The Synergic Association of hs-CRP and Serum Amyloid P Component in Predicting All-Cause Mortality in Patients With Type 2 Diabetes

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OBJECTIVE

Type 2 diabetes is characterized by increased death rate. In order to tackle this dramatic event, it becomes essential to discover novel biomarkers capable of identifying high-risk patients to be exposed to more aggressive preventive and treatment strategies. hs-CRP and serum amyloid P component (SAP) are two acute-phase inflammation proteins, which interact physically and share structural and functional features. We investigated their combined role in associating with and improving prediction of mortality in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Four cohorts comprising 2,499 patients with diabetes (643 all-cause deaths) were analyzed. The improvement of mortality prediction was addressed using two well-established prediction models, namely Estimation of Mortality Risk in Type 2 Diabetic Patients (ENFORCE) and Risk Equations for Complications of Type 2 Diabetes (RECODe).

RESULTS

Both hs-CRP and SAP were independently associated with all-cause mortality (hazard ratios [HRs] [95% CIs]: 1.46 [1.34–1.58] [$P < 0.001$] and 0.82 [0.76–0.89] [$P < 0.001$], respectively). Patients with SAP ≤33 mg/L were at increased risk of death versus those with SAP >33 mg/L only if hs-CRP was relatively high (>2 mg/L) (HR 1.96 [95% CI 1.52–2.54] [$P < 0.001$] and 1.20 [0.91–1.57] [$P = 0.20$], in hs-CRP, >2 or ≤2 mg/L subgroups, respectively; hs-CRP-by-SAP strata interaction $P < 0.001$). The addition of hs-CRP and SAP significantly (all $P < 0.05$) improved several discrimination and reclassification measures of both ENFORCE and RECODe all-cause mortality prediction models.

CONCLUSIONS

In type 2 diabetes, hs-CRP and SAP show opposite and synergic associations with all-cause mortality. The use of both markers, possibly in combination with others yet to be unraveled, might improve the ability to predict the risk of death in real-life setting.

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Type 2 diabetes is one of the leading cause of death worldwide (1). If not strenuously tackled, this tremendous burden will increase further in the near future, because of the rapidly rising prevalence of the disease (2). Identifying the highest-risk patients for the most aggressive and burdensome prevention strategies is instrumental for pursuing this ambitious goal. Unraveling novel biomarkers that improve risk stratification is a way to address this need. Additionally, such biomarkers may also be useful in pointing out pathogenic pathways that are still unknown and that may eventually become the target of novel treatments (3).

A state of chronic low-grade inflammation plays a central role in the increased risk of mortality in patients with diabetes. Chronic low-grade inflammation goes with acute phase, an orchestrated reaction to tissue injury, infection, or inflammation characterized by the induction of specific proteins, which are instrumental for restoring homeostasis (4). More recently, acute-phase proteins have been advocated to either promoting or attenuating atherosclerosis from its onset to cardiovascular events (5). Among acute-phase inflammation-induced proteins (6), CRP and serum amyloid P component (SAP) are the two short pentraxins, primarily produced by hepatocytes, mainly in response to proinflammatory cytokines (6). CRP, which colocalizes with complement deposits in human atherosclerotic lesions (7), binds to oxidized LDL and mediates their uptake by macrophages and plaque deposition, thus initiating foam cell formation and early atherosclerosis (8). Despite genetic studies questioning its causative role (9), CRP is a strong and established marker of cardiovascular events (10) and of all-cause mortality in different clinical settings (11). Conversely, the ability of CRP to improve risk stratification is still controversial (12). SAP is also found in human atherosclerotic plaques (13) and, similar to CRP, binds oxidized LDL (14). However, unlike CRP, such binding blocks LDL uptake by macrophages, thus preventing atherosclerosis (14). Unfortunately, data are sparse and conflicting on the association between SAP and cardiovascular risk factors (15) and events (15). Furthermore, not many data on the role of SAP on premature death are available (16,17). Notably, the two proteins share structural features, being organized in five identical subunits arranged in a pentameric radial symmetry and biological functions, including activation of the complement system and recognition of pathogens (6). Moreover, in Ca^{2+}-free conditions, SAP pentamers physically interact with CRP pentamers to form very stable mixed decamers (18). This interaction may have functional consequences on the activation of inflammation and atherosclerosis (19). Finally, CRP and SAP are encoded by two genes, *CRP* and *APCS*, located on chromosome 1q23, which are only 200 kb apart (20), suggesting an ancient gene duplication and possibly a common regulation and/or activation of functions related to acute-phase inflammation response, as also indicated by pathway and expression analyses (21).

