

we did have some individuals falling within that range (Table 1). Sacerdoti et al. (7) studied eight healthy individuals with 20-HETE expressed as the excretion rate (1.6 ng/min). It is difficult to compare these data with our own because these individuals were receiving an infusion of aminohippurate in 50 g/L dextrose in water at 1.5 mL/min for 5 h to determine renal plasma flow.

Information on the physiologic effects of 20-HETE in humans is limited. Sacerdoti et al. (7) showed that the rate of 20-HETE excretion was increased in individuals with hepatic cirrhosis. Laffer et al. (5) recently showed a positive correlation between diastolic BP and 20-HETE excretion rate in a group of 13 salt-sensitive hypertensive individuals. In some rat models of hypertension, high BP has been associated with increased 20-HETE production (2), but this is yet to be confirmed in human studies.

In conclusion, the sample preparation procedure using solid-phase cartridge extraction and HPLC purification would lend itself to automation and enable the convenient analysis of 20-HETE excretion in large human studies. Such studies could examine in more detail the potential role of this vasoactive CYP450 metabolite in vascular function and human hypertension.

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Rapid Genotyping for Tumor Necrosis Factor- α (TNF- α) –863C/A Promoter Polymorphism That Determines TNF- α Response, Michael Heesen,^{1*} Dagmar Kunz,² Martina Wessiepe,³ Tom van der Poll,⁴ Aeilko H. Zwinderman,⁵ and Brunhilde Blomeke⁶ (Departments of ¹Anesthesia, ⁴Experimental Internal Medicine, and ⁵Clinical Epidemiology and Biostatistics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; Departments of ²Clinical Chemistry and Pathobiochemistry, ³Transfusion Medicine, and ⁶Dermatology, University Hospital, Aachen, Germany; *address correspondence to this author at: Department of Anesthesia, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; fax 31-20-6979441, e-mail m.heesen@amc.uva.nl)

Tumor necrosis factor- α (TNF- α) plays a central role in orchestrating the inflammatory response (1). Accordingly, blocking TNF- α activity has become a standard treatment of several inflammatory diseases (2, 3). TNF- α production shows high interindividual variations, which have been assigned mainly to inherited factors (4). Several genetic polymorphisms related to TNF- α synthesis have been detected in the TNF gene (5, 6). The –308 promoter polymorphism was found to affect TNF- α production by some authors (7) but not by others (8). Similar inconsistencies have been found for the association of this polymorphic site with susceptibility to and/or outcome of sepsis (8, 9). The *NcoI* polymorphism located within the first intron of the lymphotoxin A (LTA) gene was reported to be associated with TNF- α plasma concentrations (8). In a recent report, de Jong et al. (10) found no relationship between ex vivo TNF- α production on endotoxin stimulation of human whole blood and +489, –238, and –376 single-nucleotide polymorphisms or TNF α microsatellites of the TNF- α gene (10). Thus, the genetic factors determining the TNF- α response to infection are still poorly defined.

Recently, Skoog et al. (11) identified a C/A exchange at position –863 of the TNF- α gene promoter and found higher transcriptional activity of the C allele in reporter gene assays. This polymorphic site was found to be associated with thyroid-associated ophthalmopathy (12), Crohn disease (13), juvenile rheumatoid arthritis (14), and the lumbar spine area (15).

In the present study we sought to determine the association of the –863 TNF- α promoter polymorphism with the TNF- α production capacity of human blood cells. We describe a new real-time PCR assay with specific fluorescently labeled hybridization probes for genotyping for this polymorphism. We also genotyped samples for other polymorphic sites implicated in TNF- α production capacity and evaluated the possible existence of linkage disequilibria with the –863 polymorphism under study.

Unrelated, nonsmoking, male blood donors of Caucasian origin (age range, 18–65 years) were included. The period of inclusion was 2 weeks. Exclusion criteria included acute or chronic diseases or any medication within 3 weeks before being enrolled into this study. This study

was approved by the institutional ethics committees. Written informed consent was obtained from all study participants.

