

# Complement Factor H Polymorphism, Inflammatory Mediators, and Retinal Vessel Diameters: The Rotterdam Study

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**PURPOSE.** Retinal venular dilatation is associated with systemic inflammation. The hypothesis for the current study was that larger retinal venular diameters are related to the His allele of the Tyr402His polymorphism in the complement factor H (CFH) gene, a major inhibitor of the complement pathway. Possible effect modification by smoking and inflammatory markers was examined.

**METHODS.** This cross-sectional study was performed within the Rotterdam Study, a population-based study among elderly persons aged 55 years and older. The Tyr402His polymorphism of the CFH gene was genotyped in 5066 participants and retinal arteriolar and venular diameters were graded on digitized fundus transparencies.

**RESULTS.** Genotype frequencies were 41% in TyrTyr, 45% in TyrHis, and 14% in HisHis carriers. The His<sup>402</sup> allele was associated with smaller rather than larger venular diameters (age- and sex-adjusted means and standard errors [in micrometers]

were  $222.5 \pm 0.45$  for TyrTyr,  $221.9 \pm 0.43$  for TyrHis, and  $220.6 \pm 0.78$  for HisHis carriers;  $P$ -trend = 0.03). This association was apparent only in never-smokers and was not modified by the inflammatory markers erythrocyte sedimentation rate, leukocyte count, C-reactive protein, or fibrinogen. Adjustment for cardiovascular risk factors did not change results. No associations were found with arteriolar diameters.

**CONCLUSIONS.** The findings do not support the hypothesis that the His<sup>402</sup> allele is related to larger retinal venular diameters. The association with smaller retinal venular diameters most likely is a chance finding, because it was present only among never-smokers and was not modified by inflammatory mediators of complement. These results suggest that the Tyr402His variant is not related to retinal venular diameters. (*Invest Ophthalmol Vis Sci.* 2007;48:3014–3018) DOI:10.1167/iovs.06-1460

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Retinal vessels can be visualized noninvasively and are used to assess systemic vascular damage.<sup>1</sup> In particular, retinal vessel diameters may reflect the condition of intracerebral vessels, and their investigation may help to understand the etiology of cerebrovascular disease.<sup>2</sup> Larger retinal venular diameters are associated with atherosclerosis, cholesterol levels, and markers of inflammation,<sup>3–5</sup> and with an increased risk of stroke and progression of cerebral small vessel disease.<sup>6,7</sup> These associations suggest that inflammation may be involved in the etiology of cerebrovascular disease. In addition, recent findings in a twin study and the Beaver Dam Eye Study (BDES) suggest that retinal arteriolar and venular diameters are at least in part genetically determined.<sup>8–10</sup> Moreover, the association between genetic factors and retinal vessel diameters in the BDES has been reported to be independent of hypertension,<sup>10</sup> suggesting that genetically determined processes other than hypertension may play a role in retinal vessel caliber.

Inflammation initiates and promotes atherosclerosis.<sup>11</sup> Complement plays a role in the promotion of inflammation, as complement and complement regulatory factors are deposited in atherosclerotic plaques.<sup>12</sup> Complement factor H (CFH) is an essential plasma protein in the regulation of the complement pathway and may be important in the inhibition of complement early in the process of atherosclerosis.<sup>13</sup> Recently, the His allele of the Tyr402His polymorphism (rs1061170) in the CFH gene (1q32) has been found to increase the susceptibility of aging macula disorder.<sup>14–19</sup> Because atherosclerosis has been implicated in the etiology of aging macula disorder, this association may at least in part reflect atherosclerosis.<sup>20</sup> The observation that the His<sup>402</sup> allele of the Tyr402His polymorphism was also associated with an increased risk of myocardial infarction<sup>21</sup> provides further support for this hypothesis.

A role for the Tyr402His variant in the CFH gene in atherosclerosis is biologically plausible, given its location within a

region of positively charged amino acids that are implicated in the binding of heparin and C-reactive protein.<sup>22</sup> Normally, the ability of CFH to downregulate the effects of complement is facilitated by binding to these molecules. The substitution of a positively charged histidine for a noncharged hydrophobic tyrosine at position 402 alters CFH binding properties to heparin and C-reactive protein.<sup>23</sup> As a result, heterozygous and, in particular, homozygous carriers have malfunctioning CFH, ultimately leading to increased complement-related damage to the vascular endothelium,<sup>18</sup> especially in the presence of acute and chronic inflammatory mediators of the complement pathway.<sup>19,21</sup>

We further analyzed the influence of inflammation on the retinal vasculature by examining associations between the Tyr402His polymorphism and retinal arteriolar or venular diameters, including effect modification by smoking and inflammatory mediators. We hypothesized that carriers of the His<sup>402</sup> allele had larger retinal vessel diameters, in particular the venular ones.