Unfortunately, the combined role of CRP and SAP on clinical events related to acute-phase inflammation and atherosclerosis has never been properly addressed.

To provide our contribution to this topic, we first assessed the combined association of hs-CRP and SAP with mortality rate in patients with type 2 diabetes. We then evaluated the extent to which these biomarkers improve the prediction of all-cause death when added on top of prediction models of mortality risk in type 2 diabetes (22–24), which, though well performing, still need to be improved up to outstanding discrimination ability (25).

RESEARCH DESIGN AND METHODS

Participants

Four cohorts of patients with type 2 diabetes (diagnosed according to American Diabetes Association 2018 criteria)—three from Apulia, central-southern Italy, and one from Tuscany, central Italy—were analyzed.

Gargano Heart Study-Prospective Design

The Gargano Heart Study (GHS)–prospective design comprises 368 patients with type 2 diabetes and coronary artery disease consecutively recruited from 2001 to 2008 at the Endocrine Unit of Fondazione IRCCS "Casa Sollievo della Sofferenza" in San Giovanni Rotondo (Gargano, center east coast of Italy) and followed yearly until 2011 for both cardiovascular and all-cause mortality. The vital status of study patients was ascertained by death certificates (26).

Serum hs-CRP and SAP were assessed in 358 participants (97.3%), constituting the eligible sample for this analysis.

Gargano Mortality Study

The present sample of the Gargano Mortality Study (GMS) includes 902 patients recruited from 2000 to 2005 at the Endocrine Unit of Fondazione IRCCS "Casa Sollievo della Sofferenza" in San Giovanni Rotondo for a study having all-cause mortality as end point and followed until December 2014. Serum hs-CRP and SAP were assessed in 650 participants (72%), constituting the eligible sample for this analysis. Clinical features of patients who were or were not included in the current study are very similar except for a little longer follow-up (i.e., $<$10 months) and a 5% lower estimated glomerular filtration rate (eGFR) for the former group (Supplementary Table 1).

Foggia Mortality Study

The Foggia Mortality Study (FMS) comprises 1,115 patients consecutively recruited at the Endocrine Unit of the University of Foggia (central-southern Italy) from 2002 to 2008 for a study having all-cause mortality as end point and followed until March 2015.

After excluding 70 subjects with missing variables, serum hs-CRP and SAP were assessed in 530 participants (51%), constituting the eligible sample for this analysis. No significant differences in clinical characteristics were present between participants who were and those who were not included in the current study (Supplementary Table 1).

Pisa Mortality Study

The Pisa Mortality Study (PMS) comprises 961 patients consecutively recruited at the Endocrine Unit of the University of Pisa (central Italy) from 2002 to 2008, for a study aimed at identifying predictors of incident all-cause mortality and followed until December 2017.

Serum hs-CRP and SAP were assessed in all 961 participants (100%).

For GMS, FMS, and PMS, the vital status of participants was verified by interrogating the Italian Health Card Database upon data anonymization (https://sistemats1.sanita.finanze.it/wps/portal/) (22,23,26).

For all studies, the only inclusion criterion was the presence of type 2 diabetes according to American Diabetes Association criteria, and the only exclusion criterion was the presence of poor life expectancy for non–diabetes-related diseases (22,23,26).

Biochemical Measurements

All samples from the four different cohorts were handled identically. Measurement of serum hs-CRP and SAP was centralized at the Research Unit of Diabetes and Endocrine Diseases in San Giovanni Rotondo and run in duplicate, using a Multiplex Detection 4-Plex kit also containing α2 macroglobulin and haptoglobin from Bio-Rad (Hercules, CA). The median coefficient of variation was $<$15% for all analyzed acute-phase proteins. Data analyses were performed using Bio-Plex Manager software version 6.1 (Bio-Rad). Acute-phase proteins concentrations were interpolated from an appropriate standard curve.