Genotyping for the TNF- α -308 and the LTA *NcoI* genetic polymorphisms was carried out as described previously (16). Because a previously described assay for genotyping for the TNF- α -863 polymorphism (11) is time-consuming and requires several manual steps, we developed a real-time PCR assay with specific fluorescently labeled hybridization probes. The thermocycler was a LightCyclerTM instrument (Roche Diagnostics). Genotyping of the TNF- α -863 promoter polymorphism was performed as follows: 0.5 μ M each of the primers (sense, 5'-CCTCTGGGGAGATGTGACCA-3'; antisense, 5'-AGGTCCTGGAGGCTCTTTCAC-3') and 0.2 μ M each of the detection probe specific for the A allele (5'-LC Red640-ACCCCACTTAACGAAGACAG-3') and the anchor probe (5'-CAGGGCTATGAAAGTCGAGTATGGG-3') were used. The 10- μ L PCR mixture contained 1 μ L of reaction buffer. A total of 50 PCR cycles were run, with 1 cycle consisting of denaturation at 95 °C for 10 s, annealing at 58 °C for 5 s, and extension at 72 °C for 10 s. A sample was classified as TNF -863 genotype CC or CA according to the derivative melting curves.

We confirmed the accuracy of this method by sequencing samples of all TNF- α -863 genotypes. Sequencing was performed with the Big Dye terminator technology on an ABI 3700 DNA sequencer (PE Applied Biosystems). We found no sequence differences with the two methods.

Heparin-anticoagulated venous blood was diluted 1:1 (by volume) with RPMI 1640 (Gibco BRL) and stimulated with 100 μ g/L endotoxin (*Escherichia coli* O2:B22; Sigma) for 4 h at 37 °C, as described previously (10). TNF- α concentrations (ng/L) were measured with an automated immunometric chemiluminescence assay (ImmuliteTM; DPC Biermann GmbH). The obtained CV was 6.0% at a concentration of 522 ng/L.

Monocyte CD14 expression was assessed as described previously (17). CD14 expression was analyzed by use of phycoerythrin-labeled anti-CD14 antibody (M ϕ P9; Becton Dickinson). Flow cytometry was performed with a FACSCaliburTM (Becton Dickinson).

We tested for the Hardy-Weinberg equilibrium of the polymorphisms by comparing the expected with the observed genotype frequencies using a 2 \times 3 table χ^2 test. We log-transformed the TNF- α concentrations for further calculations and tested for linkage disequilibrium between the three polymorphic sites using the likelihood ratio test (χ^2 test of 2 \times the difference in log likelihood with 1 degree of freedom) as described by Slatkin and Excoffier (18). This test uses the expectation-maximization algorithm to resolve double heterozygotes into haplotypes and then applies a likelihood ratio test to determine whether the resolutions of haplotypes are significantly nonrandom, which is equivalent to testing whether there is statistically significant linkage disequilibrium between loci (18). To assess the influence of the various TNF polymorphisms on TNF- α synthesis, we performed a multivariate regression analysis after correct-

ing for CD14 expression and monocyte count. Both procedures are incorporated in the Arlequin 2.000 software package (Arlequin), which was used for our analysis. Values are given as adjusted mean (SD). *P* values <0.05 were considered as statistically significant.

We genotyped 118 individuals for the TNF- α -863 polymorphism. Genotyping data for the TNF- α -308 and the LTA *NcoI* polymorphisms were obtained for 81 individuals (see the table in the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol50/issue1/>). Statistical analysis did not reveal a difference between the frequencies of the observed genotypes and those of the expected genotypes, indicating that the study population was in Hardy-Weinberg equilibrium for all three polymorphisms.

There was no linkage disequilibrium between the TNF- α -308 and the TNF- α -863 (*P* = 1.0) or between the LTA *NcoI* and the TNF- α -863 polymorphisms (*P* = 1.0). Statistical analysis found a linkage disequilibrium between the TNF- α -308 and the LTA *NcoI* polymorphisms (*P* <0.001). The genotype distribution is given in Table 1.

