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ARTICLE

TLR4/Asp299Gly, CD14/C-260T, plasma levels of the soluble receptor CD14 and the risk of coronary heart disease: The PRIME Study

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TLR4 and CD14 are two components of the LPS receptor complex, which are considered to play a key role in the pathogenesis of atherosclerosis. TLR4/Asp299Gly and CD14/C-260T polymorphisms are thought to modulate the activity of this complex. The aim of the study was to examine the association between the TLR4/Asp299Gly and CD14/C-260T polymorphisms, plasma levels of the soluble receptor CD14 (sCD14), and the incidence of coronary heart disease (CHD) in a prospective cohort (the PRIME Study) of 9758 healthy men aged 50–59 years recruited in France and Northern Ireland. A nested case–control design was used, comparing the 249 participants who developed a CHD event during the 5-year follow-up with 492 population- and age-matched control subjects. The two polymorphisms were genotyped and baseline plasma concentrations of sCD14 were measured. None of the two polymorphisms, or sCD14 levels, either considered alone or in combination, were associated with the risk of CHD. The CD14/C-260T allele was associated with increased plasma concentrations of soluble thrombomodulin and vascular cell adhesion molecule-1 and, to a lesser extent, sCD14. No relationship was observed between the TLR4 polymorphism and, any of the inflammatory and endothelial markers measured. The TLR4/Asp299Gly and CD14/C-260T polymorphisms and plasma sCD14 concentrations do not appear as significant predictors of the risk of CHD in healthy individuals.

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Introduction

Immune and inflammatory mechanisms are considered to play a key role in the pathogenesis of atherosclerosis.[1,2] The toll-like receptor (TLR)4 is a receptor for lipopolysaccharide (LPS) and heat-shock proteins, molecules known to initiate an innate immune response, including the production of proinflammatory cytokines by macrophages and adhesion molecules in endothelial cells through nuclear

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factor (NF)-κB activation.[3,4] TLR4 has recently been described in macrophages and endothelial cells in the atherosclerotic plaque.[5] It has been shown that a common missense mutation Asp299Gly affecting the extracellular domain of the TLR4 receptor is associated with a blunted response to inhalated LPS in humans.[6] Several studies have investigated the association of this polymorphism with the development of atherosclerosis. Three of them reported that the Gly^{299} allele of the TLR4 polymorphism might confer protection against atherosclerosis and its complications,[7–9] whereas another did not find any association between the TLR4 polymorphism and the susceptibility to and severity of coronary disease.[10]

Several lines of evidence suggest that TLR4 cannot function as a singular binding and signalling receptor but belongs to an 'LPS signalling complex'.[11] One likely component of this putative LPS receptor complex is CD14, which is present in macrophages (CD14 membrane bound, mCD14) and in plasma as a soluble protein (sCD14) that promotes the response of endothelial cells, which do not express mCD14.[12] Multiple biochemical and genetic studies support the concept that CD14 binds to LPS, but does not participate in the signalling of the NF-κB pathway.[13] Nonetheless, CD14 knockout mice have been shown to be highly resistant to LPS[14] and TLR4-mediated NF-κB activation by LPS could be enhanced by the addition of sCD14,[15] supporting a role of CD14 in the LPS receptor complex. A C-260T substitution located in the promoter region of the CD14 gene is thought to modulate the capacity to elicit inflammation through the regulation of CD14 gene expression and plasma sCD14 levels.[16–18] Studies investigating the association between the CD14/C-260T polymorphism and coronary disease brought conflicting results. The T allele was found to be associated with a higher risk of myocardial infarction (MI) in three case–control studies.[17,19,20] Conversely, the CC genotype was associated with an increased incidence of incident coronary occlusion and increased carotid artery intima-media thickness (IMT).[21,22] One prospective study[23] and three retrospective ones[18,24,25] did not find any association between the polymorphism and the risk of coronary events. No relationship was found between plasma concentrations of sCD14 and stable coronary artery disease or carotid IMT.[24,26]

Given the potential interaction between TLR4 and CD14 in mediating signal transduction of innate immunity, we investigated the relation of these two polymorphisms, as well as sCD14 plasma levels, with the risk of coronary heart disease (CHD) and the plasma levels of inflammatory markers. We used a nested case–control study design within the PRIME prospective cohort. PRIME is a large multicentre cohort with a 5-year follow-up, aimed at investigating the role of different biological and genetic markers in the development of CHD in France and Northern Ireland.

