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Abstract

Residual inflammatory risk (RIR) is defined as persistent circulating levels of high sensitivity C-reactive protein (hs-CRP) > 2 mg/L despite an optimal (<70 mg/dL) control of LDL cholesterol (C) and represents an emerging risk factor for the development of cardiovascular events in patients at high-risk for atherosclerosis. Few data are available regarding the prevalence of RIR in patients with type 2 diabetes mellitus (T2DM) and the clinical variables associated with hs-CRP elevation. Here, we report data from a well characterized cohort of patients with T2DM (n=511) stratified for statin use, LDL-C goal attainment, and prevalent T2DM complications. Statin use and having at-target LDL-C partially affect the number of patients with inflammatory risk when compared to the whole T2DM population, with a RIR prevalence of 39,2%. Among the spectra of complications, only patients with nephropathy had a higher prevalence of inflammatory risk. Total-C, non-HDL-C, triglycerides, BMI, and waist-hip ratio were associated with hs-CRP, with an increased magnitude in at-target patients. Conversely, glucose-related parameters were strongly associated with hs-CRP only in at-target patients, overall suggesting glycaemic control, insulin resistance, non-LDL-C lipid parameters, and especially central obesity as possible contributors to RIR in patients with T2DM and LDL-C<70 mg/dL.

Introduction

Residual inflammatory risk (RIR) is defined as persistently elevated circulating levels of high-sensitivity C-reactive protein (CRP) despite optimal attainment of the LDL-C goal (1-3). Indeed, among the patients with a previous cardiovascular (CV) event enrolled in multiple trials of high-dose statin therapy, those who achieved LDL-C levels <70 mg/dL and hsCRP levels <2 mg/L had substantially lower rates of recurrent CV events when compared with those who achieved only one or neither of these independent treatment targets, suggesting that the residual vascular risk observed in CV patients could be attributed to both a residual cholesterol risk and to RIR (1-3). The inflammatory hypothesis driving ischemic events has been further substantiated by the results of the CANTOS trial. Indeed, treatment with an anti-IL-1β antibody reduced the incidence of CV events in a population with high CV risk, a cohort that includes 40% of diabetic patients (4). In particular, the intervention was effective in those patients that had a hs-CRP >2mg/L at baseline and below this threshold at the end of the intervention, suggesting the tangible contribution of RIR to CV events (5).

The prevalence of RIR in cohorts of patients with high CV risk ranges from 43% of the PROVE-IT trial to the 61% of the VIRGO registry, a large database providing data from young adults with a

previous myocardial infarction (2,6). A recent publication evidenced a high prevalence of RIR also in patients with type 2 diabetes mellitus (T2DM), as shown in the cohort of the EXAMINE trial, where patients with the highest values of hs-CRP had an increased incidence of CV events, an association independent of, and additive to, achieved LDL-C levels (7). However, less data are available regarding the prevalence of RIR in T2DM patients from real-world cohorts. In addition, few information has been reported regarding the impact of statins and LDL-C goal attainment on RIR prevalence, as well as regarding the clinical variables associated with higher hs-CRP values in patients with T2DM.

Here we explored the impact of statin use, attainment of LDL-C goal, pre-existing T2DM complications on the resulting prevalence of RIR in a well characterized retrospective cohort (n=511) of patients with T2DM. In addition, we analysed the correlation between major clinical variables and hs-CRP among the different subgroups of patients. In order to better disentangle the effect of all these possible contributors, we used data from patients studied before 2009, when statin use and an LDL-C goal of <70mg/dL were considered optional in subjects with T2DM (8).

Methods

Cohort description

Samples derive from a previously published cohort composed of 511 patients with T2DM (9). The study was approved by local Institutional Ethics Committees. Written informed consent was obtained from each subject in accordance with principles of the Declaration of Helsinki. T2DM was diagnosed according to the ADA criteria, *i.e.* patients having an HbA1C ≥ 6.5% or fasting blood glucose ≥ 126 mg/dl or 2-hour blood glucose levels ≥ 200 mg/dl after OGTT, or a random blood glucose ≥ 200

mg/dl when severe diabetes symptoms are present (10). Inclusion criteria for patients with diabetes were BMI < 40 kg/m2, age 35–85years, ability and willingness to give written informed consent. Information collected included data on vital signs, anthropometric factors, medical history, and behaviours. The presence/absence of diabetic complications was established as follows: diabetic retinopathy by fundoscopy through dilated pupils and/or fluorescence angiography; incipient nephropathy, defined as a urinary albumin excretion rate >30mg/24h and a normal creatinine clearance; renal failure, defined as an estimated glomerular filtration rate (eGFR) <60 ml/min per 1.73m2; neuropathy established by electromyography; ischemic heart disease defined by clinical history, and/or ischemic electrocardiographic alterations; peripheral vascular disease, including atherosclerosis obliterans and cerebrovascular disease based on history, physical examinations, and Doppler velocimetry. Among the 511 patients, 90 were affected by neuropathy, 120 by peripheral vascular disease, 75 by major adverse cardiovascular events (MACE), 62 by nephropathy or renal failure, and 144 by retinopathy. Fasting blood samples of all subjects were processed to obtain EDTA plasma and stored at −80°C. Concentrations of presented analytes were measured by standard procedures.

