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A Comparison of Acute Phase Proteins and Traditional Risk Factors as Markers of Combined Plaque and Intima-Media Thickness and Plaque Density in Carotid and Femoral Arteries

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Objectives: *to test the hypothesis that some acute phase proteins may be better independent predictors of objective measures of arterial wall impairment than traditional risk factors.*
Design: *cross-sectional study.*
Materials and Methods: *C-reactive protein (CRP), fibrinogen, C3 complement and traditional risk factors were measured in 288 men aged 55–64 years, randomly chosen from the local registry lists. By ultrasound assessment of the bifurcations of carotid and femoral arteries, maximum combined plaque/intima-media thickness (CPIMTmax) and mean plaque density (MPD, in a grey scale from 0 to 255) were also measured.*
Results: *in multivariate analysis only traditional risk factors remained associated with the overall CPIMTmax: smoking ($r = 0.35$, $p < 0.0001$), cholesterol ($r = 0.23$, $p = 0.0001$), age ($r = 0.22$, $p = 0.0002$), glucose ($r = 0.18$, $p = 0.002$) and systolic blood pressure ($r = 0.13$, $p = 0.02$). However, with regard to carotid disease only, fibrinogen was the strongest covariate of CPIMT ($r = 0.18$, $p = 0.002$). The overall MPD was independently associated with CRP ($r = 0.25$, $p = 0.0008$), physical activity ($r = 0.19$, $p = 0.009$), triglycerides ($r = -0.18$, $p = 0.02$) and body mass index ($r = 0.15$, $p = 0.04$). CRP was mainly associated with femoral MPD, while triglycerides were the major (inverse) covariate of carotid MPD.*
Conclusions: *traditional risk factors are the main determinants of CPIMTmax, although fibrinogen seems to play a role in carotids. CRP was associated with high density femoral plaques. Finally, no acute phase protein was independently associated with low density, potentially vulnerable, plaques.*

Key Words: C-reactive protein; Fibrinogen; C3 complement; Intima-media thickness; Plaque densitometry; Risk factors.

Introduction

For reasons, as yet unknown, acute phase proteins may be better predictors of future ischaemic events than traditional vascular risk factors.[1–5] The links between acute phase proteins and such risk factors are complex and incompletely defined.[6–10] For example, tumour necrosis factor α released from the adipose tissue,[11] or from sites of chronic inflammations, may bind to insulin receptors,[12] cause insulin resistance and originate the metabolic syndrome.[13] Furthermore it is clear that atherosclerotic plaques, especially those that are at highest risk of rupture, are sites of intense inflammation.[14,15] In a random sample of 288 middle-aged men we have recently reported[16] differences between C-reactive protein (CRP), fibrinogen and C3 complement in terms of their relationship with traditional risk factors such as age, cigarette smoking, alcohol consumption, cholesterol, blood glucose, systolic blood pressure, and body mass index (BMI). As an objective assessment of the degree of atherosclerosis, in the same sample we have also employed ultrasound to measure combined plaque/intima-media thickness (CPIMT) and mean plaque density (MPD), at the carotid and femoral bifurcations.[17,18] The purpose of the present study is to determine whether there is a stronger relationship between CPIMT and MPD and acute phase proteins than there is with traditional risk factors.

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Methods

Subjects

The study population consisted of 288 unselected men aged 55–64 years as has been previously reported.[10,16] Briefly, these subjects were randomly chosen from those who agreed to participate (1111 out of 1846 invited, 60.4%) in the San Vitale Study epidemiological survey in Bologna (Northern Italy). Lifestyle information was obtained by a self-administered questionnaire. Smoking was quantified as daily number of smoked cigarettes, while alcohol consumption was expressed as number of drinks per week (where a pint of beer, a glass of wine or a small glass of spirit were considered a drink). All subjects underwent blood pressure and body weight measurement, as well as venous blood sampling and ultrasound assessment of the carotid and femoral arteries. The population included 42% hypertensives, 12% diabetics, 69% hypercholesterolemics (total cholesterol > 200 mg/dl), 34% current smokers, 30% ex-smokers, 12% with a family history of myocardial infarction (at least one first degree relative affected before 60 years of age) and 40% physically active subjects (at least 90 minutes a week of heavy work or sport). Moreover, 4% had had a previous myocardial infarction, 4% were affected by angina pectoris, 3% by peripheral arterial disease and 14% by a recent (< one month) acute inflammatory disease. Finally, 10% were taking β-blockers, 15% ACE-inhibitors, 8% antiplatelet drugs, 6% lipid lowering drugs, and 5% oral antidiabetic drugs. None of the above prevalence data differed significantly from the corresponding values obtained in the original cohort of participants.[10] Since the sample included 54 subjects who were taking lipid lowering drugs and/or who had recently suffered an inflammatory episode, the analysis was repeated after their removal, with substantially similar results to those reported further. The study was approved by the local Ethics Committee and all subjects provided written informed consent.