Statistical Analysis

Patients' baseline characteristics were reported as mean $\pm$ SD or median and interquartile range and frequency and percentage for continuous and categorical variables, respectively. Because of skewed distribution, hs-CRP and SAP were logarithmically transformed and then standardized.

Correlation and multicollinearity between hs-CRP and SAP were assessed by Pearson correlation coefficients and variance inflation factors, respectively.

In all prospective studies, time variable was defined as the time between the baseline examination and date of the event (i.e., all-cause mortality) or, for subjects who did not experience the event, the date of the last available clinical follow-up. Incidence rate for all-cause mortality was expressed as the number of events per 100 person-years.

To assess the association between hs-CRP and SAP levels, both singly and in a combined fashion, and all-cause mortality, unadjusted and adjusted Cox proportional hazard models were estimated within each study and in the pooled sample. Age at recruitment, sex, smoking habit, BMI, HbA_{1c}, diabetes duration, hypertension, and insulin and statins treatments were considered as possible confounders in the fully adjusted model. Analyses in the pooled sample were also adjusted for study cohort. Risks were reported as hazard ratios (HRs) along with their 95% CIs per 1-SD increase in standardized log hs-CRP and standardized log SAP levels.

Interaction analysis on the relationship between hs-CRP and SAP and all-cause mortality was investigated by fully adjusted multivariate Cox regression analysis, including both main effects and the interaction term.

To deeply evaluate the combined association of hs-CRP and SAP levels on all-cause mortality, the pooled sample was stratified according to hs-CRP $\leq$2 or $>$2 mg/L, a cutoff proposed for discriminating high-risk individuals for cardiovascular events (12), and SAP $\leq$33 or $>$33 mg/L (i.e., the threshold between the first and the second tertile) so to create four groups: group 1 (i.e., hs-CRP $\leq$2 mg/L and SAP $>$33 mg/L), group 2 (i.e., hs-CRP $\leq$2 mg/L and SAP $\leq$33 mg/L), group 3 (i.e., hs-CRP $>$2 mg/L and SAP $>$33 mg/L), and finally, group 4 (i.e., hs-CRP $>$2 mg/L and SAP $\leq$33 mg/L). Kaplan-Meier plots were generated, and the adjusted Cox proportional hazards model, including such subgroups as the exposure, was estimated.

As a sensitivity analysis, a competing-risk analysis was performed in which the noncardiovascular death is the competitor of cardiovascular death, using subdistribution HRs estimated through Fine and Gray proportional hazards regression (27).

To examine whether the combined associations of the two pentraxins and their interaction increase the prediction accuracy of all-cause mortality prediction models in type 2 diabetes, two different well-established prediction models were used: Estimation of Mortality Risk in Type 2 Diabetic Patients (ENFORCE) (23) and Risk Equations for Complications of Type 2 Diabetes (RECODe) (24). Predictors included in the two models are reported in the Supplementary Materials. For each of these, five models were investigated: model 1, as the reference model; model 2, adding hs-CRP on top of model 1; model 3, adding SAP on top of model 1; model 4, with both hs-CRP and SAP on top of model 1; and model 5, also adding the hs-CRP-by-SAP interaction term to model 4. Discrimination was measured by survival *c* statistic, Δ *c* statistic (28) and the improvement in discrimination by the survival version of the relative integrated discrimination improvement (rIDI) (29). In addition, the survival version of the category-free net reclassification improvement (cNRI) (30), which examines whether the predicted probabilities of individuals with and without events move in the right directions (upward and downward, respectively), from the base to the new model was evaluated. The 95% CIs for discrimination and reclassification measures were computed by bootstrap.

A *P* value $<$0.05 was considered significant. All analyses were performed using SAS Release 9.4 (SAS Institute, Cary, NC).

RESULTS

Clinical features of patients from GHS-prospective design, GMS, FMS, and PMS as well as duration of follow-up and number of events are summarized in Supplementary Table 2. None of the study patients were affected by rheumatoid arthritis or inflammatory bowel disease. Pearson correlation coefficient between hs-CRP and SAP was moderately positive (i.e., $<$0.52 in each cohort as well as in the pooled sample), with no evidence of multicollinearity (variance inflation factor of 1.22).

