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High sensitivity C-reactive protein predicts the development of new carotid artery plaques in older persons

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Abstract *Background and Aim:* Previous studies have shown that increased levels of C-reactive protein (CRP) predict cardiovascular events, including stroke, myocardial infarction and death from cardiovascular causes. Previous studies have also shown that increased levels of CRP are strong predictors of the progression of pre-existing carotid artery plaques. However, whether CRP is involved in the development of new plaques, that may or may not be associated with clinical events, in subjects with clean carotid arteries has been scarcely investigated.
Methods and Results: 486 ''InCHIANTI'' Study participants (200 men and 286 women, 72% aged 65 years and over) free from carotid artery plaques at baseline, also underwent carotid artery scan three years later. We tested the association of baseline characteristics, cardiovascular risk factors and inflammatory markers with the development of new carotid artery plaques. Older participants were significantly more likely to develop new plaques. Independent of age, the relative risks of developing new plaques associated with heavy smoking and family history of atherosclerosis were 1.7 (95%CI 1.5–1.9) and 1.9 (95%CI 1.2–3.1), respectively.

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Participants with high (>3 μg/mL) and moderate (≥1 and ≤3 μg/mL) CRP levels had a relative risk of 2.2 (95%CI 1.9—2.6) and 1.9 (95%CI 1.6—2.3) respectively, when compared with subjects with low (<1 μg/mL) CRP levels. Surprisingly, risk factors such as hypertension, diabetes, dyslipidemia and overweight/obesity were not significant predictors of the development of new carotid artery plaques.

Conclusions: High CRP levels independently predict the development of new plaques in older persons with carotid arteries free from atherosclerotic lesions.

Introduction

In the last decade several prospective studies have shown that C-reactive protein (CRP) is an independent predictor of cardiovascular events, including stroke, myocardial infarction and death from cardiovascular causes [1—5]. Though the prospective association of CRP with stroke is robust [5], whether CRP predicts the development of new plaques in subjects with clean carotid arteries has not yet been completely clarified. In fact, the longitudinal studies that consistently reported independent associations of CRP with carotid atherosclerosis [6—12] mainly addressed the progression of pre-existing plaques rather than the development of new ones.

Interestingly, studies that used the Intima Media Thickness (IMT) as an early measure of carotid atherosclerosis consistently reported no independent association of CRP with IMT [11,13—17], suggesting that IMT is scarcely affected by inflammation [17—20].

Pre-clinical research [21—23] has proposed several potential mechanisms for the initiation, the progression and the thrombotic complications of atherosclerosis. Though the link between risk factors and inflammation has not yet been completely clarified, there is agreement that inflammation might represent the final common pathway that transduces the effects of risk factors into changes within the vascular wall [23—25]. The effects of risk factors on IMT [17—20], as well as those of inflammation on the occurrence of cardiovascular events [1—5] and on the progression of pre-existing carotid artery plaques [6—12], have already been clarified. However, our understanding of carotid atherosclerosis still shows a gap concerning factors affecting the development of new plaques, that may or may not be associated with clinical events, in subjects with carotid arteries free from atherosclerotic lesions.

In this study we hypothesized that CRP might also be a predictor of the development of new carotid artery plaques. We tested this hypothesis using prospective data from a population-based cohort of predominantly older persons, who represent the portion of the population most likely to develop carotid artery plaques.

Methods

Study sample

The analysis presented in this paper is based upon data from the ''InCHIANTI'' (Invecchiare nel Chianti, Aging in the Chianti area) Study, a prospective cohort investigation on factors affecting loss of mobility in late life, whose description is reported on the web (www.inchiantistudy.net) and in Ferrucci et al. [26].