Statistical analysis

Continuous variables were tested for normality using the Shapiro Wilk's test and reported as mean ± SD. Student's t test was used to evaluate differences in continuous variables between two groups, while the chi square test was used for dichotomous variables. Pearson's correlation was used for continuous variables. Spearman coefficient was used to estimate the correlation between hs-CRP and the number of T2DM complications. A stepwise logistic regression model with forward conditional method was used to identify factors associated with inflammatory risk in T2DM. All variables

showing a significant linear correlation with hs-CRP in T2DM subjects with LDL-C ≤ 70 mg/dL were entered into the model. Model fit was assessed using the Hosmer-Lemeshow goodness-of-fit test. The analyses were carried out using IBM SPSS Statistics, version 26 (IBM Corp, Armonk, NY, USA) and R, version 3.6.1. Statistical significance was defined as a two-tailed p-value <0.05.

Results

Clinical variables of T2DM patients categorized according to the presence/absence of inflammatory risk (*i.e.* hs-CRP ≥ or < 2mg/L) are shown in **Table 1,** while **Supplementary Table 1** resumes the clinical characteristics of the entire cohort. Subjects with inflammatory risk had higher mean value of body mass index (BMI), waist-hip ratio, total-C, triglycerides, fasting glucose, insulin, HOMA index, and uric acid, while HDL-C and mean diabetes duration were lower in this group. In addition, they were more likely to have a diagnosis of hypertension and less likely to be on treatment with sulphonylureas (**Table 1**).

When considering all T2DM patients, the prevalence of inflammatory risk was 51.9%, while a slightly lower prevalence (46.9%) was observed when considering only patients on stable (>1 year) statin therapy. When considering only patients with the currently recommended LDL-C goal of 70 mg/dL, i.e. those at-target, the prevalence of RIR was 39,2% (**Figure 1A**).

Regarding the spectra of possible complications, only among patients with nephropathy there was a higher prevalence of inflammatory risk (66,1%), while patients with a previous MACE showed the same prevalence of the general T2DM cohort (50,6%) (**Figure 1B**).

Pearson's correlations among variables in different subgroups revealed that total-C, non-HDL-C, triglycerides, Apo-B, BMI, and waist-hip-ratio were positively associated with hs-CRP, while eGFR

displayed a negative correlation. These association were not significant in patients treated with statins, while the attainment of the goal of LDL<70mg/dL markedly increased the magnitude of these correlations. On the other side, HbA1c, fasting insulin, HOMA-index, serum creatinine, platelets count and the number of complications were strongly associated with hs-CRP only in patients with LDL<70mg/dL (**Figure 1C** and **Supplementary Table 2**). The progressive nature of the association was particularly apparent for non-HDL-C, HbA1c, and HOMA-index, as evidenced by linear correlation graphs (**Supplementary Figure 1**). Among other parameters, fasting glucose and age were not associated with hs-CRP in the at-target group (**Figure 1C** and **Supplementary Table 2**), despite the known pro-inflammatory effect of these two variables (11). Among immune related variables, monocytes and lymphocytes counts were positively associated with hs-CRP in at-target patients, possibly suggesting both innate and acquired immunity as potential contributors to RIR (**Figure 1C** and **Supplementary Table 2**).

To gain further insights into the variable associated with inflammatory risk in T2DM, we built a binary logistic regression predicting the likelihood of hs-CRP levels ≥ 2 mg/L. The logistic regression model with forward conditional selection was statistically significant ($\chi 2(14) = 59.120$, $p < 0.001$) and included four variables as significant predictors, *i.e.* waist-hip ratio, triglycerides, non-HDL-C, and neutrophils (**Supplementary Table 3**).

Conclusions

Our results evidence a high prevalence of RIR among patients with T2DM, comparable to what has been observed in previous cohorts of patients with CV diseases or at high risk (2,6,12). To our knowledge, this is the first manuscript showing the prevalence of RIR in a cohort with T2DM,

although many previous reports described hs-CRP elevation in T2DM (7,13).These findings also highlight a limited effect of statin use and LDL-C goal attainment on RIR prevalence in T2DM. Accordingly, lipid- and obesity-related parameters were associated with hs-CRP in the whole T2DM population, but more consistently in patients achieving the LDL-C target. Notably, glucose-related parameters were associated with hs-CRP only in at-target patients, overall suggesting that RIR might be, at least in part, attributable to non-LDL-C dyslipidaemia, central obesity, insulin resistance, and lack of glycaemic control in T2DM (13). In particular, waist-hip ratio, triglycerides, and non-HDL-C appear as the most likely variables explaining the burden of RIR in patients with T2DM. Worth mentioning, among the range of different T2DM complications, only patients with nephropathy have an increased prevalence of inflammatory risk. Consistently, diabetic nephropathy is accompanied by a complex pro-inflammatory remodelling promoting the development and progression of this complication, as evidenced by both prospective and cross-sectional studies (14).