Material and methods

The PRIME Study (Prospective Epidemiological Study of Myocardial Infarction) has been described in detail.[27] It is a population-based prospective study set-up to investigate risk factors for CHD and, more particularly, to identify those that might explain the difference in CHD incidence between France and Northern Ireland. From 1991 to 1994, 9758 men aged 50–59 years with no previous CHD events, living in the area of Lille, Strasbourg, Toulouse in France and Belfast in Northern Ireland, were recruited and followed up for over 5 years. At entry, venous blood was collected after a 12-h fast into siliconised vacutainer tubes (Vacutainer, Becton Dickinson) containing 0.11 M trisodium citrate (1 volume). Platelet-poor plasma was obtained by centrifugation at 4500 *g* and 20°C for 15 mn. Without delay, aliquots of plasma were transferred into plastic tubes and frozen on-site to −80°C, and were then sent weekly to the central laboratory at the Pasteur Institute of Lille, where they were stored in liquid nitrogen until analysis.

For subjects reporting a possible clinical event, information was sought directly from the hospital or general practitioners' files. All details of ECG, hospital admissions, enzymes, surgical operations, angioplasty, treatment, etc were collected and classified according to MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) criteria.[28] Death certificates were also used to complete information on the cause of death. A medical committee provided independent validation and classification of coronary events. CHD categories retained for analysis were nonfatal MI or coronary death (grouped together as hard coronary event), and unstable angina pectoris or effort angina (grouped as angina pectoris), yielding a total number of cases of 317.[29]

The present study was carried out using a nested case–control design. Each case was matched with two age-matched (±3 years) control subjects recruited in the same centre and on the same day (±3 days) as the corresponding case and free of CHD at the date of the coronary event of the case. Of the initial sample of 317 cases, 68 were not available for DNA analysis, either because they had not given their informed consent for the genetic part of the study or due to lack of DNA. When a case was not available for analysis, the two matched controls were excluded from the present study. When one or the two controls of a case were not available for the same reasons, they were replaced by other controls meeting the same matching criteria whenever possible. A total of 249 cases and 492 matched controls were used in the present study. Cases and controls included in the analysis did not significantly differ from those not included with respect to the main cardiovascular risk factors.

sCD14 determination

Stored plasma samples obtained at baseline were sent from the central plasma bank in dry ice to the Laboratory of

Haemostasis of La Timone Hospital in Marseilles, France. Determination of sCD14 was performed with a commercially available ELISA from R&D Systems (Minneapolis, MN, USA) according to the instructions available from the supplier. Blood specimens were analysed in blinded pairs, with the position of the case specimen randomly varied within pairs to reduce the possibility of systematic bias and minimize interassay variability. The interassay coefficient of variation was 10%. The methods used to evaluate baseline lipid parameters, inflammation and endothelial markers have been described elsewhere.[29–33]

Genotyping of TLR4/Asp299Gly and CD14/C-260T polymorphisms

Genomic DNA was extracted from peripheral blood leucocytes by the salting-out method.[34] The CD14/C-260T polymorphism was genotyped by PCR-RFLP using restriction enzyme *Hae*III. PCR was carried out in a 25 μl reaction volume, using final concentrations of 200 nM of each primer (forward: 5′-TAAGGCACTGAGGATCATCC-3′; reverse: 5′-GGCTTCACACTTGTGAACTC), 200 μM of each dNTP, 75 ng of genomic DNA, 3.5 mM MgCl2 and 0.25 U of *Taq* DNA polymerase. Conditions were: 40 cycles consisting of denaturation at 94°C, 30 s; annealing at 60°C, 45 s and elongation at 72°C 1 min, followed by a final extension step at 72°C for 5 min. The PCR product (8 μl) was digested using 2 U of *Hae*III at 37°C and the digestion products were identified by migration on a 2% agarose gel stained with ethidium bromide. Digestion of the PCR product gives a 329 bp product for the T allele and two products of 170 and 159 bp, respectively, for the C allele.