Baseline data collection started in September 1998 and was completed in March 2000. Of the 1453 interviewed participants, 1168 (523 men and 645 women, mean age 68years ± SD 15.4, 78% aged 65 and over) had complete baseline data. During the three-year follow-up, 68 of the 1168 participants (37 men and 31 women, mean age at baseline 82years ± SD 7.6, 97% aged 65 and over, 48 with and 20 without carotid artery plaques at baseline) died: of these, 31 deaths were attributable to cardiovascular causes. Of the surviving 1100 participants, 261 (109 men and 152 women, mean age at baseline 74years ± SD 14.6, 86% aged 65 and over, 137 with and 124 without carotid artery plaques at baseline) did not receive the follow-up scan due to refusal or logistic difficulties in moving from home to the ultrasound laboratory, while 839 participants (385 men and 454 women, mean age at baseline 66years ± SD 15.1, 74% aged 65 and over, 353 with and 486 without carotid artery plaques at baseline) underwent the scheduled carotid artery scan at three-year follow-up.

Consequently, the association of baseline characteristics, risk factors and inflammatory markers with the development of new carotid artery plaques at three-year follow-up was assessed in the subgroup of 486 participants (200 men and 286 women, mean age at baseline 60years ± SD 16.6, 72% aged 65 and over) free from carotid artery plaques at baseline.

Carotid artery plaques

Carotid examination was performed both at baseline and at three-year follow-up by the same expert operator using an AU3 Partner apparatus (ESAOTE, Genoa, Italy) equipped with a 7.5 MHz linear array transducer.

For our on-line analysis we adopted the protocol suggested by Bots et al. [27] for the off-line analysis of carotid artery scans in the Rotterdam Study, that was, to our knowledge, the best available and the most updated one at the time of the beginning of the InCHIANTI Study (1998). The protocol included bilateral longitudinal and cross-sectional scans of the distal part (2 cm) of the common carotid artery, of the carotid bulb and of the proximal part (1 cm) of external and internal carotid arteries. Plaques were defined as ''a focal widening relative to adjacent segments, with protrusion into the lumen'' judged visually, independent of their extension and/or degree of stenosis [27]. Participants with at least one plaque in one or both carotid arteries were classed as ''presence'', and, conversely, participants with both carotid arteries free from plaques were classed as

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''absence''. Based upon echographic pattern, each plaque was also classed as hyper-reflecting, hypo-reflecting or mixed [28].

High sensitivity CRP and other inflammatory markers

Morning, fasting blood samples were collected after a 15-min resting. Aliquots of serum were stored at −80C° and never thawed before analysis. Determination of CRP level was based on a high sensitivity enzyme-linked immunosorbent assay (ELISA), using purified protein and polyclonal anti-CRP antibodies [29]. The minimum detectable concentration was 0.03 μg/mL. The average of two measures performed on each sample was used in the analysis. Serum IL-6, soluble IL-6 receptors (sIL-6r, 80 kDa), IL-1beta, IL-1 receptor antagonist (IL-1ra) and TNF-α (kits from BIOSOURCE, Camarillo, California) were also measured in duplicate by high sensitivity enzyme-linked immuno-absorbent assays [29].

Other variables

Family history of atherosclerosis was ascertained based upon the report of death or morbidity for angina, myocardial infarction or stroke in one or both parents, or in one or more siblings, at the age under 65 years. Self-reported physical activity level in the previous year was considered as an ordinal variable and scored into five progressive grades: 0 = Sedentary or Light (<3 metabolic-equivalents, METS) physical activity < 1 h/week; 1 = Light physical activity 2−4 h/week; 2 = Light physical activity > 4 h/week or Moderate (3−6 METS) physical activity 1−2 h/week; 3 = Moderate physical activity > 2 h/week; 4 = Intense (>6 METS) physical activity several times a week [30]. The pack-years index was calculated as number of packs of cigarette smoked daily times years of smoking. Total cholesterol, HDL-cholesterol and triglycerides levels were determined by commercial assays (Roche Diagnostics, Mannheim, Germany) and LDL-cholesterol calculated using the Friedewald formula. Comorbid conditions, such as hypertension, diabetes, metabolic syndrome, peripheral artery disease, coronary artery disease and chronic infections were ascertained according to pre-established algorithms that combined information gathered from medical history, medical records, clinical examination, and blood and instrumental tests included in the ''InCHIANTI'' Study protocol [26], and expressed as categorical variables. With regard to hypertension, we considered 140 mmHg and 90 mmHg as cut-off values for systolic and diastolic blood pressure, respectively [31]. The use of statins and/or antiinflammatory drugs was based upon self-report.