In all four cohorts, hs-CRP but not SAP (Table 1, first two rows) was associated with all-cause mortality. Conversely, in a model comprising both proteins, while the association of hs-CRP within each sample did not change (Table 1, third row), SAP became negatively associated with all-cause mortality in all cohorts, reaching statistical significance in three of them (Table 1, fourth row).

In the pooled analysis, comprising a total of 2,499 individuals and 643 events, hs-CRP was positively whereas SAP was negatively associated with all-cause mortality (Table 1, fifth column), with the size effect on a log scale of hs-CRP being almost twice as much that of SAP. In a fully adjusted model, both associations remained strongly significant (Table 2). In the same model, no modifier effects were observed for sex or age at diagnosis (i.e., $<$40 or $>$40 years) (*P* of heterogeneity = 0.35 and 0.28, respectively). In the three cohorts (i.e., GMS, FMS, and PMS; n = 2,139 patients) in which eGFR was available, the addition of this covariate did not change the observed associations (HRs [95% CIs] 1.37 [1.25–1.50] vs. 1.35 [1.23–1.48] and 0.86 [0.79–0.93] vs. 0.84 [0.77–0.91]; all $P < 0.001$ before and after entering eGFR in the model for hs-CRP and SAP, respectively).

A negative hs-CRP-by-SAP interaction was observed (β value [SE] = $-$0.10 [0.04]; $P < 0.01$), with the protective effect of SAP becoming evident only in the presence of relatively high hs-CRP levels. An alternative way to address

Table 1—Univariate and bivariate associations between serum hs-CRP and SAP with all-cause mortality

	GHS-prospective design	GMS	FMS	PMS	Pooled sample*
hs-CRP†	1.83 (1.49–2.24); <0.001	1.57 (1.39–1.79); <0.001	1.19 (1.02–1.39); 0.03	1.26 (1.10–1.43); <0.001	1.40 (1.30–1.51); <0.001
SAP†	1.03 (0.83–1.29); 0.79	0.90 (0.80–1.02); 0.09	0.89 (0.77–1.03); 0.12	1.10 (0.96–1.27); 0.18	0.97 (0.89–1.04); 0.38
hs-CRP‡	2.02 (1.63–2.49); <0.001	1.76 (1.54–2.01); <0.001	1.27 (1.08–1.50); <0.01	1.28 (1.10–1.50); <0.01	1.54 (1.42–1.67); <0.001
SAP‡	0.78 (0.64–0.94); 0.01	0.75 (0.67–0.83); <0.001	0.83 (0.72–0.95); 0.01	0.96 (0.81–1.13); 0.60	0.80 (0.75–0.87); <0.001

Data are HR (95% CI); *P* value. HRs (95% CI) are given for 1-SD increase of log-transformed values of serum hs-CRP and SAP. *Adjusted for study cohort. †Univariate association. ‡Bivariate association.

this interaction is illustrated in Fig. 1, in which patients were stratified into four subgroups according to hs-CRP and SAP levels, as described in Research Design and Methods. Among patients with hs-CRP ≤2 mg/L (n = 1,152; 230 events), no significant difference in the rate of all-cause mortality by Cox regression multivariate model was observed between those with relatively low (i.e., ≤33 mg/L, the first tertile) and high (>33 mg/L) SAP levels (HR [95% CI] 1.20 [0.91–1.57]; $P = 0.20$). On the contrary, among patients with hs-CRP >2 mg/L (n = 1,347; 413 events), individuals with low SAP levels were at significantly higher risk than those with high SAP levels (HR [95% CI] 1.96 [1.52–2.54]; $P < 0.001$). Notably, the different association between low and high SAP levels across the two hs-CRP strata was highly significant (i.e., P for hs-CRP-by-SAP strata interaction = 0.002). An even stronger interaction was obtained when a higher hs-CRP cutoff (i.e., hs-CRP >3 mg/L) was used to create the four strata (P for interaction = 0.0003). In all, along the same line of what was observed when the two proteins were treated as continuous variables, these data suggest that SAP shapes the risk of all-cause mortality predominantly, if not exclusively, in patients with high hs-CRP.