The TLR4/Asp299Gly polymorphism was genotyped by allele-specific PCR. The PCR reaction conditions were as for the CD14/C-260T polymorphism, except for the *Taq* polymerase for which 0.3 U were used per reaction and the annealing temperature (58°C). Two different mixtures were carried out with forward and reverse primers in each case (forward: 5′-TTTAGACTGTCCCTGAAC-3′; reverse: 5′-AGATTTGAGTTTCAATGTGGG-3′) plus the allele-specific primer, which corresponded to the analysed genotype (A allele: 5′-CAATTAAATAAGTCAATTATATC-3′ and G: 5′-AATTAAATAAGTCAATTATACC-3′). Size of the forward-reverse product and the allele-specific were, respectively, 437 and 298 bp.

Statistical analysis

Allele frequencies were estimated by gene counting. Departure from Hardy–Weinberg equilibrium was tested separately in cases and controls and in each population by a χ^2 with 1 df. Owing to the low frequency of the TLR4/Gly^{299} allele, rare homozygotes were pooled with heterozygotes for analysis. Mean levels of continuous variables were compared between cases and controls by ANOVA adjusted for population (France/Northern Ireland). Variables with a skewed distribution were log transformed before analysis. Conditional logistic regression analysis for matched case-control studies was used to investigate the association between outcome and explanatory variables. The analysis included 243 trios and six pairs of cases–controls. Since cases and controls were matched by population, all case–control comparisons were implicitly stratified according to population. The relative risks (RRs) and 95% CIs are reported. $P<0.05$ was considered significant. All computations were performed with SAS software, version 8.1 (SAS Institute).

Results

Baseline characteristics

During the 5-year follow-up period, the annual incidence of coronary events was two-fold higher in Northern Ireland than in France (11.51/1000 *vs* 5.48/1000 per year),[29] resulting in an almost equal number of cases in the two cohorts despite the smaller size of the baseline cohort in Northern Ireland than in France ($n=2399$ and 7359, respectively). Table 1 shows the baseline clinical characteristics of the cases and controls included in the present analysis. Among the 249 cases, 128 (51.4%) had suffered from a hard CHD event and 123 (49.4%) from angina pectoris (two cases had suffered from both). As expected, initially healthy men who subsequently developed CHD (cases) were more likely at baseline to have a history of hypertension or diabetes, were more often smokers, and had an adverse lipid profile when compared to men who remained free of disease (controls). The effect of these conventional cardiovascular risk factors was similar in France and Northern Ireland.

Correlation of sCD14 with other cardiovascular risk factors

As outlined in Table 2, diabetes was associated with markedly increased concentrations of sCD14 ($P<0.001$), consistently in France and Northern Ireland. This effect remained significant ($P=0.004$) even after adjustment for C-reactive protein (CRP). In contrast, hypertension or smoking status did not significantly influence sCD14 levels. Correlations between plasma levels of sCD14 and continuous risk variables are reported in Table 3. sCD14 was not associated with body mass index or lipid parameters. It correlated with acute phase reactants such as CRP, fibrinogen and IL-6, but not with TNF-α or IL-18. With respect to endothelial cell markers, sCD14 correlated with soluble intercellular adhesion molecule (sICAM)-1 and soluble thrombomodulin (sTM), but not with soluble vascular cell adhesion molecule (sVCAM)-1, von Willebrand factor (vWF) or free tissue factor pathway inhibitor (f-TFPI). The correlation with inflammatory markers was of similar magnitude in France and Northern Ireland, whereas the relationship of sCD14 with sICAM-1 and sTM appeared stronger in Northern Ireland than in France, even though

Table 1 Baseline characteristics of cases and controls included in the nested case–control study

Characteristics	*Controls* (France/N. Ireland; $n=225/267$)	*Cases* (France/N. Ireland; $n=115/134$)	*P-value*
AGE (Y)	55.1 ± 0.1	55.3 ± 0.2	0.5
BMI (kg/m^2)	26.7 ± 0.2	27.1 ± 0.2	0.1
SBP (mm Hg)	134.6 ± 0.9	141.5 ± 1.3	<0.001
DBP (mm Hg)	83.7 ± 0.6	87.0 ± 0.8	<0.001
History (%)			
Hypertension	10.5	20.5	<0.001
Diabetes mellitus	3.4	7.6	0.02
Smoking status (%)			
Never	30.6	19.7	
Former	41.5	40.2	0.002
Current	27.9	40.2	
Lipid status			
Total cholesterol (g/l)	2.24 ± 0.02	2.34 ± 0.02	<0.001
HDL cholesterol (g/l)	0.47 ± 0.005	0.43 ± 0.007	<0.001
Triglycerides (g/l)*	1.39 (1.33–1.46)	1.54 (1.44–1.64)	0.02

BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.
Data presented are percentages for categorical variables and population-adjusted mean ± SEM for continuous variables or *population-adjusted geometric means (95% CI) for log-transformed variables.