## METHODS

### Study Population

The Rotterdam Study is a large, population-based, prospective cohort study conducted among 7983 elderly persons 55 years of age or older residing in a suburb of Rotterdam, The Netherlands, to assess incidence and determinants of chronic diseases in the elderly.<sup>24</sup> In 1990 to 1993, trained research assistants visited all participants at home and obtained information on sociodemographic characteristics, medical history, current health status, medication use, and determinants for these diseases. In addition, the participants were invited to the research center for a clinical examination by the research physicians. Because eye examinations became operational a few months after the baseline examinations had started, a smaller number ( $n = 6780$ ; response rate 78%) participated in the ophthalmic part of the study.<sup>25</sup> Fundus transparencies were available in 6436 participants and of these, 762 participants were excluded because they had ungradable fundus transparencies on both eyes. DNA was available from 5291 of the remaining 5674 participants. Genotyping failed in 225, leaving 5066 persons as the study sample for this cross-sectional study. The study was conducted according to the tenets of the Declaration of Helsinki, and the Medical Ethics Committee of the Erasmus University approved the study protocol. Written informed consent was obtained from all participants.

### Retinal Vessel Measurements

Fundus transparencies were obtained that were centered on the optic disc (20° field; Topcon Optical Company, Tokyo, Japan) after pharmacological mydriasis<sup>26</sup> and were digitized with a high-resolution scanner (LS-4000; Nikon Corp., Tokyo, Japan). For each participant the digitized image with the best quality of either eye was analyzed with a retinal vessel measurement system (Retinal Analysis; Optimate, Madison, WI; Department of Ophthalmology & Visual Science, University of Wisconsin-Madison, WI).<sup>1,3</sup> Within one-half to one disc diameter from the optic disc, all retinal arteriolar and venular diameters were measured to calculate summary values of the central retinal arteriolar and venular diameters. Four trained graders performed the assessments, masked to the clinical characteristics of the participants. Pearson's correlation coefficients for intergrader agreement were 0.67 to 0.80 for arteriolar diameters and 0.91 to 0.94 for venular diameters. For intra-grader agreement, these figures were 0.69 to 0.88 (arteriolar diameters) and 0.90 to 0.95 (venular diameters).

### Genotyping

Participants were genotyped for the Tyr402His (1277T→C) (rs1061170) polymorphism of the *CFH* gene. Genotypes were deter-

mined in 2 ng genomic DNA with an allelic discrimination assay (*TaqMan*; Applied Biosystems, Inc. [ABI], Foster City, CA). Primer and probe sequences were optimized by using the single-nucleotide polymorphism (SNP) assay-by-design service of ABI. Reactions were performed (*TaqMan* Prism 7900HT 384-well format; ABI), as described by Fang et al.<sup>27</sup>

### Covariates

Smoking status (categorized as current, former, and never smoking) and medication use were assessed at the baseline interview. At the research center, blood was drawn directly into tubes (Vacutainer; BD Biosciences, Franklin Lakes, NJ), and the erythrocyte sedimentation rate was read after 60 minutes. The leukocyte count was assessed in citrate plasma (Coulter Counter T540; Coulter Electronics, Luton, UK). High-sensitivity C-reactive protein was measured in serum samples kept frozen at -20°C, using a rate near-infrared particle immunoassay method (Immagine high-rate C-reactive protein [CRP] assay; Beckman Coulter, Fullerton, CA). Fibrinogen was measured in platelet-poor plasma kept frozen in liquid nitrogen and stored at -80°C. Fibrinogen levels were derived from the clotting curve of the prothrombin time assay by using thromborel S as a reagent on an automated coagulation laboratory (ACL 300; Instrumentation Laboratory, Lexington, MA).