Statistical analysis

Statistical analysis was performed using the STATA 7.0 software, from Stata Corporation (College Station, Texas, USA) and carried out separately for three-year incidence and prevalence of carotid artery plaques. The association of participants' baseline general characteristics, cardiovascular risk factors and inflammatory markers with the three-year incidence (or prevalence) of carotid artery plaques was first tested independent of the confounding effect of age and sex. Then, variables showing significant association with the three-year incidence (or prevalence) of carotid artery plaques were entered into a logistic regression-based model in which the three-year incidence (or prevalence) of carotid artery plaques was considered as the dependent variable. Continuous variables showing a markedly skewed distribution, such as inflammatory markers, were log-transformed before being entered into calculations. Therefore, odds ratios in the models are referred to as the difference of 1 unit in the log-transformed value. Type 1 error was set at the two-sided 0.05 level.

Results

The baseline prevalence of carotid artery plaques was 538 in 1168 participants (46%, 52% men and 41% women). Four hundred twenty-six of the 538 participants (79%) showed bilateral plaques, so that a total of 962 plaques were detected. Of these, 78% were hyper-reflecting, 5% hypo-reflecting and 17% mixed.

Table 1 (left side) shows baseline general characteristics, cardiovascular risk factors and inflammatory markers of InCHIANTI Study participants according to the presence vs. absence of carotid artery plaques at baseline.

The baseline prevalence of carotid artery plaques was significantly associated with older age, male gender, family history of atherosclerosis, higher pack-years index, diabetes, hypertension, peripheral artery disease and higher levels of CRP. When all these variables were entered into a logistic regression-based model predicting presence vs. absence of carotid artery plaques at baseline (Table 2, left side) older age, male gender, higher pack-years index and hypertension remained significantly associated with prevalent carotid artery plaques while family history of atherosclerosis, diabetes, peripheral artery disease and CRP were washed out.

The three-year incidence of carotid artery plaques was 195 in 486 participants (40%, 39% men and 41% women). One hundred fifty-six of the 195 participants (80%) showed bilateral plaques, so that a total of 351 plaques were detected. Of these, 56% were hyper-reflecting, 32% hypo-reflecting and 12% mixed.

Table 1 (right side) shows baseline general characteristics, cardiovascular risk factors and inflammatory markers of InCHIANTI Study participants according to the presence vs. absence of new carotid artery plaques at three-year follow-up among those participants without plaques at baseline.

The three-year incidence of carotid artery plaques was significantly associated with older age, family history of atherosclerosis, higher pack-years index, coronary artery disease and higher levels of CRP. When all these variables were entered into a logistic regression-based model predicting presence vs. absence of new carotid artery plaques at three-year follow-up in participants free from carotid artery plaques at baseline (Table 2, right side), older age, higher pack-years index, increased levels of CRP and family

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Table 1 Baseline general characteristics, cardiovascular risk factors and inflammatory markers of InCHIANTI Study participants according to the presence vs. absence of carotid artery plaques at baseline (left side) and of new carotid artery plaques at three-year follow-up (right side).