Table 2 Mean levels (SEM) of plasma sCD14 according to categorical risk factors[a]

	All	*France*	*Northern Ireland*
Diabetes	1.52 (0.02)	1.44 (0.03)	1.58 (0.02)
No	1.79 (0.08)	1.69 (0.06)	1.88 (0.14)
Yes	0.001	0.01	0.04
P-value			
Hypertension			
No	1.53 (0.02)	1.44 (0.03)	1.59 (0.02)
Yes	1.54 (0.05)	1.53 (0.06)	1.55 (0.08)
P-value	0.93	0.17	0.67
Smoking			
Never	1.52 (0.03)	1.52 (0.06)	1.54 (0.04)
Former+current	1.53 (0.02)	1.45 (0.03)	1.61 (0.03)
P-value	0.98	0.27	0.20

Values are in μg/ml.
[a]Means are adjusted for case–control status, and additionally adjusted for population in the column 'all'.

Table 3 Pearson's correlation coefficients of sCD14 levels with lipid parameters, inflammatory markers and endothelial-cell-derived factors

	All	*France*	*Northern Ireland*
BMI	−0.02	−0.02	−0.01
Total cholesterol	0.005	−0.04	0.03
HDL cholesterol	0.05	0.002	0.06
Triglycerides[a]	−0.004	−0.04	0.03
CRP[a]	0.19***	0.19**	0.18**
IL-6[a]	0.09*	0.14*	0.05
Fibrinogen	0.18***	0.18***	0.20**
TNF-α[a]	0.03	−0.02	0.08
IL-18[a]	0.005	0.04	−0.01
sICAM-1[a]	0.15***	0.08	0.20***
sVCAM-1[a]	−0.02	−0.11	0.05
Vwf	0.07	0.08	0.06
sTM[a]	0.16***	0.08	0.23***
f-TFPI	0.07	0.07	0.06

BMI = body mass index; CRP = C-reactive protein; IL-6 = interleukin-6; TNF-α = tumour necrosis factor-α; IL-18 = interleukin-18; sICAM-1 = soluble intercellular adhesion molecule-1; sVCAM-1 = soluble vascular cell adhesion molecule-1; vWF = von Willebrand factor; sTM = soluble thrombomodulin; f-TFPI = free-tissue factor pathway inhibitor-1.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.
Correlation coefficients are adjusted for case–control status, and additionally adjusted for population in the column 'all'.
[a]Variables were log-transformed for analysis.

the interaction did not reach significance (P for interaction = 0.30 and 0.62 for sICAM-1 and sTM, respectively). Adjustment on CRP did not modify the relation between sCD14 and sICAM-1 or sTM.

Plasma levels of sCD14 and coronary risk

Baseline concentrations of sCD14 were not significantly different between individuals who experienced a coronary event during follow-up and those who did not, either in the whole cohort or when considering each country separately (Table 4). sCD14 concentrations were similar among individuals with hard coronary events and those with angina pectoris (1.53 ± 0.02 *vs* 1.54 ± 0.04 μg/ml; $P=0.96$). Plasma concentrations of sCD14 were higher in Northern Ireland than in France, both in cases and in controls (Table 4).

Table 4 Means (SEM) of plasma sCD14 in cases and controls and RR of coronary event associated with 1 SD increase of sCD14 level

	Controls	*Cases*	*P-value**	*RR (95% CI)*
All	1.53 (0.02)	1.55 (0.03)	0.18	1.15 (0.94–1.40)
France	1.45 (0.03)	1.48 (0.04)	0.14	1.27 (0.93–1.73)
Northern Ireland	1.58 (0.03)	1.59 (0.04)	0.63	1.07 (0.82–1.39)
P-value**	<0.001	<0.001		

Values are in μg/ml. In the line 'all', means and RR are adjusted for population.
**P*-value for difference between cases and controls.
***P*-value for difference between France and Northern Ireland.