An inherent limitation of this study design is that none of the patients was treated with the recently introduced glucose-lowering drugs (15), some of which have been suggested to target also the inflammatory process (16), nor with recently introduced LDL-C-lowering drugs such as PCSK9-inhibitors. In addition, other factors beyond the large range of common variables herein presented may influence RIR.

Overall, these observations suggest that an effective targeting of modifiable risk factors, *e.g.* central obesity and triglycerides, might reduce the burden of RIR in T2DM patients, as recently suggested for patients with prevalent CV diseases (17). This hypothesis warrants further exploration, especially considering that an aggressive, multidimensional reduction of risk factors is already known to reduce the incidence of CV events in patients with T2DM (18).

Author contribution

FP, FO and AC contributed to the conception and design of the study and drafted the manuscript. AG and JS contributed to acquisition and interpretation of data and drafted figures. GM, DR, ARB, MRR, PdC, and ADP critically revised the manuscript. All authors gave final approval.

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Disclosures

None of the authors have competing interests.

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	High-sensitivity C- reactive protein stratification		
Variables	**Hs-CRP < 2 mg/L (N=246)**	**Hs-CRP ≥ 2 mg/L (N=265)**	**p-value**
hs-CRP (mg/L)	1.06 (0.55)	5.91 (3.59)	**<0.0001**
Age (years)	65.8 (8.6)	65.6 (7.7)	0.796
Gender (males, %)	150 (61%)	132 (49.8%)	**0.011**
Diabetes duration (years, SD)	16.3 (11.4)	13.25 (11.5)	**0.005**
BMI (Kg/m^2)	27.3 (3.8)	29.7 (4.3)	**<0.0001**
Waist-hip ratio	0.92 (0.07)	0.94 (0.07)	**0.001**
Total cholesterol (mg/dL)	202.0 (38.7)	210.7 (37.4)	**0.010**
LDL-C (mg/dL)	114.2 (32.2)	117.8 (31.1)	0.202
HDL-C (mg/dL)	54.9 (15.4)	50.3 (16.0)	**<0.0001**
Triglycerides (mg/dL)	112.4 (58.1)	160.5 (134.4)	**<0.0001**
Fasting glucose (mg/dL)	155.8 (42.7)	167.4 (51.2)	**0.005**
HbA1C (%)	7.30 (1.13)	7.50 (1.31)	0.062
Insulin (UI/mL)	6.30 (9.26)	7.69 (5.70)	**0.043**
HOMA index	2.39 (3.35)	3.29 (7.24)	**0.002**
WBC (n/mm^3)	6.29 (1.49)	6.87 (1.59)	**<0.0001**
Platelets (n/mm^3)	212.4 (82.1)	221.4 (55.6)	0.147
Creatinine (mg/dL)	0.89 (0.25)	0.92 (0.33)	0.224

Azotemia (mg/dL)	40.6 (11.3)	39.7 (12.2)	0.356
eGFR (mL/min)	80.9 (18.8)	73.2 (22.2)	0.069
Uric acid (mg/dL)	4.62 (1.11)	4.94 (1.24)	**0.002**
Hypertension (N, %)	140 (56.9%)	180 (67.9%)	**0.010**
Smokers (N, %)	37 (15%)	42 (15.8%)	0.800
Medications (N, %)			
Metformin	84 (34.1%)	100 (37.7%)	0.398
Sulphonylureas	136 (55.3%)	113 (42.6%)	**0.004**
Glinides	4 (1.6%)	7 (2.6%)	0.429
Insulin	44 (17.9%)	46 (17.4%)	0.876
Statins (N, %)	51 (20.8%)	45 (17%)	0.278

Table 1. Clinical variables of patients categorized according to the presence/absence of residual inflammatory risk (i.e. hs-CRP < or ≥ 2 mg/L). Data are expressed as mean (SD) and p values refer to Student's t test for continuous variables or to chi square test for dichotomous variables. Significant differences ($p<0.05$) are highlighted in bold.

Figure legend

Figure 1. Prevalence of inflammatory risk (hs-CRP ≥ 2 mg/L) in all patients with T2DM, only in those treated or not with statins, and only in patients with at-target (< 70 mg/dL) or not at-target LDL-C (A). Prevalence of RIR in patients affected by different complications(B). Correlation plot showing the significant correlations among the different variables and hs-CRP in the whole population (left), in patients on statins therapy (middle), and in patients with at-target LDL-C (right). The intensity of

the color and the size of the circles depend on the magnitude of the correlation, while the symbol X indicates a non-significant correlation. Blue = positive correlation/Red= negative correlation (C).

PAD = peripheral artery disease. MACE = major adverse cardiovascular events. * $p<0.05$ chi square test