	Carotid artery plaques at baseline (*n.* 1168)[a]			New carotid artery plaques at follow-up (*n.* 486)[b]		
	Presence	Absence	p^c	Presence	Absence	p^c
	n. 538 (46.1%)	*n.* 630		*n.* 195 (40.1%)	*n.* 291	
General characteristics						
Age (years) (mean ± SD)	75.5 ± 8.3	61.7 ± 17.2	**< 0.001**	71.2 ± 7.2	56.6 ± 16.9	**< 0.001**
Sex (% females)	49.6	60.0	**< 0.001**	59.5	58.4	0.576
Chronic respiratory, urinary tract and dental Infections (%)	49.7	33.4	0.316	43.7	24.7	0.260
Use of statins and/or antiinflammatory drugs (%)	13.0	8.9	0.451	11.8	7.2	0.987
Cardiovascular risk factors						
Family medical history of atherosclerosis (%)	51.0	43.5	**0.011**	55.9	37.1	**0.024**
Physical activity level previous year (score 0–4, 4 best) (mean ± SD)	1.29 ± 0.89	1.51 ± 0.87	0.612	1.35 ± 0.74	1.71 ± 0.86	0.337
BMI (kg/m2) (mean ± SD)	27.5 ± 3.7	27.1 ± 4.4	0.628	27.9 ± 4.2	26.7 ± 4.4	0.891
Pack-years Index (mean ± SD)	15.1 ± 22.1	7.6 ± 14.4	**< 0.001**	10.5 ± 17.2	5.5 ± 9.9	**0.001**
Total Cholesterol (mg/dL) (mean ± SD)	219.2 ± 39.4	212.6 ± 40.1	0.173	221.1 ± 36.3	210.1 ± 41.6	0.637
LDL-Cholesterol (mg/dL) (mean ± SD)	138.5 ± 33.7	132.0 ± 35.7	0.138	141.1 ± 32.2	129.8 ± 37.4	0.818
HDL-Cholesterol (mg/dL) (mean ± SD)	54.5 ± 15.1	56.0 ± 14.4	0.687	54.2 ± 12.5	56.9 ± 14.8	0.269
Triglycerides (mg/dL) (mean ± SD)	130.6 ± 72.4	123.3 ± 82.6	0.795	128.5 ± 65.9	117.2 ± 69.4	0.416
Diabetes (%)	13.5	6.3	**0.021**	9.7	2.6	0.104
Hypertension (%)	77.1	49.2	**< 0.001**	62.2	36.0	0.780
Metabolic Syndrome (%)	25.6	18.5	0.135	20.8	16.4	0.280
Coronary artery disease (%)	9.5	3.2	0.080	6.6	0.3	**0.037**
Perpheral artery disease (%)	16.3	4.0	**0.001**	5.2	1.0	0.094
Inflammatory markers						
C-reactive protein high sensitivity (μg/mL) (mean ± SD)[d]	5.89 ± 10.81	3.76 ± 6.56	**0.006**	4.45 ± 5.30	2.57 ± 3.56	**0.005**
Interleukin 1-β (pg/mL) (mean ± SD)[d]	0.28 ± 1.34	0.32 ± 1.80	0.342	0.29 ± 1.77	0.29 ± 1.48	0.286
Interleukin 1 receptor antagonist (pg/mL) (mean ± SD)[d]	162.9 ± 124.0	152.6 ± 125.0	0.798	154.4 ± 94.8	143.0 ± 106.6	0.192
Interleukin 6 (pg/mL) (mean ± SD)[d]	2.32 ± 4.41	1.52 ± 1.80	0.516	1.70 ± 1.51	1.21 ± 1.77	0.172
Interleukin 6 soluble receptor (ng/mL) (mean ± SD)[d]	105.0 ± 55.6	99.3 ± 58.6	0.148	102.7 ± 59.7	99.5 ± 61.7	0.341
Tumor necrosis factor α (pg/mL) (mean ± SD)[d]	3.85 ± 6.00	3.02 ± 3.92	0.891	3.22 ± 4.14	2.69 ± 3.55	0.672

Bold value indicates statistically significant *p*-values.
[a] Participants with complete data at baseline.
[b] Participants free from carotid artery plaques at baseline who received a carotid artery scan at three-year follow-up.
[c] From age- and sex-adjusted linear or logistic regressions, as appropriate.
[d] Variables log-transformed before being entered into regressions due to marked skewness.

history of atherosclerosis remained significant independent predictors of incident carotid artery plaques.

