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Associations of Proinflammatory Cytokines With the Risk of Recurrent Stroke

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Background and Purpose—There are few reports on proinflammatory cytokines and risk of primary or recurrent stroke.

We studied the association of interleukin (IL)-6, IL-18, and tumor necrosis factor- α (TNF- α) with recurrent stroke in a nested case-control study derived from the Perindopril Protection Against Recurrent Stroke Study (PROGRESS).

Methods—We performed a nested case-control study of 591 strokes (472 ischemic, 83 hemorrhagic, 36 unknown subtype) occurring during a randomized, placebo-controlled multicenter trial of perindopril-based therapy in 6105 patients with a history of stroke or transient ischemic attack. Controls were matched for age, treatment group, sex, region, and most recent qualifying event at entry to the parent trial.

Results—IL-6 and TNF- α , but not IL-18, were associated with risk of recurrent ischemic stroke independently of conventional risk markers. Adjusted odds ratios comparing the highest to lowest third of their distributions were 1.33 (95% CI, 1.00 to 1.78) for IL-6 and 1.46 (1.02 to 2.10) for TNF- α . No inflammatory marker was associated with hemorrhagic stroke risk. In multivariable models, IL-6 and TNF- α fully explained observed associations of C-reactive protein and fibrinogen with risk of ischemic stroke, but TNF- α retained borderline significance after full adjustment.

Conclusions—Inflammatory markers associated with the acute-phase response (IL-6, TNF- α , C-reactive protein, and fibrinogen, but not IL-18) are associated with risk of recurrent stroke. These markers are dependent on each other in multivariable models, and once all were included, only TNF- α retained a borderline association. Markers of generalized inflammation of the acute-phase response are associated with recurrent stroke, rather than IL-6, C-reactive protein, or fibrinogen in particular. (*Stroke*. 2008;39:2226-2230.)

Key Words: stroke ■ inflammation ■ acute-phase response ■ epidemiology

There is increasing evidence that inflammatory variables are associated with atherogenesis and predict risk of cardiovascular disease (CVD).^{1,2} The acute-phase reactants C-reactive protein (CRP)³ and fibrinogen⁴ have been shown to predict risk of primary ischemic stroke. One recent report suggests that CRP may be a better risk predictor for stroke than for coronary heart disease (CHD),⁵ another that the associations are similar,⁶ and 1 that CRP is a moderate predictor of ischemic stroke risk and adds no information to the Framingham assessment.⁷ The association between stroke risk and proinflammatory markers therefore requires further investigation. It is possible that inflammatory markers “upstream” of the acute-phase response (APR) may explain associations between acute-phase reactants (such as CRP and fibrinogen) and CV events.¹ Prediction of recurrent stroke is clinically important. We have previously demonstrated that CRP and fibrinogen predict risk of recurrent ischemic stroke independently of conventional stroke risk markers,⁸ and a

meta-analysis of 3 other studies has also shown this association with fibrinogen.⁹

The proinflammatory cytokines interleukin (IL)-6, IL-18, and tumor necrosis factor- α (TNF- α) have all been implicated as risk markers for CHD.^{10–19} This association may reflect observed experimental roles in atherogenesis and atherothrombosis for these markers,^{20–25} although caution must be used in implicating causality.² Despite recent interest in positive associations of IL-6 gene polymorphisms with stroke,^{26–29} this biomarker has been understudied as a risk marker for ischemic stroke, and IL-18 and TNF- α have only been prospectively studied as risk markers of stroke in cohort studies with composite end points and few stroke patients.^{17–19} We aimed to extend current data by examining the association between IL-6, IL-18, and TNF- α and recurrent stroke, as well as relating these to the associations of fibrinogen and CRP with recurrent stroke, which we have previously reported.⁸

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Table 1. Baseline Summary Data for All Subjects in the Nested Case-Control Study Classified by Whether or Not They Developed Either of the Major Subtypes of Stroke During PROGRESS