Finally, in GHS-prospective design, the risk of cardiovascular mortality (57 events) in a model comprising both markers was 1.90 (1.48–2.44; $P < 0.001$) and 0.78 (0.61–0.98; $P = 0.04$) for a 1-SD increment of hs-CRP and SAP, respectively. The associations observed when performing a competing-risks analysis, with noncardiovascular death (n = 24 events) used as competitor (HR 1.78 [95% CI 1.41–2.24], $P < 0.001$ and 0.79 [0.64–0.98], $P = 0.01$, for hs-CRP and SAP, respectively), were similar to those with all-cause mortality (Table 1, first column), thus suggesting the latter were not exclusively driven by cardiovascular mortality.

The Addition of hs-CRP and/or SAP to the ENFORCE and RECODe Mortality Prediction Models

We then tested the effect of adding hs-CRP and/or SAP on top of ENFORCE, a well-performing, validated, and freely available prediction model for 6-year all-cause mortality in patients with type 2 diabetes that we recently developed. To this purpose, GMS, FMS, and PMS, comprising a total of 2,139 patients and 233 deaths (at 6 years), in which our ENFORCE model was applicable, were used.

Four models, as described in Research Design and Methods, were then likened (Table 3). As compared with model 1, the reference model, model 2, but not 3, showed an improved c statistic and a significant percentage of rIDI (Table 3). A further rise of both measures was observed in model 4 (Table 3). When risk reclassification was investigated, results were along the same line, with cNRI being significant in model 2 and even more in model 4, but not in model 3 (Table 3). In both models 2 and 4, most cNRI was driven by nonevents correctly reclassified (Table 3). Notably, when model 2 was used as the reference model, both percentage of rIDI ($P < 0.01$) and cNRI ($P = 0.04$) measures were significantly improved. A similar trend, though not reaching statistical significance, was observed for Δ c statistic ($P = 0.08$).

As compared with model 4, no difference was observed in model 5, in which an interaction term between hs-CRP and SAP was also added (data not shown).

The ability of hs-CRP and SAP in improving all-cause mortality prediction was also tested in RECODe, a well-performing and validated prediction model for 10-year mortality in patients with type 2 diabetes. To this purpose, a total of 1,169 patients and 415 deaths (at 10 years) from GMS and PMS, the only two cohorts with a median follow-up of >10 years, were studied by using the same four models described above. As compared with model 1, model 2, but not 3, showed a significant percentage of rIDI and cNRI increase (Table 3). An improved c statistic and a further rise of percentage of rIDI and cNRI were observed in model 4 (Table 3). In both

Table 2—Multivariable associations with all-cause mortality in the pooled sample

	HR (95% CI)	*P*
hs-CRP	1.46 (1.34–1.58)	<0.001
SAP	0.82 (0.76–0.89)	<0.001
Males vs. females	1.69 (1.42–2.01)	<0.001
Age at recruitment (per 10 years)	2.20 (1.98–2.45)	<0.001
Smoking habit (yes vs. no)	1.34 (1.08–1.68)	0.01
BMI (per 1 unit)	1.00 (0.99–1.02)	0.69
HbA_{1c} (per 1 unit)	1.08 (0.99–1.18)	0.08
Disease duration (per 10 years)	1.01 (1.00–1.02)	0.08
Hypertension (yes vs. no)	1.54 (1.23–1.94)	<0.001
Insulin treatment (yes vs. no)	1.63 (1.37–1.95)	<0.001
Statins treatment (yes vs. no)	0.89 (0.75–1.06)	0.21

HRs (95% CI) for serum hs-CRP and SAP are given for 1-SD increase of log-transformed values. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or antihypertension therapy. Analyses are adjusted for study cohort. HbA_{1c}, glycated hemoglobin A_{1c}.