Association of CD14 and TLR4 genotypes with conventional cardiovascular risk factors and plasma concentrations of inflammatory and endothelial markers

The two genotypes were not associated with any of the conventional cardiovascular risk factors (hypertension, smoking status, diabetes, body mass index, total cholesterol and HDL cholesterol) (data not shown).

As. shown in Table 5, a weak association between the CD14/C-260 T polymorphism and sCD14 concentrations was observed, the T allele being associated with increased levels in a codominant manner ($P=0.05$ for the association when assuming additive allele effects). The CD14/C-260T polymorphism was also associated with CRP ($P=0.04$), the T allele being associated with decreased levels of this marker. No relation was observed with TNF-α or fibrinogen. The CD14/C-260T polymorphism was associated with endothelial parameters such as sTM ($P=0.003$) and sVCAM-1 ($P=0.003$). After adjustment for CRP, the association between the C-260T polymorphism and sCD14 remained significant. Adjustment for sCD14 did not modify the relation between endothelial markers and the CD14 polymorphism.

No association was observed between the TLR4/Asp299Gly polymorphism and any of the inflammatory or endothelial markers studied (Table 5).

Association of CD14 and TLR4 polymorphisms with coronary risk

The genotype distributions of the CD14 and TLR4 polymorphisms did not deviate from Hardy–Weinberg expectations in any group or population (all $P>0.1$). Genotype and allele frequencies were similar in France and Northern Ireland (Table 6).

Genotype distributions of the CD14 polymorphism did not significantly differ between cases and controls, either in the whole cohort or in each country considered separately (Table 6). When splitting cases according to the type of event (hard CHD event and angina pectoris), no association was observed between either category and genotype (Table 6).

With respect to the TLR4 polymorphism, genotype distributions did not significantly differ between cases and controls in the whole sample (Table 6). However, carriers of the Gly^{299} allele were significantly more represented among cases with a hard CHD event than among their respective controls (16 *vs* 9%, $P=0.04$), whereas no difference was observed between cases with angina pectoris and their controls. The difference observed in the group of hard CHD events was seen only in Northern Ireland (data not shown).

We next explored the combined effect of the two polymorphisms on coronary risk (Figure 1). Since, in the literature, TLR4/Gly^{299} has been shown to be associated with a blunted response of monocytes to LPS[6] and CD14/CC with a lower density of CD14 receptors on monocytes and lower sCD14 plasma levels,[16,17,24] individuals who carry both genotypes might be expected to have a decreased sensitivity to LPS and, hence, to be at lower risk of CHD. However, when we analysed the two polymorphisms in combination in a logistic regression model, neither did we find any evidence for an interaction between them ($P=0.94$ for the interaction) nor did we observe a significant decreased risk in individuals carrying both the Gly^{299} allele and the CC genotype compared to all others (RR = 1.40; $P=0.33$). However, it should be noted that the group of individuals carrying both the Gly^{299} allele and the CC genotype was small ($n=34$) and that the study might lack power to detect such an interaction. When the CD14/C-260T polymorphism was replaced by sCD14 concentrations in the model, the interaction with the TLR4 polymorphism did not reach significance either ($P=0.28$).

Discussion

Several recent studies have suggested that the TLR4/Gly^{299} allele might confer a protective effect against atherosclerosis and its complications. This allele has been associated with a lower risk of carotid atherosclerosis in a population-based survey,[7] a decreased risk of acute coronary events in a case–control study,[8] and a lower incidence of cardiovascular events in the REGRESS study, a prospective cholesterol-lowering trial in subjects with documented coronary artery disease.[9] However, it should be stressed that in the REGRESS study, the protective effect of the Gly^{299} allele was

Table 5 Mean levels of inflammatory and endothelial parameters according to CD14/C-260 T and TLR4/Asp299Gly genotypes