Variable	Hemorrhagic Stroke			Ischemic Stroke		
	Cases (n=83)	Controls (n=199)	P Value	Cases (n=472)	Controls (n=1011)	P Value
Mean (95% CI)						
Age, y*	62.5 (60.6–64.5)	62.4 (61.2–63.6)	0.92	66.5 (65.7–67.3)	66.2 (65.7–66.7)	0.48
SBP, mm Hg	149.7 (145.4–154.0)	145.9 (143.4–148.4)	0.11	149.9 (148.1–151.6)	147.4 (146.3–148.6)	0.02
Fibrinogen, g/L	3.58 (3.40–3.76)	3.76 (3.63–3.89)	0.11	3.87 (3.79–3.96)	3.73 (3.70–3.78)	0.005
CRP, mg/L†	1.47 (1.10–1.96)	1.62 (1.35–1.94)	0.57	2.20 (1.98–2.45)	1.91 (1.77–2.05)	0.03
IL-6, pg/mL†	2.00 (1.70–2.37)	2.00 (1.83–2.18)	0.99	2.44 (2.31–2.59)	2.20 (2.11–2.29)	0.003
IL-18, pg/mL†	455 (399–521)	474 (436–517)	0.59	484 (459–511)	481 (463–500)	0.84
TNF- α , pg/mL†	2.61 (2.24–3.07)	2.48 (2.35–2.62)	0.54	2.56 (2.44–2.69)	2.45 (2.38–2.53)	0.15
No. (%)						
Men*	63 (76)	154 (77)	0.88	348 (74)	750 (74)	0.85
Smokers	15 (18)	43 (22)	0.63	110 (23)	173 (17)	0.006
PAD	1 (1)	7 (4)	0.94	32 (7)	39 (4)	0.02
Statin use	5 (6)	13 (7)	1.00	36 (8)	77 (8)	0.99
Diabetes	6 (7)	19 (10)	0.53	85 (18)	113 (11)	0.0003
Antiplatelet use	39 (47)	104 (52)	0.42	362 (77)	780 (77)	0.85

SBP indicates systolic blood pressure; PAD, peripheral arterial disease.

*Matching variable.

†Analysed on a logarithmic scale and back-transformed to show estimates.

Subjects and Methods

The design,³⁰ principal results,³¹ and case-control study results⁸ of the Perindopril Protection Against Recurrent Stroke Study (PROGRESS) have been published previously. In brief, in the principal study, 6105 patients with a history of stroke or transient ischemic attack were recruited, and each patient was randomly assigned active treatment (n=3051) or placebo (n=3054). For those deemed suitable by the responsible physician, mono or dual therapy was allocated: perindopril and/or indapamide, or single/double placebo. Patients were followed up for a mean of 3.9 years, during which time stroke occurred in 307 (10%) in the active treatment group and in 420 (14%) in the placebo group (relative risk reduction=28%; 95% CI, 17 to 38; $P<0.0001$). At baseline, venous blood samples collected from 5918 (97%) patients were anticoagulated with K₂EDTA and centrifuged at 2000g for 10 minutes at 4°C. Aliquots (1.5 mL) of plasma were stored at -80°C. Owing to the high proportion of patients from whom blood samples were taken, no imputation of the missing samples was necessary.

The base population for the case-control study comprised all those who had plasma frozen and stored within 48 hours of venipuncture and whose most recent qualifying event (classified as ischemic stroke, hemorrhagic stroke, or transient ischemic attack) at baseline in the clinical trial occurred >1 month ago. These restrictions give protection against deterioration of the blood samples and confounding attributable to acute-phase reactant increases in blood variables as a result of the incident stroke or its acute complications.

Stroke cases comprised anyone with a stroke recorded during follow-up. By standard methodology for nested case-control studies and as previously described,⁸ each case was randomly matched to between 1 and 3 controls selected from all those free of recurrent stroke at the time of recurrent stroke for that case. Matching variables were age, sex, treatment allocation, therapy, region, and most recent qualifying event at trial baseline, the latter variable being the best classification available to us to describe a patient's previous CV history. Prespecified ischemic and hemorrhagic stroke subtypes isolated those cases and their matched controls who had ischemic or hemorrhagic strokes as their first recorded stroke event.