Ultrasound measurements were performed by a single operator (CM) with a Hewlett Packard Sonos 2500 duplex scanner using a high-resolution 7.5 MHz linear transducer. In each subject the carotid and femoral bifurcations were examined along a 3 cm portion straddling the flow divider. At each of the four sites, the maximum intima-media thickness (CPIMTmax)[17] or, in the presence of one or more plaques, the maximum plaque thickness, was recorded. Then, the maximum carotid value, the maximum femoral value and the maximum value in the four sites were used in the subsequent assessments. Preliminary analyses (data not shown) and other studies[17] had suggested that maximum, rather than mean, values provide optimal correlations with risk factors. Moreover, this combined parameter is more influenced by the presence of plaques than by intima-media thickness,[19] and plaque thickness correlates with a greater number of risk factors, and with higher correlation coefficients, than intima-media thickness.[19] On the other hand, with respect to plaque thickness alone, the combined parameter offers the advantage of being measurable in all subjects, even those without plaques. Intima-media thickness was measured on the basis of a longitudinal view of the artery, in the "far" wall with respect to the transducer. When a plaque (defined as a localized region of increased intimal thickness and density) was found, the probe was rotated by 90°, so that a transversal view was obtained: the maximum plaque thickness was sought, and the densitometric analysis was performed, within this view.

Densitometric analysis was performed following the methods of El-Barghouty *et al.*,[20] Beletski *et al.*[18] and Grønholdt *et al.*[21] The main ultrasound parameters (dynamic range, depth range, power output and frame rate) were kept constant, while gain control was adjusted so that optimal B-mode images could be obtained. All investigations were recorded on a S-VHS videotape. In the presence of plaques the operator outlined the whole cross section of the plaque, as well as the lumen (black) area, using the software incorporated in the ultrasonographer, and avoiding the inclusion of possible areas with acoustic shadowing. These images were printed out by a Sony Video Graphic printer (UP-890MD) and then digitized by a Hewlett Packard Scanjet 4P scanner interfaced with the program Visioneer PaperPort, version 3.0.1, from Visioneer Communications, Inc. The digitized images were then processed by a second operator (L.Bal.) with the program Photoshop, version 5.0, from Adobe Systems, Inc. This program assigned a density value to each pixel in the outlined region, in a grey scale ranging from 0 to 255, and then generated the frequency distribution of these densities. The median of this distribution (median grey scale, MGS) was the numeric parameter reassuming the density of the region of interest. This parameter has been shown to correlate directly with the fibrous and calcified component of plaques and, inversely, with the intraplaque content in lipid, haemorrhagic or thrombotic material.[18,21] Moreover, carotid plaque density, as assessed by median grey scale, is inversely associated with the occurrence of cerebral infarction.[20,22,23] To obtain comparable values, blood density (i.e. the median density

in the lumen area) was considered the background density, and this value was subtracted from the density in the plaque area.[21] The so normalized density value of each plaque was then used to obtain the three main parameters for the following analyses: the mean density in carotid plaques, the mean density in femoral plaques, and the mean plaque density in all the four sites explored.

Six to eight months after the first assessment, the videotape recordings concerning 53 randomly chosen plaques were re-examined by the same operator (CM). Plaque thickness was measured and, similarly, plaque images (plus the corresponding arterial lumen) were outlined, printed and digitized for the second time. The digitized images were then processed by another operator (L.Bal.), as previously described, until normalized density values were obtained. The mean of the differences between the first and the second measurement of plaque thickness was 0.03 mm (95% C.I.: -0.01 to 0.06), with a repeatability coefficient[24] of 0.26 mm. For plaque density, the mean difference, in the MGS, was -0.7 (95% C.I.: -3.7 to 2.4), with a repeatability coefficient of 22.0. Although correlation analysis does not provide a reliable estimate of repeatability,[25] the correlation between the first and the second series of values was good for both parameters ($r = 0.98$ for plaque thickness and $r = 0.94$ for plaque density).