Genotype	*sCD14 (μg/ml)*	*CRP*[a] *(mg/l)*	*IL6*[a] *(pg/ml)*	*IL18*[a] *(pg/ml)*	*TNFα*[a] *(pg/ml)*	*Fibrinogen (mg/ml)*	*sTM*[a] *(ng/ml)*	*sICAM-1*[a] *(ng/ml)*	*sVCAM-1*[a] *(ng/ml)*	*f-TFPI (ng/ml)*	*vWF (IU/ml)*
CD14/C-260T											
CC ($n=157$)	1.49 (0.03)	1.79 (1.54–2.08)	1.61 (1.46–1.79)	207.6 (190.0–226.8)	3.93 (3.68–4.19)	3.54 (0.07)	39.3 (37.3–41.3)	603.1 (580.4–626.8)	764.5 (729.9–800.6)	18.2 (0.42)	1.20 (0.03)
CT ($n=275$)	1.53 (0.03)	1.75 (1.56–1.97)	1.50 (1.39–1.62)	227.0 (212.0–243.1)	3.80 (3.62–3.99)	3.50 (0.05)	38.7 (37.2–40.3)	612.0 (594.6–630.0)	816.1 (788.1–845.1)	18.5 (0.32)	1.24 (0.02)
TT ($n=138$)	1.58 (0.04)	1.40 (1.19–1.63)	1.40 (1.26–1.56)	222.2 (2.02.8–243.4)	4.01 (3.75–4.29)	3.49 (0.07)	43.5 (41.2–46.0)	608.3 (584.9–632.7)	859.4 (819.6–901.1)	18.5 (0.45)	1.21 (0.03)
P-value	0.16	0.04	0.18	0.29	0.44	0.86	0.003	0.84	0.003	0.86	0.44
Homogeneity between populations	0.25	0.09	0.02	0.70	0.48	0.60	0.98	0.78	0.25	0.10	0.04
TLR4/Asp299Gly											
Asp/Asp ($n=502$)	1.54 (0.02)	1.68 (1.54–1.82)	1.50 (1.42–1.59)	219.8 (209.1–231.1)	3.92 (3.78–4.06)	3.51 (0.04)	40.0 (30.8–41.2)	609.1 (596.4–622.1)	818.2 (797.4–839.6)	18.38 (0.24)	1.22 (0.02)
Gly+($n=64$)	1.46 (0.05)	1.60 (1.26–2.03)	1.52 (1.30–1.78)	224.0 (195.1–257.1)	3.65 (3.30–4.04)	3.51 (0.10)	39.5 (36.4–42.9)	611.0 (575.8–648.4)	771.9 (718.1–829.8)	18.97 (0.65)	1.26 (0.05)
P-value	0.10	0.70	0.86	0.80	0.20	0.94	0.78	0.92	0.14	0.39	0.40
Homogeneity between populations	0.60	0.85	0.60	0.56	0.81	0.56	0.46	0.13	0.89	0.68	0.13

sCD14 = soluble CD14; CRP = C-reactive protein; IL-6 = interleukin-6; IL-18 = interleukin-18; TNF-α = tumour necrosis factor-α; sTM = soluble thrombomodulin; sICAM-1 = soluble intercellular adhesion molecule-1; sVCAM-1 = soluble vascular cell adhesion molecule-1; f-TFPI = free-tissue factor pathway inhibitor-1; vWF = von Willebrand factor. Means (SEM) were adjusted for population and case–control status.
[a]For log-transformed variables, geometric means (95% CI) are given.

Table 6 Genotype and allele frequencies of the CD14/C-260 T and TLR4/Asp299Gly polymorphisms in men with and without a CHD event during the follow-up period