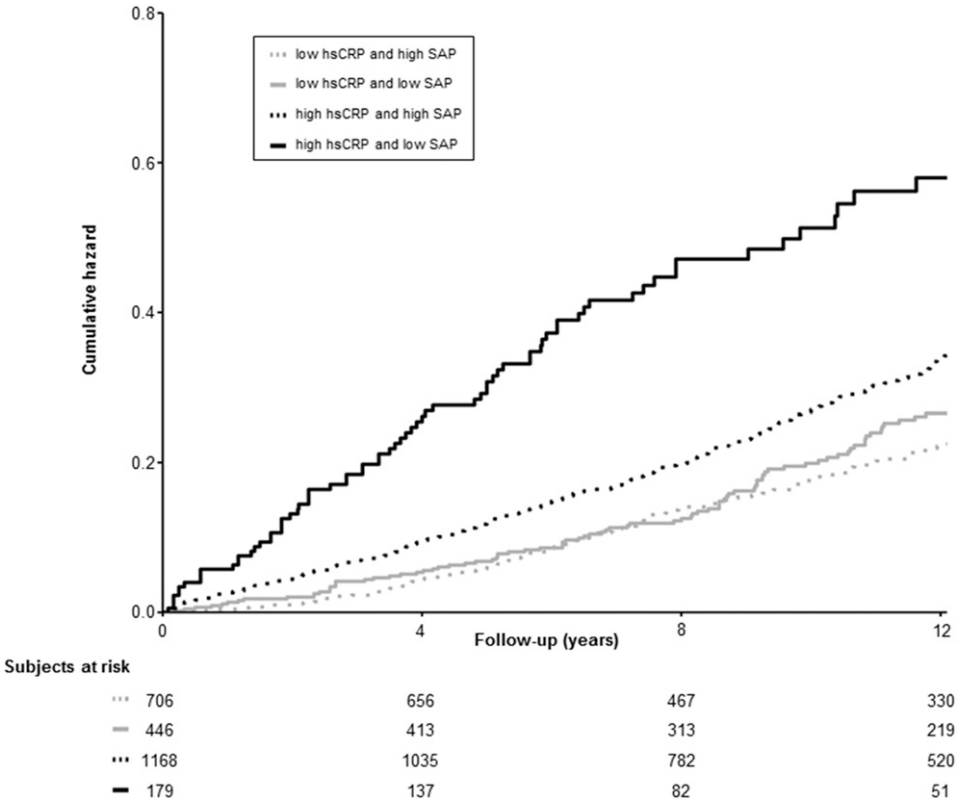

Figure 1—Kaplan-Meier survival curves for all-cause mortality in the pooled sample divided into four groups according to hs-CRP levels ≤2 mg/L (gray lines) or >2 mg/L (black lines) and SAP ≤33 mg/L (the first tertile, solid lines) or >33 mg/L (dotted lines): group 1, indicated in the inset as low hs-CRP and high SAP (gray dotted line; i.e., hs-CRP ≤2 mg/L and SAP >33 mg/L; $n = 706$; 132 events); group 2, indicated in the inset as low hs-CRP and low SAP (gray solid line; i.e., hs-CRP ≤2 mg/L and SAP ≤33 mg/L; $n = 446$; 98 events); group 3, indicated in the inset as high hs-CRP and high SAP (black dotted line; i.e., hs-CRP >2 mg/L and SAP >33 mg/L; $n = 1{,}168$; 338 events); and finally, group 4, indicated in the inset as high hs-CRP and low SAP (black solid line; i.e., hs-CRP >2 mg/L and SAP ≤33 mg/L; $n = 179$; 75 events). The number of subjects at risk for each group for each time point (i.e., 0, 4, 8, and 12 years) is detailed at the bottom of the figure.

models 2 and 4, most cNRI was driven by nonevents correctly reclassified (Table 3). Notably, when model 2 was used as the baseline model, Δ *c* statistic ($P = 0.03$), percentage of rIDI ($P = 0.01$), and cNRI ($P = 0.01$) measures were all significantly improved.

In all, these data consistently show the ability of the combined use of hs-CRP and SAP to improve both discriminatory and reclassification measures of well-established prediction models of all-cause mortality in patients with type 2 diabetes.