Multiple regression analysis of all three markers after adjustment for CD14 and monocytes showed that only the -863 marker was significantly associated with ln(TNF) values; the regression weights were 0.46 (SE = 0.22; *P* = 0.04) for the -863 marker, 0.02 (SE = 0.23; *P* = 1.0) for the -308 marker, and 0.06 (SE = 0.22; *P* = 0.8) for the LTA *NcoI* marker. None of the two-way interactions was statistically significant, nor was the three-way interaction (*P* >0.37). Fig. 1 in the online Data Supplement shows the TNF- α concentrations for the 81 individuals genotyped for TNF- α -863, corrected for CD14 expression (10³ abc) and monocyte count (10³ cells/ μ L). Individuals homozygous for the C allele had significantly higher TNF- α concentrations than the other genotypes. The percentage differences of corrected TNF- α concentrations were 39% (95% confidence interval, 8–60%; *P* = 0.02) between TNF- α -863 CC and CA/AA carriers and 19% (15–66%;

Table 1. Genotype frequencies of the TNF- α -863, TNF- α -308, and LTA *NcoI* polymorphisms.^a

Polymorphism	Genotype	Observed frequency, n (%)	Expected frequency, n (%)	<i>P</i>
TNF- α -863 (n = 118)	AA	5 (4.2)	2 (1.6)	0.20
	CA	20 (16.9)	26 (22.2)	
	CC	93 (78.8)	90 (76.2)	
TNF- α -308 (n = 81)	AA	2 (2.5)	3 (3.7)	0.42
	AG	28 (34.6)	26 (32.3)	
	GG	51 (62.9)	52 (64.0)	
LTA <i>NcoI</i> (n = 81)	B1B1	5 (6.2)	8 (9.6)	0.16
	B1B2	40 (49.4)	35 (42.8)	
	B2B2	36 (44.4)	38 (47.6)	

^a Observed and expected frequencies were compared to test for presence of Hardy-Weinberg equilibrium.

$P = 0.31$) between TNF- α -308 GG and GA/AA carriers. The percentage difference of corrected TNF- α concentrations between the B2B2 and the B2B1+B1B1 genotypes of the LTA *NcoI* marker was 19% (15–66%).

The mean (SD) monocyte CD14 density of TNF- α -863 CC carriers [$57.0 (20.4) \times 10^3$ abc] and carriers of an A allele [$56.5 (19.7) \times 10^3$ abc] did not differ significantly ($P = 1.0$). The difference in monocyte counts was $0.46 (0.14) \times 10^3$ cells/ μL for C homozygotes vs $0.39 (0.13) \times 10^3$ cells/ μL for CA/AA carriers ($P = 0.11$).

The allele frequencies found in our study for the three TNF polymorphisms were in agreement with those reported in previous studies (11, 16). We also demonstrated that the -863 C/A polymorphism is associated with the TNF- α response of human whole blood of healthy volunteers to endotoxin stimulation. In particular, C homozygotes had significantly higher TNF- α values. Although they are located on the same chromosome, we found no linkage disequilibria between the TNF- α -863 and the TNF- α -308 or the LTA *NcoI* sites. We observed a linkage disequilibrium between the TNF- α -308 and the LTA *NcoI* polymorphisms, in agreement with a previous report by our group (16).

CD14, a mediator of endotoxin activity and monocyte count, was measured to exclude that these factors could influence the results. No difference in CD14 density was observed among the TNF- α -863 genotypes.

Interestingly, our data obtained with human cells are in line with a previous report (11). These authors used reporter gene assays and found remarkably higher transcription for the -863 C allele. This result was challenged by Kaijzel et al. (19), who could not establish a functional relevance for this polymorphic site. Another study (20) found higher transcriptional activity of the A allele after endotoxin stimulation when the reporter construct contained the 3' untranslated region. A construct without this region had higher activity for the C allele (20). This study illustrates the variability of results obtained with reporter gene assays and underlines the need of studies using human whole blood.

In summary, our findings suggest that the TNF- α -863 C/A polymorphism is a genetic factor influencing TNF- α synthesis that is not in linkage disequilibrium with the TNF- α -308 or the LTA *NcoI* polymorphisms. The genotyping assay described here is rapid, accurate, and suitable for routine laboratories with a high sample throughput.

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Effect of Thyroxine Replacement on Creatinine, Insulin-Like Growth Factor 1, Acid-Labile Subunit, and Vascular Endothelial Growth Factor, Christoph Schmid, Michael Brändle, Cornelia Zwimpfer, Jürgen Zapf, and Peter Wiesli* (Department of Internal Medicine, Division of Endocrinology and Diabetes, University Hospital of Zurich, CH-8091 Zurich, Switzerland; * author for correspondence: fax 41-1-255-4447, e-mail peter.wiesli@dim.usz.ch)

Hypothyroidism is associated with endothelial dysfunction, arterial hypertension, and impaired kidney function