</td>
</tr>
<tr>
<td><strong>P_down in survived</strong></td>
<td>68.80</td>
<td>63.90</td>
</tr>
<tr>
<td><strong>Event NRI</strong></td>
<td>39.20</td>
<td>35.30</td>
</tr>
<tr>
<td><strong>Non-event NRI</strong></td>
<td>31.60</td>
<td>27.70</td>
</tr>
<tr>
<td><strong>NRI</strong></td>
<td>70.8±12.8</td>
<td>63.1±12.8</td>
</tr>
</tbody>
</table>

GRK2 indicates G protein-coupled receptor kinase-2; NRI, net reclassification improvement, NT-proBNP, N-terminal pro-brain natriuretic peptide; P_up, proportion of subjects increasing probabilities of death; and P_down, proportion of subjects decreasing probabilities of death.
Prognostic Value of Lymphocyte G Protein-Coupled Receptor Kinase-2 Protein Levels in Patients With Heart Failure

Giuseppe Rengo, Gennaro Pagano, Pasquale Perrone Filardi, Grazia Daniela Femminella, Valentina Parisi, Alessandro Cannavo, Daniela Liccardo, Klara Komici, Giuseppina Gambino, Maria Loreta D’Amico, Claudio de Lucia, Stefania Paolillo, Bruno Trimarco, Dino Franco Vitale, Nicola Ferrara, Walter J. Koch and Dario Leosco

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Model building strategy

The assessment of the impact on the outcome (survival) of several potentially influential variables requires their simultaneous evaluation in a single model in order to explain the effect of each factor independently of the others. This avoids the misleading conclusion that multiple single factor (univariate) models may cause, even if these are a good starting point. Thus, a multivariate analysis was planned and a multivariable model-building strategy was carried out for the present study.

Aware that there is no consensus among researchers on the ‘best’ strategy to find a ‘good’ model, we chose a pragmatic approach as proposed by Royston and Sauerbrei (1). In accord with these Authors, “by ‘good’ we mean a model that is satisfactory from the subject-matter point of view, robust with respect to minor variation of the current data, predictive in new data, parsimonious and useful beyond the dataset on which it was created” (chap. 1.1.1 page 1 of ref 1).

The strategy used can be summarized as follow.

1. The initial step involves, first, the selection of the criteria employed to choose the pool of variables among which the subsequent analysis had to identify the factors relevant to the outcome and, second, the selection of the model class to be used, i.e., the Cox model, being the outcome of interest the survival time. Having in mind the main aim of our model (assess the effect of a new factor of interest, adjusting for some established factors in a multivariable model) we selected a pool of variables representing known factors affecting the outcome (1). Parsimonious selection criteria were used to avoid overfitting bias. The rule of “at least 10 observed events for each tested variable” (chap. 2.9.1 page 47 of ref 1) adopted at an early stage of the analysis was relaxed to “at least 10 observed subjects for each tested variable”. This allows the assessment of a wider spectrum of candidate factors in a framework with an acceptable balance between the number of observations and the size of the model tested.

2. The multivariable analysis follows. The first aim of the analysis is the selection of the ‘important’ factors that independently affect the outcome picking them out as a subset of the initial pool by means of a stepwise selection algorithm. The second aim is the clarification of the functional form (linearity or non-linearity) of the continuous predictors since the linearity assumption “may prevent one from recognize a strong effect or lead one to mismodel the effect” (chap 1.2.1 pag.8 of ref 1). The Multivariable Fractional Polynomial (MFP) modelling algorithm developed by Sauerbrei and Royston (2) addresses these two key tasks in multivariable model building: elimination of ‘unimportant’ variables and selection of a ‘reasonable’ dose-response function for continuous variables (chap. 1.7.3 page 19 of ref 1). The MFP algorithm combines a backward variables elimination with a search for the best functional form (linear or not linear) of continuous variables (ref. 1, 2).

3. The weight of each significant factor in the model is evaluated by both the hazard ratio and by the contribution to the global explained variation ($R^2$). The partition of the global $R^2$ is accomplished by the Shapley-Owen decomposition algorithm (3). The hazard ratio of continuous variables is related to clinically meaningful variation (e.g. 10 years period for age, 5% units for left ventricular ejection fraction). For variables without a range of definite clinical meaning the hazard ratio relative to the interquartile range (75th-25th percentile difference) is employed. This allows comparison of the relative weight between factors and give a measure of the factor relevance on the studied population (e.g. NT-proBNP, GRK2, and norepinephrine). The interquartile range was preferred over the standard deviation given the non normal distribution of the variables.

4. Since the Cox model was adopted, it was mandatory to verify the proportionality assumption inherent with the basic formulation. To this end we used a modified version of the MFP (the MFPT) devoted
to comprehensively explore the time-variable(s) interaction in order to check the assumption (chap. 11.1.1 page 242-243 of ref 1).