In a separate analysis, we also calculated relative risks, along with their 95% confidence intervals (CI), for family history of atherosclerosis and for categories of variables previously reported as continuous variables. When compared with participants aged less than 65 years, the relative risk of developing new carotid artery plaques was 5.48 (95%CI: 5.02–5.94) for participants aged 65–69 years, 6.89 (95%CI: 6.44–7.35) for participants aged 70–74 years, 8.04 (95%CI: 7.58–8.50) for participants aged 75–79 years and 9.57 (95%CI: 9.12–10.02) for participants aged 80 years or more. Heavy smokers (pack-years index >20) had a relative risk of developing new plaques of 1.70 (95%CI: 1.48–1.93) when compared with subjects with no or light smoking history (pack-years index ≤10). On the contrary, moderate smoking (pack-year index, 11–20) was not associated with differential risk. Participants who reported family history of atherosclerosis had a relative risk of developing new plaques of 1.57 (95%CI 1.35–1.79). Finally, subjects with high (>3 μg/mL) or moderate (≥1 and ≤3 μg/mL) CRP levels had a relative risk of developing

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Table 2 Multivariate logistic regressions modeling the probability of having carotid artery plaques at baseline (left side) and of developing new carotid artery plaques at three-year follow-up (right side).

	Carotid artery plaques at baseline (n° 1168)		New carotid artery plaques at follow-up (n° 486)	
	LR chi^2 = 355.98; Prob > chi^2 = 0.0000; Pseudo r^2 = 0.2352		LR chi^2 = 226.85; Prob > chi^2 = 0.0000; Pseudo r^2 = 0.3468	
	OR (95%CI)	p	OR (95%CI)	p
Age (years)	1.10 (1.08–1.12)	**<0.001**	1.14 (1.10–1.18)	**<0.001**
Female sex (Y/N)	0.62 (0.45–0.87)	0.005	[a]	–
Family medical history of atherosclerosis (Y/N)	1.12 (0.85–1.49)	0.416	1.79 (1.12–2.86)	**0.015**
Pack-years Index	1.02 (1.01–1.03)	**<0.001**	1.03 (1.01–1.05)	**0.002**
Diabetes (Y/N)	1.54 (0.90–2.45)	0.093	[a]	–
Hypertension (Y/N)	1.77 (1.30–2.42)	**<0.001**	[a]	–
Coronary artery disease (Y/N)	[a]	–	4.04 (0.49–33.10)	0.193
Peripheral artery disease (Y/N)	1.3 (0.87–1.94)	0.200	[a]	–
C-reactive protein high sensitivity (μg/mL)[b]	1.12 (0.97–1.28)	0.101	1.30 (1.08–1.64)	**0.021**

Bold value indicates statistically significant p-values.
[a] Variables not entered into the model due to the lack of association with the outcome after age- and sex-adjustment.
[b] Variables log-transformed before being entered into regressions due to marked skewness.

new carotid artery plaques of 2.23 (95%CI: 1.89–2.57) and 1.91 (95%CI: 1.56–2.26), respectively, when compared with subjects with low (<1 μg/mL) CRP levels.

Discussion

Using data collected in a predominantly elderly population-based cohort living in the Tuscany countryside we found that older age, higher pack-years index, increased levels of CRP and family history of atherosclerosis were strong independent predictors of the development of new plaques within three years among participants free from carotid artery plaques.

The three-year incidence of carotid artery plaques was 40% (39% men and 41% women), corresponding to an average incidence of 13% per year. To the best of our knowledge, this is the first prospective study addressing the issue of the development of new carotid artery plaques in older persons. Therefore, our findings cannot be directly compared with the existing literature. Indeed, the 40% incidence of carotid artery plaques over 3 years we found in our study might appear surprisingly high. However, Johnsen et al. [32] also reported a 40% incidence of carotid artery plaques. Though in the above study the follow-up was longer (7 vs. 3 years), the proportion of participants aged 65 years or older was remarkably smaller than in our sample (25% vs. 72%). Accordingly, the relevant presence of younger participants might have diluted the incidence of carotid artery plaques among older persons.