The methods used to measure the biochemical variables in the 288 men of our population sample have been described in detail elsewhere.[10,16] In particular, fibrinogen was measured with the method of Clauss,[26] C3 complement with nephelometry (Behring kits, Behringwerke AG, Marburg, Germany) and CRP with a high-sensitivity nephelometric method (N Latex CRP mono, Dade Behring, Liederbach, Germany).

The statistical analysis mainly consisted of multivariate analysis techniques. Univariate analysis (simple regressions and some unpaired *t*-tests) was performed essentially as a preliminary step to multivariate analysis (multiple linear regressions): in each initial multivariate model all the variables were included, which, in univariate analysis, were associated with CPIMTmax or MPD with p values < 0.30. Thereafter, multiple regression was repeated several times, removing every time from the model the variable with the highest p value, until all of the remaining variables were significantly associated with their dependent variable (backward elimination procedure). All the variables that were not normally distributed were logarithmically transformed. Two tail tests were performed and p values < 0.05 were considered significant.

Results

Figure 1 shows the frequency distributions of CPIMTmax in all the 288 subjects, and of MPD in the 183 subjects with plaques, considering all the four arterial sites examined. The distribution of both variables had a positive skewness, which was greater for MPD. The median and interquartile range for CPIMTmax were 2.29 mm (1.47–3.00 mm). The same values for MPD were 32 (16 – 50, MGS). There was a weak inverse correlation between the two variables, in both carotids ($r = -0.25$; $p = 0.011$; $n = 104$) and femorals ($r = -0.17$; $p = 0.025$; $n = 163$).

Factors associated with CPIMT

Table 1 shows the univariate associations (simple linear regressions) of the natural logarithm of CPIMTmax with traditional risk factors and the three acute phase proteins, in all the 288 men of our sample. The assessments in carotids alone, in femorals alone, or in all the four arteries together, are separately reported.

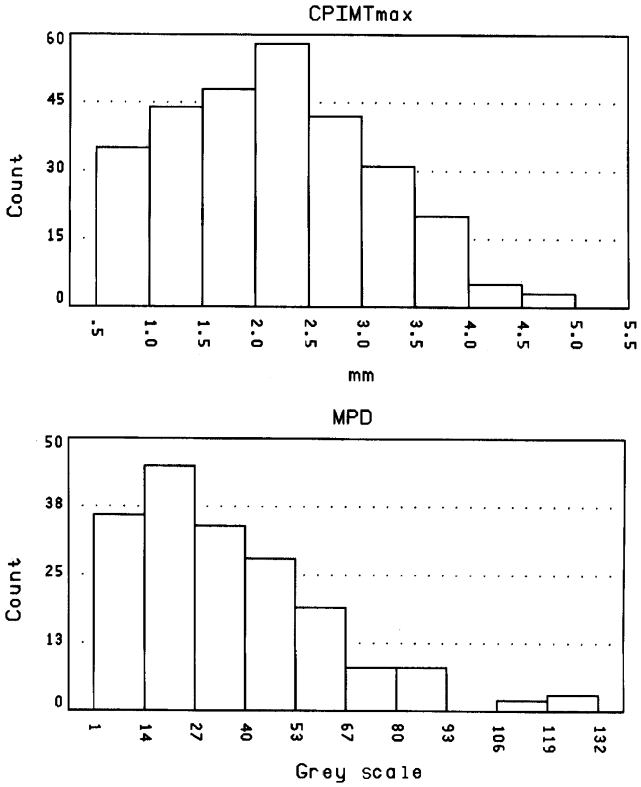

Fig. 1. Frequency distribution of maximum combined plaque/intima-media thickness (CPIMTmax) in all the 288 subjects (upper panel), and of mean plaque density in the 183 subjects with plaques (lower panel), considering all the four arterial sites examined.

