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The Asp^{299}Gly Toll-like receptor 4 polymorphism in advanced aortic atherosclerosis

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ABSTRACT

Background: Recently, the common Asp^{299}Gly polymorphism of the Toll-like receptor 4 (TLR-4) was found to be associated with a reduced incidence of acute myocardial infarction and carotid atherosclerosis. As TLR-4 signalling is causally involved in atherogenesis, the polymorphism was postulated to impart protection from atherosclerosis. To explore a potential atheroprotective effect, we studied the association between the Asp^{299}Gly polymorphism and atherosclerosis in hypertensive patients undergoing angiography for suspected renovascular disease.

Methods: 140 hypertensive subjects underwent intra-arterial digital subtraction angiography, during which the presence of atherosclerotic lesions was assessed at the level of the abdominal aorta and renal arteries. Extensiveness of disease was classified as follows: atherosclerosis confined to the abdominal aorta, unilateral renal artery stenosis or bilateral renal artery stenosis. Subsequently, genotyping for the +896 A>G (Asp^{299}Gly) single nucleotide polymorphism was performed in all patients. In statistical analyses 17 patients were excluded because of incomplete data (n=3) or a diagnosis of fibromuscular disease (n=14).

Results: 21 patients were found heterozygous for the ^{299}Gly allele, whereas none of the subjects were ^{299}Gly homozygous (^{299}Gly allele frequency 7.8%). The prevalence of the ^{299}Gly allele in atherosclerotic patients was not different from the prevalence observed in subjects without atherosclerotic lesions (16.9 *vs* 15.5%, p=0.83). Moreover, ^{299}Gly carriership was not associated with the extensiveness of (advanced) aortic atherosclerosis (p=0.64).

Conclusion: Our results suggest that the Asp^{299}Gly TLR-4 receptor polymorphism is not associated with the prevalence nor extensiveness of (advanced) aortic atherosclerosis.

KEYWORDS

Angiography, atherosclerosis, hypertension, Toll-like receptor

INTRODUCTION

Mounting evidence suggests that specific (infectious) agents enhance arterial inflammation during atherogenesis, based on their interaction with receptor signalling pathways of the innate immune system.[1,2] Consequently, Toll-like receptor 4 (TLR-4) induced signalling has been described in chronic low-grade arterial inflammation.[3]

TLR-4 is well known as a pattern-recognition receptor for exogenous lipopolysaccharide (LPS) derived from gram-negative bacterial infection.[4,5] Although several other ligands such as fibrinogen,[6] fibronectin,[7] heat-shock protein,[8] hyaluronan oligosaccharide[9] and minimally modified low-density lipoproteins (LDL)[10,11] have also been described, the exact nature of TLR-4 engaged signalling in atherosclerosis remains elusive.

Although rather speculative, several authors[12,13] have postulated that the advantages of a prominent TLR-4 mediated inflammatory response and subsequent containment of pathogens are outweighed by the unremitted receptor response to endogenously derived epitopes (e.g. oxidised LDL) during atherogenesis. Thus, an attenuated TLR-4 response might confer a potential advantage, as progression of atherosclerosis will decline.

In this context, recent clinical research has described a common Asp^{299}Gly TLR-4 receptor polymorphism associated with a blunted receptor activity and a subsequently diminished inflammatory response.[2,5,14] According to an Asp^{299}Gly based attenuated receptor signalling and a subsequently hypothesised reduced atherogenesis, ultrasound analysis of carotid arteries in the

Bruneck study[14] showed that the Asp[299]Gly polymorphism was found less frequently in patients with progressive carotid lesions, when compared with a control group.

Although a potential Asp[299]Gly mediated protective cardiovascular effect has since been studied extensively, clinical research has focused on acute coronary events,[12,15-22] while data regarding Asp[299]Gly prevalence in peripheral atherosclerosis have remained remarkably scarce.[14,23-25] (See *figure 1* for an overview of published case-control studies).

Yet critical appraisal of clinical reports merely demonstrates a consistent trend towards a reduced frequency of the Asp[299]Gly TLR-4 polymorphism in patients with acute myocardial infarction,[15,18,20] whereas progression of coronary stenosis was found unaffected by genetic TLR-4 variants.[12,21] Moreover, a protective effect based on [299]Gly carriership in early atherosclerosis remained inconclusive.[14,23,24]

Therefore, the present study was conducted to explore the association between the Asp[299]Gly polymorphism and atherosclerosis in hypertensive patients undergoing angiography for suspected renovascular disease. Since renovascular disease is generally considered to be advanced systemic atherosclerosis,[26] we hypothesised a higher frequency of the Asp[299]Gly TLR-4 polymorphism in patients without angiographically demonstrated atherosclerotic lesions in the abdominal aorta and/or renal artery.