For the blood pressure measurement, we took the average of two measurements in sitting position with random zero sphygmomanometry at the brachial artery. Hypertension was defined as a systolic blood pressure  $\geq 160$  mm Hg, a diastolic blood pressure  $\geq 95$  mm Hg, or the use of blood pressure-lowering medication. An ultrasound carotid artery atherosclerotic plaque score (range: 0-6) reflected the number of locations with plaques at the bifurcation, common, and internal carotid artery on both sides.<sup>28</sup> Nonfasting serum total and high-density lipoprotein (HDL) cholesterol concentrations were determined by an automated enzymatic procedure.<sup>29</sup> The body mass index (BMI) was calculated by dividing body weight (kg) by height squared (m<sup>2</sup>). Diabetes mellitus was considered present if participants reported use of antidiabetic medication or when the random or postload serum glucose level was greater than 11.1 mM.

### Data Analysis

Hardy-Weinberg equilibrium of the Tyr402His polymorphism was tested with a  $\chi^2$  test. We used analysis of covariance to compute age- and sex-adjusted mean vessel diameters within genotype. To examine effect modification by complement activators, we subsequently performed separate analyses stratified on smoking status, and the inflammatory markers erythrocyte sedimentation rate, leukocyte count, serum C-reactive protein, and fibrinogen. For smoking, participants were categorized as never, former, and current smokers. For the inflammation markers, the median value was used to divide participants into two subgroups: one below and one above the median value of the variable. These analyses were also adjusted for blood pressure, carotid artery plaque score, serum total and HDL cholesterol, BMI, and diabetes mellitus. We next performed additional analyses stratifying on hypertensive status, diabetes, carotid artery plaque score, and BMI. For hypertension and diabetes, participants were grouped into those with hypertension or diabetes and those without. For the carotid artery plaque score and BMI the median value was used to divide participants into two subgroups: one below and one above the median value of the variable. All analyses were performed with commercial software (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL).

## RESULTS

Genotype distributions were in Hardy-Weinberg equilibrium. The overall frequency of the His allele was 36%, and genotype frequencies were 41% in TyrTyr, 45% in TyrHis, and 14% in HisHis carriers. Baseline characteristics for the 5066 participants in this study are shown in Table 1.

TABLE 1. Baseline Characteristics of the Study Population

	Total (n = 5066)
Age (y)	67.9 (8.1)
Sex (% female)	58.2
Systolic blood pressure (mm Hg)	138.4 (21.9)
Diastolic blood pressure (mm Hg)	73.6 (11.2)
Body mass index (kg/m <sup>2</sup> )	26.3 (0.4)
Total cholesterol (mM)	6.6 (1.2)
HDL cholesterol (mM)	1.3 (0.4)
Diabetes mellitus (%)	9.5
Smokers (%)	
Never	33.2
Current	23.6
Former	43.2
Erythrocyte sedimentation rate (mm/h)	12.9 (10.7)
Leukocyte count (10 <sup>9</sup> /L)	6.7 (1.9)
Serum Fibrinogen (g/L)	2.8 (0.7)
Serum C-reactive protein (mg/L)*	1.79 (0.87-1.79)

Data are the mean (SD) or percentages.

\* Median (interquartile range) are presented because of the skewed distribution.

Table 2 shows the association of the Tyr402His polymorphism with retinal vessel diameters, both in all participants and after stratification on complement activators or cardiovascular risk factors. The His<sup>402</sup> allele was not associated with arteriolar diameters, whereas it was associated with smaller venular diameters in an allele-dose-dependent manner. Stratification on the participants' smoking status showed that this association

was present among never-smokers, but was absent in current or former smokers, although the interaction was statistically nonsignificant (*P* for interaction = 0.15). No effect modification was observed when we stratified on serum inflammatory markers (*P* for interaction all >0.65). Additional adjustment for cardiovascular risk factors did not change the results. When stratified on cardiovascular risk factors, the association of the His<sup>402</sup> allele with larger venular diameters was stronger among those without hypertension or diabetes, but the interaction terms were statistically nonsignificant (*P* for interaction = 0.62 and 0.93, respectively). Also, no effect modification was observed when the association was stratified on the carotid artery plaque score or BMI (*P* for interaction = 0.52 and 0.32, respectively).

## DISCUSSION

Carriers of the His<sup>402</sup> allele had smaller retinal venular diameters in an allele-dose-dependent manner. This association was present only in never-smokers and was not modified by inflammatory mediators of complement or adjustment or stratification for established cardiovascular risk factors. Retinal arteriolar diameters were not related to the Tyr402His variant. Thus, we could not support our hypothesis that larger retinal venular diameters are associated with the His<sup>402</sup> allele of the Tyr402His polymorphism.