CONCLUSIONS

To the best of our knowledge, this is the first study to show that serum hs-CRP and SAP are associated in an independent, opposite, and synergic fashion with mortality rate in patients with type 2 diabetes. Both associations were independent of age at diabetes diagnosis, a known modulator of mortality risk in these patients (31). Notably, statistically significant associations were observed in all (for hs-CRP) or in three out of the four

Table 3—Survival discrimination and reclassification measures of the ENFORCE and RECODe models

		Discrimination			Reclassification		
Prediction models		*c* statistics (95% CI)	Δ *c* statistics (*P*)	Percent of rIDI (*P*)	Percent of one-half cNRI (*P*)	Percent of events (*P*)	Percent of nonevents (*P*)
ENFORCE	Model 1 (ENFORCE)	0.75 (0.72–0.79)					
	Model 2 (ENFORCE + hs-CRP)	0.76 (0.73–0.80)	0.01 (0.01)	9.6 (0.01)	10.5 (<0.01)	3 (0.35)	18 (<0.001)
	Model 3 (ENFORCE + SAP)	0.75 (0.72–080)	0.00 (0.23)	2.3 (0.60)	2.5 (0.43)	—	—
	Model 4 (ENFORCE + hs-CRP + SAP)	0.77 (0.74–0.81)	0.02 (<0.01)	19.4 (0.01)	14.5 (<0.001)	6 (0.25)	23 (<0.001)
RECODe	Model 1 (RECODe)	0.73 (0.71–0.76)					
	Model 2 (RECODe + hs-CRP)	0.73 (0.71–0.76)	0.00 (0.25)	7.8 (<0.001)	7.0 (<0.01)	1 (0.81)	13 (<0.001)
	Model 3 (RECODe + SAP)	0.73 (0.71–0.76)	0.00 (0.12)	1.0 (0.78)	2.0 (0.44)	—	—
	Model 4 (RECODe + hs-CRP + SAP)	0.74 (0.72–0.77)	0.01 (<0.05)	14.5 (0.01)	10.0 (<0.001)	4 (0.09)	16 (<0.001)

All *P* values in the table are referred to comparisons vs. model 1. Predictors included in the ENFORCE and RECODe models are described in the Supplementary Materials.

(for SAP) study cohorts, thus making both findings replicable across independent samples. Competing-risk analysis, conducted in one of our samples, in which information on cardiovascular and noncardiovascular death was available, strongly suggests that the associations of hs-CRP and SAP with all-cause mortality are not solely driven by cardiovascular disease.

While the role of hs-CRP as a marker of cardiovascular disease and all-cause mortality is established (10,11), that of SAP, which is mainly related to fibrotic clinical conditions and related treatments (32–34), is scarce and conflicting. In fact, while SAP circulating levels positively predict angina and myocardial infarction (but not stroke and cardiovascular death) in elderly subjects (15), they are inversely related to the risk of all-cause mortality in some peculiar clinical conditions, including sepsis (16) and HIV (17), thus resembling our present findings in type 2 diabetes.

Although the association of all-cause mortality with hs-CRP was also observed when singly considered, the one with SAP was not, becoming evident when only both pentraxins were included in the model. This is likely due to the significant interaction observed between the two molecules, which clearly indicates that hs-CRP plays a permissive role on the protective effect of SAP on the risk of mortality. The biology behind this interesting phenomenon is unknown. Considering that genetic studies have questioned a biological role of CRP in directly shaping the risk of cardiovascular disease and death (9), even generating hypotheses becomes difficult. Thus, only speculations can be offered in this study. For example, it is worth noticing that a physical interaction between the two proteins has been previously reported (18) and that this could have functional consequences. Furthermore, *CRP* and *APCS* genes encoding for the two proteins are located in the same region on chromosome 1q23 (20), probably sharing common regulation and functions related to acute-phase inflammation response as also indicated by pathway and expression analyses (21). All of this makes it possible that activation and regulation of CRP and SAP and their biological effects may be interdependent.

Remarkably, regardless of the underlying biology, the interaction between CRP and SAP is clinically relevant because it improves the stratification of the individual risk of death. In fact, in patients already considered at risk because of hs-CRP $>$2 mg/L (12), having low SAP levels is particularly deleterious, further increasing their risk. Conversely, SAP levels are not decisive for modeling the risk of death in individuals with hs-CRP $\leq$2 mg/L.

The prediction analyses have highlighted that the addition of hs-CRP, but not SAP, improves the discrimination ability of both ENFORCE (23) and RECODe (24), two well-established prediction models of all-cause mortality in patients with type 2 diabetes.