MATERIALS AND METHODS

Subjects

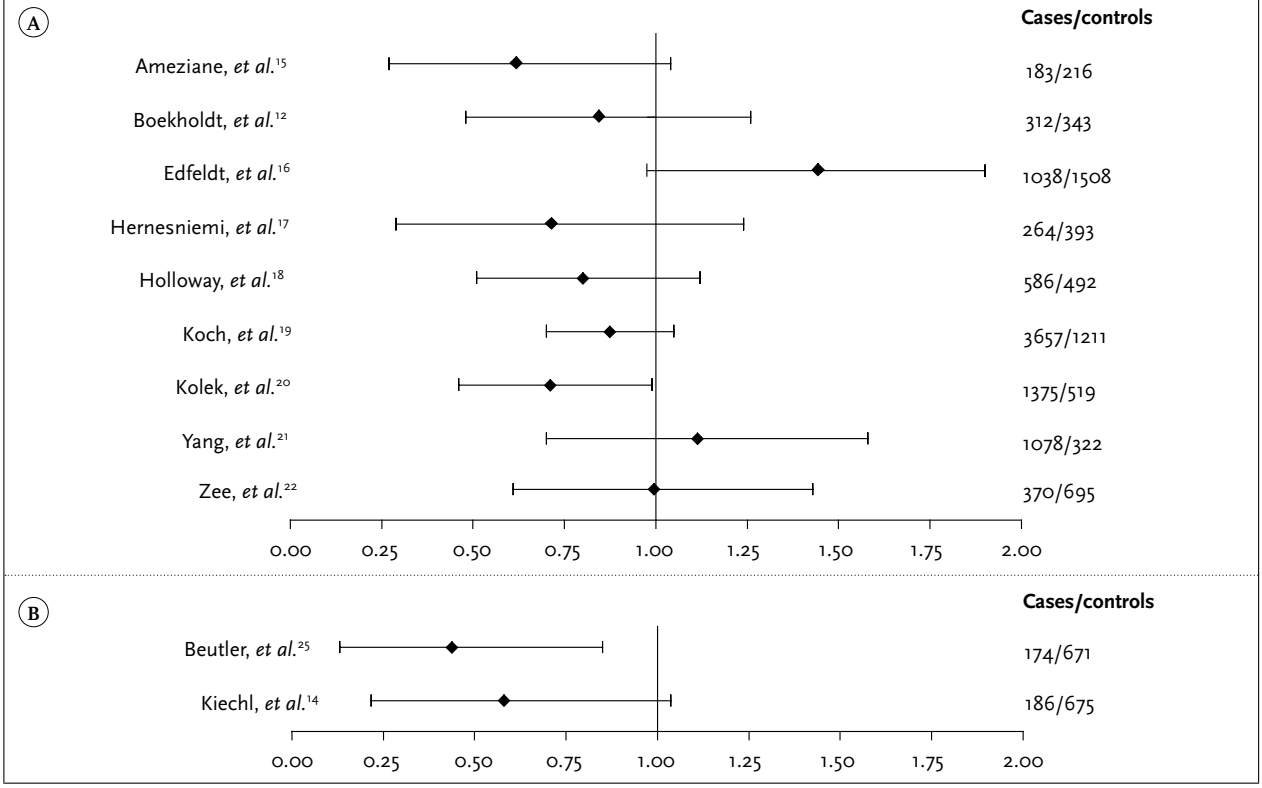
All 140 hypertensive patients included in the present study underwent angiography when one or more of following criteria were present: treatment-resistant hypertension (elevated blood pressure despite ≥3 adequately dosed antihypertensive drugs), >20% increase in serum creatinine concentrations induced by an angiotensin-converting enzyme inhibitor, smoking and diastolic blood pressure >110 mmHg, malignant or accelerated hypertension, extra-renal atherosclerosis in ≥2 different vascular beds.[27-29]

Other causes of secondary hypertension were excluded biochemically before patients underwent angiography of the renal arteries. Written informed consent was obtained from all patients and the Medical Ethical Committee of Maastricht University Hospital approved the study protocol.

Angiography

The angiographic procedure was carried out in the angiosuite of the Department of Radiology in Maastricht University Hospital. Intra-arterial digital subtraction angiography (DSA) was performed with a commercially available digital subtraction system (Integris 5000; Philips Medical Systems;

Figure 1. *Relative risk of acute coronary events (A) and extracoronary atherosclerosis (B) (crude odds ratio and 95% confidence interval) in carriers of the [299]Gly TLR-4 variant allele*



Best, the Netherlands). Angiographic images of the abdominal aorta and renal arteries were obtained in anteroposterior, and left and right oblique views with injection of 30 ml iohexol (Omnipaque 300; Nycomed, Oslo, Norway) through a 4-F Universal Flush catheter (Cordis Europe, Roden, the Netherlands) positioned at the level of the renal arteries.