Plasma fibrinogen and high-sensitivity CRP were both measured by immunonephelometry as previously described.⁸ IL-18, high-

sensitivity IL-6, and high-sensitivity TNF- α were all assayed by ELISA (R&D Systems, Oxon, UK). Intra-assay variation was 5.6% for IL-18, 7.5% for IL-6, and 8.4% for TNF- α . Corresponding interassay coefficients were 10.4%, 8.9%, and 12.5%, respectively.

We compared inflammatory markers and potential confounding variables between cases and unique controls by *t* tests or the χ^2 test, as appropriate. Odds ratios (ORs) were calculated according to equal thirds of the distributions of each of the inflammatory markers, and trends across these thirds were tested by using conditional logistic-regression models.³² These were computed both with and without adjustment for potential confounding factors (systolic blood pressure, smoking, prevalence of peripheral artery disease, statin use, and antiplatelet use) not controlled for in the matching variables. A previous report from this study⁸ that used identical methods found fibrinogen and CRP to have independent effects on ischemic stroke, but not on hemorrhagic stroke. Thus, another model added these 2 additional inflammatory variables to the adjustment set, together with the inflammatory variables measured for the current report, to assess the relative independent contributions of the inflammatory markers to risk of ischemic stroke.

Results

In this nested case-control study, there were 591 cases (472 ischemic strokes, 83 hemorrhagic strokes, and 36 of unknown type) and 1182 controls who did not subsequently become cases. In this substudy of PROGRESS, there were few missing values for IL-6, IL-18, fibrinogen, or CRP (<1%) but several for TNF- α , because of its being the last of the assays performed on the limited available plasma samples. Sample sizes for TNF- α were 433 cases and 859 controls (73% of the total).

Table 1 summarizes the baseline demographic data and shows risk factor data according to whether the subject became a case or not during PROGRESS. In the ischemic stroke group, IL-6 was significantly ($P=0.003$) higher in cases than in controls (but not IL-18 or TNF- α), as were

Table 2. ORs (95% CIs) for Hemorrhagic, Ischemic, and Total Stroke by Thirds of Each Variable

Variable/Third	Hemorrhagic Stroke		Ischemic Stroke		Total Stroke	
	Matched	Adjusted*	Matched	Adjusted*	Matched	Adjusted*
IL-6, pg/mL						
1 (<1.70)	1	1	1	1	1	1
2 (1.70–2.94)	1.10 (0.58–2.06)	1.15 (0.60–2.21)	1.36 (1.03–1.79)	1.33 (1.01–1.77)	1.29 (1.01–1.65)	1.28 (1.00–1.64)
3 (>2.94)	1.17 (0.61–2.24)	1.18 (0.61–2.27)	1.43 (1.08–1.90)	1.33 (1.00–1.78)	1.37 (1.06–1.76)	1.31 (1.01–1.69)
<i>P</i> for trend	0.62	0.60	0.02	0.06	0.02	0.04
IL-18, pg/mL						
1 (<366)	1	1	1	1	1	1
2 (366–582)	0.70 (0.37–1.33)	0.78 (0.40–1.50)	1.09 (0.83–1.44)	1.07 (0.81–1.41)	1.02 (0.79–1.30)	1.02 (0.79–1.30)
3 (>582)	0.74 (0.38–1.45)	0.77 (0.39–1.53)	1.04 (0.78–1.39)	0.97 (0.72–1.29)	0.99 (0.77–1.27)	0.94 (0.72–1.21)
<i>P</i> for trend	0.36	0.44	0.78	0.84	0.92	0.63
TNF-α, pg/mL						
1 (<2.17)	1	1	1	1	1	1
2 (2.17–2.89)	0.88 (0.41–1.88)	0.81 (0.37–1.80)	0.98 (0.68–1.40)	0.96 (0.67–1.39)	1.01 (0.74–1.39)	1.00 (0.73–1.38)
3 (>2.89)	1.43 (0.66–3.12)	1.23 (0.55–2.75)	1.47 (1.03–2.11)	1.46 (1.02–2.10)	1.49 (1.08–2.05)	1.47 (1.07–2.03)
<i>P</i> for trend	0.35	0.58	0.02	0.03	0.01	0.01

*Adjusted for systolic blood pressure, smoking, prevalence of peripheral artery disease, statin use, diabetes, and antiplatelet use.

several conventional risk factors for stroke (complete results shown previously⁸). In the hemorrhagic stroke study, there were no significant differences in cases and controls for the cytokines, nor for any conventional CV risk factors.