Table 1. Univariate associations of CPIMTmax with risk factors and acute phase proteins in carotid and femoral arteries.

	Any arteries		Carotid arteries		Femoral arteries	
	r coeff.	*p* value	*r* coeff.	*p* value	*r* coeff.	*p* value
Age	0.19	**0.001**	0.17	**0.004**	0.21	**0.0002**
log smoking	0.30	**<0.0001**	0.12	**0.033**	0.34	**<0.0001**
log alcohol	0.01	0.856	−0.04	0.514	−0.03	0.640
BMI	0.06	0.303	0.10	**0.084**	0.04	0.473
Systolic blood pressure	0.19	**0.001**	0.15	**0.008**	0.14	**0.014**
Diastolic blood pressure	0.05	0.347	0.04	0.457	0.01	0.860
log insulin	0.00	0.955	0.01	0.892	0.01	0.800
log blood glucose	0.18	**0.001**	0.08	**0.150**	0.20	**0.0005**
Cholesterol	0.22	**0.0001**	0.12	**0.043**	0.21	**0.0002**
HDL cholesterol	−0.07	**0.249**	−0.07	**0.217**	−0.07	**0.228**
log triglycerides	0.21	**0.0003**	0.10	**0.104**	0.19	**0.001**
Fibrinogen	0.16	**0.005**	0.20	**0.0004**	0.14	**0.020**
log CRP	0.15	**0.010**	0.15	**0.008**	0.15	**0.012**
C3 complement	0.14	**0.014**	0.10	**0.084**	0.11	**0.057**

	No.	CPIMTmax (mm)	*p* value	CPIMTmax (mm)	*p* value	CPIMTmax (mm)	*p* value
FHMI							
No	254	2.20 (1.46–2.95)	**0.173**	1.30 (0.90–2.01)	**0.194**	2.00 (1.20–2.70)	**0.167**
Yes	34	2.35 (1.90–3.10)		1.40 (0.96–2.10)		2.18 (1.60–2.87)	
Physical activity							
No	172	2.30 (1.50–3.00)	**0.198**	1.33 (0.95–2.00)	**0.286**	2.10 (1.30–2.82)	**0.236**
Yes	116	2.18 (1.42–2.82)		1.20 (0.90–2.08)		2.00 (1.06–2.56)	

All subjects ($n = 288$) were considered in the assessment of each association. Values for CPIMTmax are median (interquartile range). FHMI = family history of myocardial infarction. HDL = high density lipoprotein. *p* values of the variables included in the subsequent multivariate analysis ($p < 0.30$) are marked in bold. For linear regressions the logarithm of CPIMTmax was used as dependent variable.

The strongest correlation was the one with cigarette smoking, in femoral arteries. Similarly, there was a progressive and highly significant increase in the medians of femoral CPIMTmax from never smokers (1.5 mm), to ex-smokers (2.0 mm), up to current smokers (2.5 mm, $p < 0.0001$ by variance analysis of log-transformed data). The median of the overall CPIMTmax was significantly greater in the subjects with previous ischaemic events (especially myocardial infarction, $n = 13$, 3.1 mm) and in the subjects who were taking antiplatelet drugs ($n = 24$, 3.0 mm) or oral antidiabetic drugs ($n = 15$, 3.1 mm), than in the subjects who did not present these characteristics (2.2 mm, $p < 0.001$ for all comparisons).

To ascertain which associations were independent, some multiple linear regressions were performed with a backward elimination procedure, initially including in the models all the variables associated with CPIMTmax, in univariate analysis, with *p* values < 0.30. The final results are reported in Table 2. Considering the four arteries together, the relationships with cigarette smoking, cholesterol, age, blood glucose and systolic blood pressure (in decreasing order of partial *r*) were confirmed. Overall, these five traditional risk factors explained 23% of CPIMTmax variance. Instead, the correlations of CPIMTmax with the three acute phase proteins and triglycerides were not confirmed.

In carotids alone the associations with age, smoking and cholesterol were also confirmed, but in this site the strongest association of CPIMTmax was with fibrinogen. The univariate non significant association with BMI, in multivariate analysis became borderline-significant. However, these variables explained only 11% of CPIMTmax variability in carotids. The correlations with systolic blood pressure and CRP were not confirmed.