5. The next step involve the computation of model performance measures, i.e., calibration, discrimination ability and internal validity.
   a) The first is a goodness of fit assessment that we accomplished with the Gronnesby and Borgan calibration test (4). This test verifies the concordance between the observed survival (Kaplan Meier) and the survival estimated with the Cox model in five risk groups of the studied population. Five contiguous strata of the prognostic index (the linear combination of the factors with their Cox coefficients) identify the risk groups. Non-significant test indicates good calibration (4).
   b) The discrimination ability refers to how well the model can distinguish between patient outcomes. We used two indices to quantify it, first the Harrel’s C as a natural extension of the binary logistic C statistic (the area under the ROC curve), specifically we used a Harrel’s C version corrected for the censoring bias as suggested by Gonen and Heller (5). Second, we used a measure of the explained variance in the natural scale of the Cox model (R2) as proposed by Royston and Sauerbrei (6).
   c) Adhering to the suggestion expressed by Royston and Sauerbrei (chap 2.2 page 24 of ref 1) we measured the internal validity of the model by assessing the stability of the model characteristics with nonparametric bootstrap sampling (chap 8 page 183-186 of ref 1). Briefly, given the parameters of a model obtained applying the described model-building procedure, the stability of each factor tested in the model is measured by the frequency that this factor is selected as ‘significant’ in a series of bootstrap replications of the dataset by applying the same procedure. Each bootstrapped dataset may be considered as a random replicate of the original dataset.

External validity cannot be evaluated since it requires an independent dataset (i.e. an independent HF population) on which verify the model obtained in the studied population. Splitting the available dataset in ‘test’ and ‘training’ groups is also not feasible since it would require a greater dataset dimension. Therefore, as we pointed out in the limitations, the external validity had to be deferred to future studies.

6. The assessment of the clinical utility of a new marker is a mandatory step before its employment in the clinical practice and requires an ‘ad hoc’ study design oriented to the specific characteristics of the marker and of the clinical pathology involved. However, great interest has been raised by the possibility of gather measures of clinical utility from the same dataset used to assess the impact of a new biomarker. Several measures of usefulness have been suggested and gained popularity. These include the Net Reclassification Improvement (NRI) (7), weighted NRI (wNRI) (8), Net Benefit (NB) (9) and Relative Utility (RU) (10). A study comparing the performance of all these indices (11) concluded that the three utility measures that take into account misclassification cost (wNRI, NB and RU) are preferable over the NRI and, “being a mathematical transformations of each other, lead to equivalent information”. Notably, the Authors that first introduced the named indices jointly conducted this study. The Authors concluded recommending the use and report of these decision-analytic measures for a range of risk thresholds, thus grounding the deduction over a meaningful range of risk.

We, therefore, adopted the NB plots of different models over a wide risk range to enlighten the utility of the new biomarker.

As regards the way to illustrate graphically the effect of the factor of interest on the outcome (survival), since the stratified Kaplan Meir graphs cannot take into account the presence of confounding, survival curves adjusted by influential covariate have to be employed.

We adopted the directly adjusted survival curve method (12), namely, for each subject in the data set a survival curve is computed using the estimated Cox model. Each curve is obtained using the covariate values specific of each subject except for the factor of interest that is set to a given value for all curves. An average curve is then computed. This curve represent an estimate of the Kaplan Meier survival curve that would be observed if all subjects in the study population had had the factor of interest at the chosen value. Actually, if the process is carried out using the values observed for each subject for all covariates (including the one of interest) the curve obtained indeed ‘is’ the overall Kaplan Meier.
Thus a plot of ‘directly adjusted curves’ at the appropriate factor of interest values along with the standard overall Kaplan Meier will give graphical view of the strength of the effect of the factor of interest independent from confounding.

STATA (v.13.0) was used to perform all analyses.
Supplemental Table I. Adjudicated causes of death and rates of death by cause

<table>
<thead>
<tr>
<th>Cause</th>
<th>N</th>
<th>% of pts</th>
<th>% of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Cause Deaths</td>
<td>131</td>
<td>51.0%</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular deaths</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudden</td>
<td>42</td>
<td>16.3%</td>
<td>32.1%</td>
</tr>
<tr>
<td>Worsening HF</td>
<td>26</td>
<td>10.1%</td>
<td>19.8%</td>
</tr>
<tr>
<td>Fatal Stroke</td>
<td>5</td>
<td>1.9%</td>
<td>3.8%</td>
</tr>
<tr>
<td>Other Cardiovascular</td>
<td>29</td>
<td>11.3%</td>
<td>22.1%</td>
</tr>
<tr>
<td>Non Cardiovascular</td>
<td>27</td>
<td>10.5%</td>
<td>20.6%</td>
</tr>
<tr>
<td>Infection</td>
<td>14</td>
<td>5.4%</td>
<td>10.7%</td>
</tr>
<tr>
<td>Malignancy</td>
<td>11</td>
<td>4.3%</td>
<td>8.4%</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>0.8%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>0.8%</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

HF= heart failure; MI= myocardial infarction; pts=patients.
Supplemental Table II. Beta blocker dose distribution in patients with lymphocyte GRK2 below and above the median value.

<table>
<thead>
<tr>
<th>Beta Blockers therapy dose</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pts below lymphocyte GRK2 median value 1.31 D.U.), % (n) ≤</td>
<td>44.8% (30)</td>
<td>43.3% (29)</td>
<td>11.9% (8)</td>
</tr>
<tr>
<td>Pts above lymphocyte GRK2 median value 1.30 D.U.), % (n) ≥</td>
<td>50.0% (33)</td>
<td>39.4% (26)</td>
<td>10.6% (7)</td>
</tr>
<tr>
<td>p=0.833</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GRK2 = G protein-coupled receptor kinase 2. Pts = patients
Supplemental Figure I.

Lymphocyte GRK2 levels in patients not assuming beta-blocker therapy and in patients at low, medium and high doses of beta-blocker therapy. BBlocker= beta-blocker
Supplemental References