Strengths of this study are the population-based design, the detailed assessment of vessel diameters on 20° stereoscopic transparencies leading to larger magnified images, and the adjustment for refractive errors of the eye. Our method en-

TABLE 2. The Tyr402His Variant in the *CFH* Gene and the Association with Mean Summarized Retinal Arteriolar and Venular Diameters, Stratified According to the Parameters Shown

	Estimated Central Retinal Arteriolar Diameter				Estimated Central Retinal Venular Diameter			
	TyrTyr	TyrHis	HisHis	<i>P</i> -trend	TyrTyr	TyrHis	HisHis	<i>P</i> -trend
<i>n</i> (%)	2088 (41)	2279 (45)	699 (14)		2088 (41)	2279 (45)	699 (14)	
All	146.8 (0.31)	147.0 (0.30)	146.8 (0.54)	0.94	222.5 (0.44)	222.0 (0.43)	220.5 (0.77)	0.03
Smoking								
Never	145.1 (0.54)	145.7 (0.51)	145.0 (0.93)	0.80	218.2 (0.77)	217.1 (0.72)	213.8 (1.33)	0.01
Former	146.5 (0.48)	146.2 (0.47)	146.8 (0.85)	0.99	222.1 (0.67)	221.8 (0.65)	221.3 (1.18)	0.53
Current	150.1 (0.66)	150.8 (0.61)	149.7 (1.09)	0.97	229.8 (0.95)	229.5 (0.88)	228.7 (1.57)	0.57
Sedimentation rate								
≤10 mm/h	148.3 (0.51)	147.4 (0.49)	148.9 (0.89)	0.98	225.3 (0.72)	222.3 (0.69)	224.3 (1.26)	0.08
>10 mm/h	147.4 (0.57)	148.3 (0.52)	146.5 (0.93)	0.79	222.2 (0.78)	222.6 (0.72)	218.5 (1.28)	0.06
Leukocyte count								
≤10 (10 <sup>9</sup> /L)	146.7 (0.46)	146.2 (0.44)	146.2 (0.82)	0.43	220.6 (0.64)	219.3 (0.61)	218.0 (1.14)	0.03
>10 (10 <sup>9</sup> /L)	147.6 (0.46)	148.0 (0.43)	147.6 (0.76)	0.79	224.8 (0.65)	224.3 (0.62)	222.6 (1.09)	0.11
C-reactive protein								
≤1.78 mg/L	147.0 (0.46)	146.9 (0.44)	147.0 (0.80)	0.98	221.5 (0.66)	220.7 (0.63)	219.1 (1.13)	0.08
>1.78 mg/L	147.0 (0.46)	147.0 (0.44)	145.9 (0.80)	0.31	223.5 (0.65)	223.0 (0.62)	221.4 (1.13)	0.14
Fibrinogen								
≤2.6 g/L	146.0 (0.65)	146.7 (0.62)	147.3 (1.14)	0.27	223.1 (0.95)	220.3 (0.91)	221.5 (1.66)	0.13
>2.6 g/L	146.9 (0.67)	146.4 (0.62)	148.6 (1.15)	0.43	222.6 (0.99)	221.8 (0.92)	220.0 (1.70)	0.19
Hypertension								
No	148.3 (0.42)	148.9 (0.40)	147.6 (0.40)	0.68	223.6 (0.60)	223.4 (0.56)	220.8 (1.01)	0.05
Yes	143.8 (0.65)	143.6 (0.63)	143.9 (1.11)	0.99	220.4 (0.93)	220.2 (0.91)	219.0 (1.59)	0.53
Diabetes mellitus								
No	146.7 (0.33)	147.0 (0.32)	146.7 (0.57)	0.90	222.6 (0.47)	221.9 (0.45)	220.6 (0.81)	0.04
Yes	147.8 (0.94)	147.9 (0.86)	147.6 (1.61)	0.95	221.8 (1.50)	223.2 (1.37)	219.4 (2.55)	0.70
Carotid plaque score								
≤1	148.0 (0.46)	148.0 (0.42)	147.8 (0.79)	0.82	223.4 (0.64)	222.3 (0.58)	220.9 (1.08)	0.04
>1	146.3 (0.54)	146.4 (0.53)	145.7 (0.94)	0.68	223.1 (0.77)	221.9 (0.76)	219.6 (1.35)	0.03
Body mass index								
≤26.0 kg/m <sup>2</sup>	147.4 (0.46)	147.6 (0.43)	147.5 (0.78)	0.87	222.7 (0.65)	222.0 (0.61)	220.5 (1.11)	0.10
>26.0 kg/m <sup>2</sup>	146.3 (0.43)	146.4 (0.42)	145.9 (0.75)	0.78	222.3 (0.61)	222.1 (0.60)	220.4 (1.07)	0.19