Older age was a significant and independent predictor of the development on new atherosclerotic plaques. This finding confirms the established notion of the progressive increase of cardiovascular risk with advancing age, that reflects the cumulative nature of atherosclerosis [33]. The lack of association between gender and three-year incidence of carotid artery plaques is consistent with previously reported data from the Rotterdam Study on the progression of extra-coronary atherosclerosis in older persons [7,34]. The development of new carotid artery plaques was also significantly and independently associated with the pack-years index, a measure of lifelong exposure to smoking, and this is also consistent with the cumulative nature of atherosclerosis [33].

Worthy of note, other cardiovascular risk factors, such as hypertension, diabetes, dyslipidemia and overweight/obesity, whose prevalence was moderately high in our cohort (62.1%, 9.6%, 78.9% and 69.0% respectively), failed to identify elderly subjects at risk of incident carotid artery plaques.

Finally, increased levels of CRP and family history of atherosclerosis independently predicted the prospective development of new carotid artery plaques in older persons. This finding is consistent with recently published papers that have shown that by adding information on inflammation, namely CRP, and genetic risk, namely family history of cardiovascular events, the algorithms for the assessment of global cardiovascular risk were considerably improved both in women [2] and in men [3].

Interestingly, in spite of the predictive value of increased levels of CRP on the development of new carotid artery plaques, levels of several cytokines (CKs) were not associated with differential risk. This finding could be explained by the lack of precision in CKs measurement methods or by the wide short-term fluctuation in CKs circulating levels. However, it may also be hypothesized that CRP might be a summary measure of inflammation capturing the overall burden more than the specific aspects of it, that are mostly expressed by specific CKs. Alternatively, it may be hypothesized that the moderately increased levels of CRP, as those we found in participants that developed new carotid artery plaques, result from local production. Accordingly, they might reflect the early stage of the vessel inflammation that

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precedes the macroscopic appearance of the plaques [21,22]. Histological investigations have, in fact, shown that CRP frequently co-localizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries [35], and that elongated muscle-like cells positive for CRP can be found inside atherosclerotic plaques [36]. Further, though the majority of CRP commonly detected during the acute-phase response to microbial infections, tissue injury or autoimmune disorders is produced by the hepatocytes stimulated by increased levels of IL-6 [37], recent papers have shown that small amounts of CRP can also be produced by vascular smooth muscle cells [38,39]. Noteworthy, CRP has been shown to be produced by the vascular smooth muscle cells of atherosclerotic lesions with active disease but not in end-stage plaques [36] and this might also explain the lack of association of increased levels of CRP with prevalent carotid artery plaques, 78% of which showed a hyper-reflecting echographic pattern, usually found in end-stage plaques.

Our study has limitations. In particular, as in all prospective studies that use a single baseline measurement to predict future events, it is likely that random fluctuations of risk factors over time led to a systematic underestimation of the true association between risk factors and the development of new carotid artery plaques [33]. Further, the lack of intra-observer reliability assessment might also appear as a potential limitation of this study. Actually, due to logistic reasons, we were unable to obtain an appropriate set of participants to be re-evaluated. In fact, to undergo carotid artery scan, older participants had to move from home to the ultrasound laboratory. However, the operator who performed all carotid artery scans confirmed the presence of carotid artery plaques in 347 over 353 (98%) participants with carotid artery plaques at baseline who also underwent carotid artery scan at follow-up. This might be considered an indirect measure of the intra-observer reliability. Accordingly, the lack of intra-observer reliability assessment should not have significantly affected our results.

In conclusion, our findings provide the evidence that increased levels of CRP predict the prospective development of new plaques in older persons with carotid arteries free from atherosclerotic lesions. Future studies are needed to confirm our results on a broader scale and to verify whether an aggressive medical treatment targeting inflammatory pathways might also effectively prevent the development of new plaques in older persons free from carotid artery lesions and with increased levels of CRP.

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