On analysis by thirds of their distributions, none of the cytokines showed significant associations with hemorrhagic stroke (Table 2). The highest third of TNF- α had a moderately elevated risk compared with the lowest third, the adjusted OR being 1.23, although the 95% CI was wide (0.55 to 2.75) owing to small numbers. For ischemic stroke, IL-6 showed a stepwise increase in OR across thirds, and although the trend was reduced to borderline significance after adjustment for conventional risk factors, the highest third still had an increased risk compared with the lowest: OR=1.33; 95% CI, 1.00 to 1.78. TNF- α showed somewhat similar associations, although the significance of its trend was entirely due to the excess risk comparing the highest with the lowest third: OR=1.46; 95% CI, 1.02 to 2.10, after adjustment. For total stroke, both IL-6 and TNF- α had a more significant trend in risk across thirds compared with the major subtypes of stroke (owing to increased case numbers and smaller CIs). Estimates of associations were relatively unaffected by the addition of hemorrhagic and unknown strokes to ischemic stroke (largely owing to the predominance of ischemic stroke). IL-18 showed no association with risk of hemorrhagic, ischemic, or total stroke in any model.

Previously,⁸ we found significant independent effects of fibrinogen and CRP: comparing the extreme thirds, the OR for fibrinogen was 1.34 (95% CI, 1.01 to 1.78) and for CRP it was 1.39 (95% CI, 1.05 to 1.85), after adjustment for systolic blood pressure, smoking, prevalent peripheral artery disease, statin use, and antiplatelet use. A summary of a multivariable model, including classic risk markers and all 5 inflammatory markers at once, is shown in Table 3. Inclusion of all 5 markers in the same model produced ORs for any of the markers with ischemic stroke to near unity, with the exception of the OR comparing the extreme thirds of TNF- α , which was 1.43 (95% CI, 0.98 to 2.10) and hence borderline significant.

Discussion

To our knowledge, this is the first reported study of the association between the proinflammatory cytokines IL-6, IL-18, and TNF- α and risk of recurrent stroke. We found that IL-6 and TNF- α , but not IL-18, were significant risk predictors of recurrent ischemic stroke. IL-6 appeared to show stepwise increases in risk through thirds of this population, although the trend was less notable after adjusting for potential confounders. For TNF- α , only the top third of the population was at statistically increased risk after adjusting for classic risk factors. This finding is consistent with a

Table 3. ORs (95% CIs) for Ischemic Stroke by Thirds for Each of the 5 Variables Adjusted for the Other 4 and for Systolic Blood Pressure, Smoking, Peripheral Artery Disease Prevalence, Statin Use, Diabetes, and Antiplatelet Use

	IL-6	IL-18	TNF- α	Fibrinogen	CRP
First third					
Second third	1.11 (0.76–1.61)	0.88 (0.62–1.24)	0.93 (0.64–1.34)	1.07 (0.76–1.53)	0.95 (0.65–1.38)
Third third	0.99 (0.65–1.50)	0.92 (0.63–1.33)	1.43 (0.98–2.10)	1.02 (0.68–1.53)	1.13 (0.73–1.76)
<i>P</i> for trend	0.92	0.15	0.04	0.89	0.50

previous study of recurrent myocardial infarction, which found that only those with very high TNF- α expression were at increased risk.¹⁵

The absence of an association of any of the inflammatory markers with hemorrhagic stroke may be due to small numbers, although we believe it is more likely that the observed differences are due to differing underlying pathologies of ischemic and hemorrhagic stroke. Chronic low-grade inflammation is believed to be a key factor in atherogenesis,^{1,2,20–25} which may ultimately lead to the atherothrombotic complications of plaque rupture such as ischemic stroke. In contrast, hemorrhagic stroke often occurs as a result of rupture of small intraparenchymal vessels, and such events are often the result of hypertension or other factors not necessarily directly causally related to atherosclerosis or inflammation.³³