Although the improvement of *c* statistic is rather small, it is of note that adding both hs-CRP and SAP provided a much larger discrimination improvement, as indicated by percentage of rIDIs. The observed improvements are all well above the threshold of 6%, which is the cutoff suggested by the 2013 American College of Cardiology/American Heart Association Guideline on the Assessment of Cardiovascular Risk for adding new biomarkers on top of already well-performing prediction models (12). In addition, as indicated by statistically significant cNRI values, adding hs-CRP, but not SAP, to ENFORCE and RECODe also causes an important improvement in reclassification, mainly driven by the ability to reclassify nonevents. Finally, adding both hs-CRP and SAP to the models provided a further reclassification improvement. This may be useful in clinical practice to help reducing the risk of overestimation, especially in high-risk individuals, as are patients with diabetes duration of $>$10 years. The improvement of discrimination and reclassification observed when both markers were used is the likely consequence of the interaction between the two pentraxins in shaping the risk of all-cause mortality.

Our study has some strengths as follows. We used well-established prospectively analyzed cohorts with a completeness of information, including standardized clinical evaluations and mortality validated by death certificates. In addition, all samples were handled identically, and measurements of both hs-CRP and SAP were centralized. Moreover, the usefulness of both pentraxins for risk stratification purposes was tested on top of ENFORCE and RECODe, two prediction models of all-cause mortality in patients with diabetes (23,24). This makes our current findings possibly implementable in the routine clinical set, expecially if in combination with other markers yet to be unraveled. At this time, an updated version of ENFORCE is available at https://www.operapadrepio.it/enforce/enforce.php. In all, this study belongs to those efforts needed to turn precision medicine based on objective algorithms into reality and may therefore eventually represent a service for the entire community with diabetes, including patients, their families, and their caregivers.

A major limitation of our study is intrinsic to its very nature. As mentioned before, an observational study does not provide mechanistic information, thus leaving unanswered any question about the biology underlying our findings. Secondly, with the only exception being the GHS-prospective design, no data on cardiovascular mortality were available in our cohorts, thus making it impossible to robustly extend our observation to death of cardiovascular origin. In addition, baseline information on cardiovascular disease/vascular risk factors, majorly responsible for all-cause mortality (35), is only present in the PMS, while the GHS-prospective design comprises only patients with previous cardiovascular events. Such incompleteness of information makes it impossible to address whether this clinical condition affects our results. Keeping this in mind, however, it should be recognized that information on previous cardiovascular events is obtained primarily by self-reporting, which might provide unreliable data that affect the final results (36–38). Another limitation is the lack of information on renal function in one of our cohorts (i.e., the GHS-prospective design). However, such data are available in the remaining cohorts constituting the 85.6% of our study patients in whom they do not affect our findings. Furthermore, although apparently no patients were affected by rheumatoid arthritis or inflammatory bowel disease, we cannot completely exclude that inflammatory diseases have played some role in the reported associations.

Finally, it is not known whether our present findings can be generalizable to patients with a diabetes duration shorter than that of our study individuals or to different ethnic populations with different environmental and/or genetic

backgrounds as well as to different clinical settings. For example, both ENFORCE and RECODe have been reported to perform better in predicting all-cause mortality in real life rather than in a clinical trial setting (23,24,39). Therefore, if the use of CRP and SAP can help to ameliorate the planning of future clinical trials, improving the prediction of all-cause mortality and using such predicted risk as a criterion for inclusion and randomization, it is a possibility that deserves to be addressed in further studies. Particular caution is needed for the role of SAP, which, in the context of cardiovascular disease and mortality rate, needs to be further clarified (15–17). In this regard, it is rewarding that both discrimination and reclassification measures were significantly improved even after adding only hs-CRP to the prediction models.

In conclusion, as far as we know, this is the first study reporting data on the opposite and synergic association of hs-CRP and SAP on mortality rate in a large, unselected sample of real-life patients with type 2 diabetes. Moreover, in this clinical setting, hs-CRP and SAP provided a statistically significant improvement of several measures of established prediction models of all-cause mortality.

Further studies are definitively needed to better understand the strong intertwined relationship between these two short pentraxins in shaping the risk of mortality in patients with type 2 diabetes as well as to investigate the transportability of their role in improving well-established prediction models in other clinical and environmental contexts.

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