Values are age and sex adjusted means (standard errors).

abled us to estimate the intraluminal arteriolar and venular diameters more in detail, where others reported uncorrected vessel diameters in pictures with smaller magnification.<sup>1,30,31</sup> Another advantage is the use of a genetic marker. This approach deals with reverse causation, as genotype is determined before disease onset.<sup>32</sup> Limitations related to the semi-automated system assessing the retinal vessel diameters have been described.<sup>1</sup> For instance, photographs were taken independent of the cardiac cycle and effects of pulsatility on vessel width cannot be ruled out. Most likely, these limitations led to an underestimation of our effects due to random misclassification because photography and assessment of vessel diameters were unrelated to clinical characteristics of the participants. Allele frequencies were in correspondence with previous reports from the Rotterdam Study.<sup>19,21</sup> We therefore consider selection bias due to selective nonparticipation of HisHis carriers unlikely.

CFH is an important regulator of the complement pathway. Activation of this pathway initiates a proteolytic cascade that releases proinflammatory anaphylatoxins and causes formation of a membrane-attack complex, ultimately leading to cell lysis. CFH is a potent inhibitor of the complement pathway by binding and inactivating complement component C3b. This prevents the production of C3 convertase in the alternative cascade and production of C5 convertase in the common pathway. As a result, CFH interferes with progression of the entire cascade.<sup>23</sup> A malfunctioning CFH may lead to less inactivation of the complement component C3b and as a result of the entire complement cascade, presumably by altered binding to C-reactive protein and heparin. Heterozygous and, in particular, homozygous carriers of the His<sup>402</sup> variant are genetically predisposed to malfunctioning CFH. This disorder seems of importance in particular when the complement cascade is switched on by acute and chronic inflammatory mediators including C-reactive protein, leukocyte count, white blood cell count, fibrinogen, and smoking.<sup>19,21</sup>

Because higher levels of the above mentioned inflammation markers and smoking are all associated with larger retinal vessel caliber and, in particular, larger venular diameters,<sup>3-5</sup> we hypothesized that carriers of the His<sup>402</sup> allele would have larger venular diameters, especially in the presence of inflammatory mediators. In contrast, we found an association of the His<sup>402</sup> allele with smaller venular diameters than in carriers of the Tyr<sup>402</sup> allele. This association was not modified by inflammatory mediators and was present in never rather than current smokers. Given that only 222 participants among the never-smokers were carriers of the HisHis allele, we consider this result most likely to be a chance finding due to the relatively low number of subjects.

Alternative explanations should be discussed. First, the His<sup>402</sup> allele could be related to smaller venular diameters through mechanisms other than inflammation. The Tyr<sup>402</sup>His variant is located within a cluster of positively charged amino acids that is implicated in the binding of CFH, not only to C-reactive protein but also to heparin,<sup>22</sup> which normally increases the affinity of CFH for cell-surface-bound C3b.<sup>23</sup> Carriers of the His<sup>402</sup> allele may have a less-effective binding to heparin. The association of larger retinal venular diameters with inflammation has been ascribed to damage to the endothelial surface layer caused by oxidized low-density lipoproteins and activated leukocytes.<sup>3</sup> Heparin attenuates leukocyte and low-density lipoprotein-related damage to the venular endothelial surface.<sup>33</sup> If less effective binding of CFH to heparin leads to higher plasma levels of heparin, it could indirectly have led to the observed decrease in venular diameter. However, the potential influence of the Tyr<sup>402</sup>His polymorphism on CFH function and interaction with heparin is currently unknown.<sup>23</sup> Also, we cannot explain why this mechanism

would be of importance among never-smokers only. Second, it must be determined whether the Tyr<sup>402</sup>His polymorphism is the variant that actually underlies the association with venular diameters, rather than merely reflecting another marker within the CFH gene in complete or partial linkage disequilibrium.

In conclusion, our findings do not support the hypothesis that larger retinal venular diameters are related to the His<sup>402</sup> allele of the Tyr<sup>402</sup>His polymorphism in the CFH gene. The association of the His<sup>402</sup> allele with smaller retinal venular diameters is most likely a chance finding, because it was present only among never-smokers and was not modified by inflammatory mediators of complement. Taken together, our findings suggest that the Tyr<sup>402</sup>His variant is not related to retinal vessel diameters.

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