A major biologic property of IL-6 and TNF- α is that these cytokines may actively participate in the hepatic stimulation of the APR.¹ There is little evidence to suggest that IL-18, which was not associated with any major stroke subtype in this study, directly contributes to the APR.³⁴ As we have previously noted in this study,⁸ the acute-phase reactant behavior of fibrinogen, CRP, or any inflammatory marker may be viewed as a potential confounder of associations with recurrent stroke. This is because the underlying association may be due to “generalized inflammation” (as proximally measured by the APR) rather than the causal behavior of any individual inflammatory marker per se.² However, adjusting acute-phase reactants (CRP and fibrinogen) only for each other to remove potential APR confounding assumes that the APR is an absolute event, with roughly equal effects on circulating CRP and fibrinogen levels. This may not be an accurate assumption.³⁵

By simultaneously adjusting for CRP, fibrinogen, IL-6, TNF- α , and IL-18 (not associated with risk), we have shown that markers of the APR (fibrinogen and CRP) and the chief stimulator of the APR (IL-6) almost completely explain each other’s association with recurrent stroke. This suggests that despite evidence that IL-6, IL-18, and TNF- α are involved in experimental atherogenesis and atherothrombosis,^{20–25} potential associations with recurrent stroke are almost fully explained by conventional risk markers and proximity to the APR. Equally, this suggests that CRP and fibrinogen themselves are associated with recurrent stroke because they are acute-phase reactants rather than that they necessarily make additional inflammatory contributions to atherothrombosis. The exception to this trend is TNF- α , which retained borderline associations with risk of recurrent ischemic stroke in this cohort after adjusting for conventional risk factors and the APR. TNF- α is a pluripotent cytokine and may be involved in atherogenesis and atherothrombosis in mechanisms independent of the APR, perhaps particularly in those with very high circulating concentrations of the cytokine,¹⁵ eg, in those with rheumatoid arthritis.³⁶ Despite this, the risk association in the current cohort by thirds of the population is modest and unlikely to provide appreciable clinical utility, although this requires verification in larger cohorts. The lack of association of IL-18 with risk of recurrent stroke is in contrast to some studies that prospectively showed that IL-18 was associated

with risk of CHD events.^{13,14} However, this finding has not been universal in cohorts after full adjustment for potential confounders in multivariable models.³⁷ Thus, IL-18 may be related to CHD as a heavily confounded variable, such as by associations with lipid parameters, which are not strongly related to ischemic stroke.³⁸ Residual associations of IL-18 with CHD risk may be due to specific roles in inflammatory pathways (eg, interferon- γ -dependent atherogenesis³⁹), which have yet to be directly related to risk of recurrent ischemic stroke.

Just as caution must be used in drawing conclusions of causality in epidemiologic studies,² it is equally important not to exclude causality on the same basis. It is quite possible (perhaps probable) that some inflammatory markers actively participate in atherogenesis and atherothrombosis, at least partially through other conventional risk factors (eg, the association of inflammation with smoking or cholesterol levels). However, associations of inflammatory markers of the APR (IL-6, CRP, and fibrinogen) with recurrent stroke were fully explained by conventional risk markers and APR confounding in this model. This suggests that from the inflammatory markers measured in this study, it is only “general inflammation” rather than a particular marker that is strongly associated with secondary ischemic stroke. More studies are required to expand on these findings, both for primary and secondary stroke as well as CHD.

Summary

Inflammatory markers associated with the APR (IL-6, TNF- α , CRP, and fibrinogen, but not IL-18) are associated with risk of recurrent stroke. These markers are dependent on each other in multivariable models, and once all were included, only TNF- α retained a borderline association. This suggests that markers of generalized inflammation of the APR are associated with recurrent stroke, rather than IL-6, CRP, or fibrinogen in particular.

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Disclosures

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