Radiological evaluation

DSA images were reviewed by two independent radiologists for the presence or absence of atherosclerotic lesions in both renal arteries and the abdominal aorta (celiac truncus up to iliac bifurcation). Subsequently, extensiveness of atherosclerosis was scored as either lesions confined to the abdominal aorta, unilateral renal artery stenosis or bilateral renal artery stenosis. Final results were based on consensus. Patients in whom fibromuscular dysplasia had been diagnosed were excluded from analysis.

Laboratory measurements

Blood samples were drawn after an overnight fast. Plasma cholesterol and glucose were determined using standard methods with commercially available kits. Serum creatinine was measured on the Beckman Coulter Synchron LX-20 system (Beckman Coulter, Inc Fullenton, CA, USA). Creatinine clearance was calculated using the Cockcroft and Gault formula.[30]

Moreover, peripheral blood cells were obtained by standard procedures involving ultracentrifugion and the cell fraction obtained was stored on a phosphate buffered saline or a nucleic acid sequence-based amplification buffer (QIagen, CA, USA) at - 80°C until analysis.

DNA isolation and polymerase chain reaction

DNA isolation from peripheral blood cells was performed using the WIZARD method (Promega, CA, USA), according to the manufacturer's instructions. Amplification was performed in 40 cycles, starting at 94°C for four minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C (30 seconds) and extension at 72°C (30 seconds). The polymerase chain reaction (PCR) was performed in a reaction mixture containing 2 µl $MgCl_2$, 1 µl dNTPs, 0.25 µl Taq, 1 µl forward primer and 1 µl reverse primer (New England Biolabs, MA, USA).

PCR fragments were digested using the NCO_1 enzyme (New England Biolabs, MA, USA) and the digested products were tested on a 2.5% agarose gel stained with ethiumbromide. Restriction fragments were visualised using the Bio-Rad Multi-Analyst ™/PC version 1.1 (BioRad, CA, USA). Two researchers independently scored the genotype in a blinded fashion.

Statistical analysis

All data are represented either as mean and standard deviation, or median and ranges in case of nonparametric distribution. In case of normally distributed data, differences were assessed using a two-sided t-test and Mann-Whitney testing was applied in case of deviation. Dichotomous data were compared using χ^2 statistics. The Hardy-Weinberg equilibrium was tested using standard methods.[31]

To assess the association between TLR-4 genotype and advanced atherosclerosis, subjects were stratified based on the presence/absence of angiographically demonstrated lesions in either aorta or renal arteries. Subsequently, a distinction was made based on the presence of unilateral or bilateral renal artery stenosis (extensiveness of atherosclerosis). To explore potential interactions between Asp^{299}Gly genotype and atherosclerosis logistic regression analysis was adapted.

A two-sided p value <0.05 was considered statistically significant. Analyses were performed with SPSS software (SSPS version 11.0, IL, USA).

RESULTS

Among 140 hypertensive subjects genotyped, 21 patients were heterozygous for the Asp^{299}Gly TLR-4 allele. None of the subjects were ^{299}Gly homozygous. Subsequently, an overall Asp^{299}Gly allele frequency of 7.8% was calculated. Allele frequencies did not deviate from the Hardy-Weinberg expectations (p=0.64).

To determine whether the presence of the Asp^{299}Gly polymorphism decreased susceptibility to and extensiveness of atherosclerotic disease, subsequent statistical analyses were based on 123 patients. Seventeen patients were excluded from analysis because of missing data (n=3) or a diagnosis of fibromuscular dysplasia (n=14). Clinical characteristics of all patients analysed are presented in *table 1*.

Angiographic imaging revealed atherosclerotic lesions in 65 patients (52.8%). In 24 cases lesions were confined to the abdominal aorta, while most patients (n=30) displayed lesions of both aorta and renal artery. In 25 patients bilateral renal artery stenosis as part of advanced atherosclerosis was diagnosed.

When patients were stratified according to the presence of atherosclerotic lesions in either aorta or renal arteries, a 16.9% prevalence of the Asp^{299}Gly mutation in atherosclerotic subjects *vs* 15.5% in subjects without atherosclerotic lesions was found (p=0.83, *table 2*). Moreover, an association between Asp^{299}Gly carriership and the extensiveness of advanced atherosclerosis appeared to be lacking (R=0.89, p=0.64).

Although distribution of several cardiovascular risk factors differed significantly between patients with and those without atherosclerotic lesions (*table 1*), none of these factors showed an interaction with both atherosclerosis and the Asp^{299}Gly genotype.