	All		*By population*				*By type of events*			
			France		*Northern Ireland*		*Hard CHD*		*Angina pectoris*	
	Controls	*Cases*[a]	*Controls*	*Cases*	*Controls*	*Cases*	*Controls*	*Cases*	*Controls*	*Cases*
	N (%)	*N (%)*	*N (%)*	*N (%)*	*N (%)*	*N (%)*	*N (%)*	*N (%)*	*N (%)*	*N (%)*
CD14/C-260T										
CC	129 (26)	73 (29)	64 (28)	36 (31)	65 (24)	37 (28)	69 (27)	43 (34)	61 (25)	31 (25)
CT	235 (48)	116 (47)	107 (48)	55 (48)	128 (48)	61 (45)	113 (45)	59 (46)	124 (51)	58 (47)
TT	128 (26)	60 (24)	54 (24)	24 (21)	74 (28)	36 (27)	71 (28)	26 (20)	58 (24)	34 (28)
Allele freq (C/T)	0.50/0.50	0.53/0.47	0.52/0.48	0.55/0.45	0.48/0.52	0.50/0.50	0.50/0.50	0.57/0.43	0.51/0.49	0.49/0.51
P-value	0.34		0.40		0.60		0.08		0.70	
RR[b] for genotype CT	0.88 (0.61–1.27)		0.92 (0.55–1.55)		0.84 (0.50–1.42)		0.85 (0.52–1.38)		0.91 (0.52–1.58)	
RR[b] for genotype TT	0.84 (0.56–1.27)		0.79 (0.42–1.47)		0.87 (0.50–1.51)		0.60 (0.33–1.07)		1.14 (0.63–2.05)	
TLR4/Asp299Gly										
Asp/Asp	439 (89)	211 (85)	201 (90)	102 (89)	238 (89)	109 (82)	230 (91)	108 (84)	213 (88)	105 (87)
Asp/Gly	50 (10)	35 (14)	22 (10)	13 (11)	28 (10)	22 (17)	22 (9)	19 (15)	28 (11)	16 (13)
Gly/Gly	1 (1)	1 (1)	0	0	1 (1)	1 (1)	0	1 (1)	1 (1)	0 (0)
Allele freq (Asp/Gly)	0.95/0.05	0.92/0.08	0.95/0.05	0.94/0.06	0.94/0.06	0.91/0.09	0.96/0.04	0.92/0.08	0.94/0.06	0.93/0.07
P-value[c]	0.10		0.67		0.08		0.04		0.78	
RR[b] for genotype Gly+	1.46 (0.93–2.30)		1.17 (0.58–2.37)		1.72 (0.94–3.14)		2.00 (1.03–3.87)		1.09 (0.58–2.08)	

[a]Two cases had both types of events (ie angina pectoris then hard CHD) during follow-up and were included together with their respective matched controls ($n=4$) in both hard CHD and angina pectoris groups.
[b]RR (95% CI) were calculated by reference to the most frequent genotype (CC or Asp/Asp).
[c]Rare homozygotes for the Gly^{299} allele were pooled with heterozygotes for test.

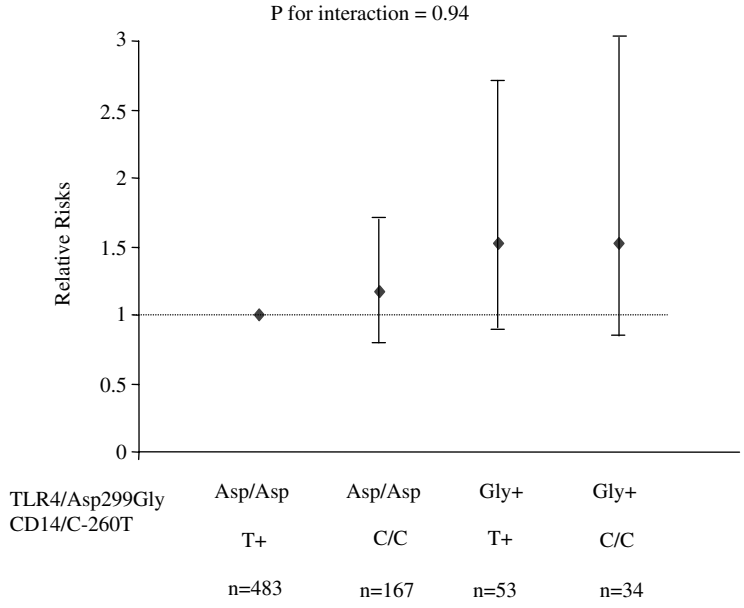

Figure 1 RR (95% CI) for coronary event according to the combination of TLR4/Asp299Gly genotype and CD14/C-260T genotype. The combination of the Asp/Asp and T+ genotypes was taken as the class of reference.

not observed in the entire cohort but was confined to the group treated with pravastatin.[9] In the present study, assessing for the first time the effect of the TLR4/Asp299Gly polymorphism in a prospective cohort of initially healthy subjects, we could not confirm the protective effect of the Gly^{299} allele on coronary risk. The power of the present study for detecting an RR of 0.5 in carriers of the Gly^{299} allele (who represent 10% of the population) was 0.63, so we cannot exclude that the study was underpowered to detect such an effect, and *a fortiori* a weaker effect. However, data from the present study rather suggest that the Gly^{299} allele is associated with an increased - and not decreased - risk of coronary events, a finding at variance with previous results. Since this effect was observed only in the population from Northern Ireland and not in France, and it concerns only hard CHD events (MI or coronary death) and not angina pectoris, it has to be interpreted with caution. Further prospective studies are required to gain more insight into the role of the Gly^{299} allele on cardiovascular risk in various clinical settings.