Finally, considering the femorals alone, cigarette smoking, age, blood glucose and cholesterol remained independently and strongly associated with CPIMTmax (these four factors alone explained 27% of CPIMTmax variance in femorals), while the univariate associations with systolic blood pressure, triglycerides, fibrinogen and CRP were not confirmed.

Factors associated with MPD

Overall, 408 arterial plaques (143 in the carotids and 265 in the femorals) were demonstrated in the 288 subjects studied. Table 3 reports the univariate correlations of the natural logarithm of MPD with traditional risk factors and acute phase proteins. MPD was not associated with previous ischaemic events or the use of any type of drug, although almost in all

Table 2. Significant multivariate associations of CPIMTmax with risk factors and acute phase proteins in carotid and femoral arteries (multiple linear regressions).

	Any arteries			Carotid arteries			Femoral arteries		
	Partial r	Seq. R^2	p value	Partial r	Seq. R^2	p value	Partial r	Seq. R^2	p value
Age	0.22	0.03	0.0002	0.16	0.03	0.007	0.28	0.05	<0.0001
log smoking	0.35	0.14	<0.0001	0.14	0.05	0.014	0.41	0.18	<0.0001
BMI			n.t.	0.11	0.06	0.051			n.t.
Systolic blood pressure	0.13	0.16	0.024			N.S.			N.S.
log blood glucose	0.18	0.19	0.002			N.S.	0.24	0.23	<0.0001
Cholesterol	0.23	0.23	0.0001	0.14	0.08	0.015	0.23	0.27	<0.0001
Fibrinogen			N.S.	0.18	0.11	0.002			N.S.

All subjects ($n = 288$) participated in the three regressions. Only variables associated with CPIMTmax with p values <0.30 in univariate analysis were tested. N.S. = not significant. n.t. = not tested. Seq. R^2 = sequential R^2. For all regressions the logarithm of CPIMTmax was used as a dependent variable.

Table 3. Univariate associations of MPD with risk factors and acute phase proteins in carotid and femoral arteries.

	Subjects with any plaques ($n = 183$)		Subjects with carotid plaques ($n = 104$)		Subjects with femoral plaques ($n = 163$)	
	r coeff.	p value	r coeff.	p value	r coeff.	p value
Age	0.05	0.512	0.20	**0.046**	−0.00	0.948
log smoking	0.05	0.465	0.09	0.369	−0.02	0.792
log alcohol	0.02	0.742	−0.10	0.319	0.09	**0.239**
BMI	0.16	**0.024**	0.16	**0.093**	0.10	**0.184**
Systolic blood pressure	0.01	0.932	−0.07	0.473	0.04	0.592
Diastolic blood pressure	0.01	0.867	−0.06	0.522	0.08	0.324
log insulin	0.08	0.306	0.12	**0.219**	0.06	0.425
log blood glucose	−0.05	0.503	−0.06	0.523	0.00	0.982
Cholesterol	−0.13	**0.084**	−0.21	**0.036**	−0.10	**0.214**
HDL cholesterol	0.02	0.756	−0.00	0.980	0.01	0.929
log triglycerides	−0.09	**0.227**	−0.18	**0.064**	−0.06	0.430
Fibrinogen	0.10	**0.159**	0.01	0.910	0.08	**0.278**
log CRP	0.18	**0.013**	−0.01	0.929	0.16	**0.046**
C3 complement	0.01	0.892	−0.02	0.799	0.04	0.646

	No.	MPD	p value	No.	MPD	p value	No.	MPD	p value
FHMI									
No	159	33.0 (16.0–50.0)	0.587	89	31.5 (16.0–57.0)	0.419	140	31.0 (14.0–46.7)	0.871
Yes	24	25.7 (19.1–56.5)		15	29.0 (24.0–67.0)		23	22.0 (16.5–43.0)	
Physical activity									
No	113	26.7 (16.0–43.0)	**0.086**	64	28.0 (15.5–57.2)	**0.079**	102	26.7 (15.0–43.0)	0.569
Yes	70	38.3 (22.0–57.0)		40	39.0 (22.5–62.2)		61	33.0 (14.0–52.0)	

Values for MPD are median (interquartile range). FHMI = family history of myocardial infarction. HDL = high density lipoprotein. p values of the variables included in the subsequent multivariate analysis ($p < 0.30$) are marked in bold. For linear regressions the logarithm of MPD was used as a dependent variable.

these categories there was a non-significant tendency towards higher density values (data not shown).