Table 1. *Patients characteristics*

	All patients (n=123)	Atherosclerosis (n=65)	No atherosclerosis (n=58)	P value
Age (years)	55.0±13	61±11	48±12	0.000*
Gender (male/female)	82/41	52/13	30/28	0.001*
Body mass index (kg/m^2)	27.6±5.0	26.7±5.9	28.7±3.9	0.037*
Current smokers (%)	36.6	43.1	29.3	0.130
Diabetes (%)	9.8	14.1	5.1	0.100
Cholesterol (mmol/l)	5.7	5.5	5.7	0.156
Range	2.6-8.7	2.6-8.7	3.5-8.7	
Glucose (mmol/l)	5.9	6.0	5.6	0.015*
Range	3.4-14.7	3.4-14.7	4.1-10.0	
Serum creatinine (µmol/l)	102.0	116.0	90.5	0.000*
Range	51-404	64-404	51-173	
Creatinine clearance (ml/min)	81.1±33.7	66.7±25.2	97.3±34.9	0.000*

* **Difference is statistically significant.**

Table 2. *Genotype and allele frequencies of the Asp299Gly polymorphism in subjects with and without documented atherosclerosis*

Genotypes	Atherosclerosis n (%)	No atherosclerosis n (%)	Odds ratio	95% CI
Asp/Asp	54 (83.9)	49 (84.4)		
Asp/Gly	11 (16.9)	9 (15.5)	1.1	0.42-2.90
Gly/Gly	0 (0)	0 (0)		
Frequency of the Gly allele	0.085	0.078		

Post hoc analysis showed that exclusion of patients with fibromuscular dysplasia did not influence the main results of this study.

DISCUSSION

Based on the assumption of an attenuated Asp^{299}Gly receptor function and a consequently blunted inflammatory response in atherogenesis, we expected carriers of the ^{299}Gly allele to be less prone to develop atherosclerosis. In contrast to our hypothesis and in spite of the intriguing findings described in previous reports,[14,15,17,18,20,25] the present study obtained no association between the Asp^{299}Gly TLR-4 receptor polymorphism and advanced aortic atherosclerosis.

Although an explanation for these negative findings is not readily apparent, our results might reflect the absence of Asp^{299}Gly homozygosity in our study. In this context, *in vitro* research[1,4,5,32-35] has obtained contradictory results regarding the functional relevance of a heterozygous Asp^{299}Gly TLR-4 mutation, whereas a functional association between impaired receptor function and Asp^{299}Gly homozygosity is rather well established.[1,4,5,35] In keeping with these findings, some clinical studies have described an impaired inflammatory response,[20,36] a lower incidence of coronary plaques[15,17,20] and a reduced progression of carotid atherosclerosis[36] in heterozygous carriers of the ^{299}Gly allele, while other reports could not corroborate these observations.[16,21,22] Given that the present study, like most other associated studies,[12,15,17,18,21] hones in on an attenuated receptor function without exploring *in vivo* cytokine production, the above alluded discrepancies may thus have consequences for the interpretation of our data.

However, considering the complex nature of atherosclerosis and the fact that the totality of the reported data does not unequivocally demonstrate a decreased atherosclerotic burden in carriers of the Asp^{299}Gly polymorphism (*figure 1*), it seems conceivable that Asp^{299}Gly carriership has only a minor impact in atherosclerosis. As a corollary, large epidemiological studies are required in order to address the impact of the Asp^{299}Gly polymorphism in atherosclerosis and hence the setting for genetic association studies, such as the present one, is rapidly disappearing.

Although all patients recruited in the present study underwent angiography based on clinical criteria creating a population with a rather constant prevalence of atherosclerosis, we recognise that there are potential drawbacks to our study which might have yielded false-negative results.

Most conspicuous is the rather small study size and a consequently restricted statistical power to detect minor differences. Exclusion of patients with fibromuscular dysplasia made our study even smaller, but it was imperative in order to reduce potential bias due to the inevitable misclassification of these subjects as not having atherosclerosis. Moreover, exclusion did not affect the main results of this study.

Another caveat might be the recruitment of high-risk patients as controls. Despite the careful characterisation of both cases and controls, a potential prevalence of subclinical atherosclerosis outside the angiographically assessed vasculature cannot be excluded. However, given the fact that we did not observe an association between

the occurrence of evident cardiovascular risk factors and Asp[299]Gly heterozygosity, we expect that a potential distortion due to subclinical atherosclerosis is less likely.

In conclusion, our results suggest that Asp[299]Gly heterozygosity has no effect on the prevalence nor extensiveness of advanced aortic atherosclerosis.

NOTE

Part of this work was presented at the European Society of Hypertension meeting in Madrid in June 2006.

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