The TLR4 polymorphism is thought to play a role in atherosclerosis through modulation of expression of proinflammatory cytokines. Carriers of the Gly^{299} allele have been shown to have lower plasma concentrations of IL-6, fibrinogen or sVCAM-1 than individuals with the Asp/Asp genotype.[7,8] In the present study, we evaluated numerous inflammatory and endothelial markers but did not find any relationship with the TLR4 polymorphism.

Besides the TLR4, the endotoxin-sensing complex includes another LPS receptor, CD14. This receptor exists in a soluble form (sCD14) and a membrane-bound form (mCD14). Few data are available on the association between sCD14 and the risk of coronary disease. In a large case–control study, plasma sCD14 levels did not differ between individuals with stable angina pectoris and controls.[24] The present prospective study did not provide evidence for an association of sCD14 with a future coronary event in a cohort of healthy men. Moreover, individuals with hard coronary events during follow-up had plasma sCD14 concentrations similar to those of cases suffering from angina pectoris. Paradoxically, sCD14 concentrations were increased in the high-risk population of Northern Ireland compared to France, as also observed for lipid variables and inflammatory markers (CRP, fibrinogen, IL6, IL18).[31,32,35]

It has been suggested that sCD14 plays a role in innate immunity by promoting the response of endothelial cells that do not express mCD14.[12] This hypothesis is supported in the present study by the correlation observed between plasma concentrations of sCD14 and endothelial markers such as sICAM-1 and sTM. Given the strong relationship between sCD14 and acute-phase reactants, sCD14 might be responsible at least in part for the endothelial dysfunction observed during the inflammatory process. Plasma concentrations of sCD14 have been shown to be increased in individuals with diabetes, independent of CRP levels, suggesting that sCD14 *per se* might play a role in endothelial dysfunction observed in diabetes. A potential functional role of the CD14/C-260T has been suggested as it alters an Sp1 transcription factor binding site and modulates the activity of the promoter, the T allele being associated with higher transcription.[36] Consistent with this

finding, the T allele has been found to be associated with increased plasma sCD14 concentration or CD14 density on monocytes in some[16,17,24] but not all studies.[19,20] The present study confirmed a mildly increasing effect of the T allele on sCD14 levels. This allele was also associated with higher levels of endothelial cell markers such as sTM and sVCAM-1. Conversely, CRP levels were lower in the presence of this allele.

This result is unexpected since, in the presence of the -260T allele, monocytes are predicted to have higher levels of CD14 on their surface and hence to be more sensitive to activation.

In the PRIME Study, the CD14/C-260T polymorphism was not associated with future coronary events. The power of the study for detecting an RR of 1.5 associated with the T allele was 0.95, so it is unlikely to be a problem of lack of power. This lack of association is in accordance with the negative results of the prospective evaluation of the CD14 polymorphism on the risk of future MI in the Physicians' Health Study.[23] Given the potential interaction between TLR4 and CD14 in mediating signal transduction of innate immunity, we hypothesized that the CD14 polymorphism or plasma sCD14 concentrations might modify the relation between TLR4 polymorphism and the risk of coronary event. Since in the literature, the TLR4/Gly^{299} allele is associated with a blunted response by monocytes to LPS[6] and the CD14/CC genotype is associated with a lower density of CD14 receptors on monocytes[17] and lower sCD14 plasma levels,[16,24] individuals who carry both genotypes should be less sensitive to LPS than others, and hence at lower risk of CHD. However, this hypothesis was not supported by the present data.

In conclusion, this prospective study conducted in healthy individuals failed to find any association between TLR4 polymorphism, CD14 polymorphism or plasma sCD14 concentrations and the risk of future coronary events. The importance of both sCD14 and CD14 polymorphism should however not be dismissed, given their impact on the levels of other molecules that have been related to the atherosclerotic process, such as sTM and sVCAM-1.

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Appendix A1. The PRIME Study Group

The PRIME Study is organised under an agreement between INSERM and the Merck, Sharpe and Dohme-Chibret Laboratory, with the following participating Laboratories:

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The Vitamin Research Unit, The University of Bern, Bern, Switzerland (F Gey).

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The Coordinating Center, INSERM U258, Paris-Villejuif, France (P Ducimetière, A Bingham).