As for CPIMTmax, three multivariate analyses were performed in order to identify the independent associations of MPD in the three sites considered (Table 4). In the subjects with plaques of any site, CRP was the parameter most strongly associated with MPD, together with physical activity, triglycerides (inversely) and BMI. Considering the carotids alone, the inverse association with triglycerides was the strongest one, followed by the correlations with age, BMI and physical activity, while the univariate association with cholesterol was not confirmed. Finally, the only variable independently associated with MPD in the femoral arteries was CRP. The proportion of MPD variability explained by these variables was 11% in the four arteries, 15% in the carotids and only 2% in the femorals.

Discussion

This study has compared acute phase proteins and traditional vascular risk factors as markers of the thickness and density of atherosclerotic lesions in a random sample of middle-aged men. It should be

Table 4. Significant multivariate associations of MPD with risk factors and acute phase proteins in carotid and femoral arteries (multiple linear regressions).

	Subjects with any plaques ($n = 183$)			Subjects with carotid plaques ($n = 104$)			Subjects with femoral plaques ($n = 163$)		
	Partial r	Seq. R^2	p value	Partial r	Seq. R^2	p value	Partial r	Seq. R^2	p value
Age			n.t.	0.24	0.04	0.026			n.t.
BMI	0.15	0.02	0.040	0.24	0.06	0.028			N.S.
log triglycerides	−0.18	0.04	0.017	−0.26	0.12	0.014			n.t.
log CRP	0.25	0.08	0.0008			n.t.	0.16	0.02	0.046
Physical activity	0.19	0.11	0.009	0.23	0.15	0.034			n.t.

Only variables associated with MPD with p values <0.30 in univariate analysis were tested. N.S. = not significant. n.t. = not tested. Seq. R^2 = sequential R^2. For all regressions the logarithm of MPD was used as a dependent variable.

noted that this population sample was not selected for having advanced or complicated atherosclerotic lesions. In general, the factors associated with plaque/intima-media thickness were not the same as those associated with plaque density. Moreover, there were differences among the acute phase proteins as far as their associations with plaque characteristics were concerned.

The only acute phase protein to be independently associated with CPIMTmax after adjustment for traditional risk factors was fibrinogen. However, for reasons unknown, this was only observed at the carotid bifurcation.[19,27,30] Hyperfibrinogenaemia may be a consequence of intra-plaque inflammation. Alternatively, fibrinogen may play a role in plaque development through the formation of mural thrombi, the stimulation of smooth muscle cell proliferation,[28] or the intimal trapping of lipoproteins.[29] No relationship between fibrinogen and MPD was detected.

The relationship between CRP and CPIMTmax disappeared after adjustment for traditional risk factors, which is in keeping with the earlier work of others.[31–33] On the other hand, CRP does seem to be associated with plaque density, especially at the femoral level, which supports the previously described link between CRP and peripheral arterial disease[34] but not carotid lesions.[35] Relevant to this association may be the fact that fibroblasts are important sources of interleukin-6, the main trigger for CRP production in the liver.[36]

With regard to C3, present data, unlike those from earlier studies,[37] are based on objective ultrasound parameters, and fail to show any dependence on the thickness or density of femoral and carotid plaques. Thus, as previously suggested,[10,16] the relationship between C3 and the risk of myocardial infarction is probably due to the role played by this cytokine/acute phase protein upstream in the atherosclerotic process, with a possible participation in the appearance of the metabolic syndrome and its related cluster of risk factors.

Overall traditional risk factors remain the main determinants of plaque/intima-media thickness. Several earlier studies have reached similar conclusions, although in general acute phase proteins were not measured, and the investigations were limited to the carotid arteries.[17,19,38] The association between elevated triglyceride levels and low density carotid plaques has already been reported by Grønholdt *et al*.[21,39] Furthermore, fibrinogen was the variable most closely associated with the thickness of carotid plaques, and CRP was the only specific marker, albeit weak, of high plaque density in femoral arteries. However, no acute phase protein was independently associated with the presence of low density, potentially "vulnerable", plaques in the sites explored.

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