Letters

Comments

– to: F. M. Nakhoul et al. (2001) Haptoglobin phenotype and diabetes. *Diabetologia* 44: 602–604

To the Editor: A recent publication in *Diabetologia* from Nakhoul et al. [1] found that the haptoglobin phenotype was correlated with the occurrence of diabetic nephropathy. Similar to the correspondence from the same group in *The New England Journal of Medicine* [2], no patient with a haptoglobin 1–1 phenotype had macroalbuminuria or even microalbuminuria. Thus, these reports concluded that the haptoglobin 1–1 phenotype protects against the development of diabetic nephropathy.

Because we have a large collection of DNA from patients with Type I (insulin-dependent) diabetes mellitus, well classified by renal status, we decided to examine this hypothesis in our population. Our study population has been described in detail [3]. Briefly, Type I diabetes patients with persistent proteinuria or end-stage renal disease were considered patients, and those without nephropathy despite having diabetes for 15 or more years were considered the control subjects. A total of 312 patients and 290 control subjects were available for the study. Only Caucasian patients were included. The patients were classified into stages of diabetic nephropathy on the basis of questionnaires, medical records, and measurements of the albumin-to-creatinine ratio. Patients without a history of diabetic nephropathy having an albumin-to-creatinine ratio of less than 17 µg/mg in men and 24 µg/mg in women, were considered normoalbuminuric (control subjects). Patients with clear evidence of renal disease (renal transplant or dialysis) or persistent proteinuria were considered (after review of all information for evidence of non-diabetic renal disease) to have diabetic nephropathy. Persistent proteinuria was diagnosed if a patient had two out of three positive sequential urinalyses: reagent strip $\geq 1+$ (Multistix, Ames Division, Miles Laboratories, Elkhart, Ind., USA) or an albumin-to-creatinine ratio of 300 µg/mg or higher. Patients who did not fit one of these categories because they had microalbuminuria or intermittent proteinuria were considered unclassifiable and were excluded.

All studies done up to now determined the haptoglobin phenotype analysing the haemoglobin-enriched serum by gel

Table 1. Distribution of haptoglobin genotypes in patients with Type I (insulin-dependent) diabetes mellitus with and without nephropathy

	Normo-albuminuria	Proteinuria/ ESRD	OR (95 % CI)
Genotype			
1/1	34 (11.7 %)	37 (11.9 %)	0.99 (0.60–1.62)
2/1 or 2/2	256 (88.3 %)	275 (88.1 %)	reference

electrophoresis. Because the haptoglobin phenotype is determined by three common alleles Hp^{1F} , Hp^{1S} and Hp^2 [4], we have designed a PCR-based protocol to determine the presence of the Hp^2 allele. The following primers were used in a multiplex PCR reaction: Hptgl2 5'-GCT GTC ACT GCT GCG TAA AGT AG-3', Hptgl3 5'-GCATGGAGACAGTTGTCCACA-3' and Hptgl4 5'-CTG CTC TTA CTG GTG TGG AAA TG-3'. The PCR mixture (total volume 50 µl) contained 40 ng of genomic DNA together with 0.2 µmol/l of primer Hptgl3 and Hptgl4 and 0.05 µmol/l of primer Hptgl2, 2.0 mmol/l $MgCl_2$, 5 µl of 10xPCR buffer, 0.2 mmol/l of each dNTP and 1.25 units of GeneChoice TAQ DNA Polymerase (PGC Scientifics Corp., Frederick, Md., USA). The PCR mixture was denatured at 94°C for 1 min followed by annealing at 56°C for 1 min and extension at 72°C for 1.5 min. The PCR amplified fragments were electrophoresed on a 1.5 % agarose gel stained with ethidium bromide. When the Hp^2 allele was absent (1/1 genotype) only a single 386 bp band was observed. When present (2/2 or 1/2 genotype), an additional 451 bp band was observed together with a 386 bp band. The 1/1, 1/2, and 2/2 genotypes correspond to the 1–1, 1–2, and 2–2 phenotypes, respectively. In comparing carriers of the Hp^2 allele (1/2 or 2/2 genotype) to non-carriers (1/1 genotype), we did not observe any difference in the frequency of genotypes between the patients and the control subjects (OR = 0.99, 95 % CI 0.60–1.66, Table 1). We found 37 patients with proteinuria or end-stage renal disease who had the 1/1 genotype, contrary to the previously reported observation that no patients with the 1–1 phenotype had macroalbuminuria or even microalbuminuria [1, 2]. Thus, our results do not support the theory that the haptoglobin 1/1 phenotype protects against the development of diabetic nephropathy. Our study population is about five times bigger than that reported by

Nakhoul et al. [1] and we did not even observe a small distortion in the genotype distribution. The positive results found by Nakhoul et al. might first be caused by population stratification, second, be false positive due to small number of patients, or third, apply only to the studied population.

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References

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The author's reply

To the Editor: We have read the report and comments of Moczulski et al with considerable interest and offer the following response regarding potential reasons for differences between our studies in the ability of haptoglobin 1–1 to protect against diabetic nephropathy.

The critique of unrecognized population stratification raised by Moczulski et al is not appropriate in this setting. Unrecognized population stratification refers to a confounding effect in a population-based study in which an association based on linkage disequilibrium is inferred between different alleles at a polymorphic site (genetic marker) and disease predisposing variations or mutations within or at a nearby locus. Under

such circumstances, it is indeed the case that differences in allele distributions could reflect underlying unrecognized population stratification and confound the analysis of linkage disequilibrium. However, in our study the alleles are actually the products of distinct phylogenetic histories at this locus. Our interest is precisely in the human phylogenetic history for this locus, and association of the alleles with the disease (nephropathy) in question. In this regard, family-based association studies which are powerful at uncovering linkages between loci will not necessarily serve as the best tool for our studies. Furthermore, the inference of a causal relation between the haptoglobin phenotypes and predisposition for the disease has not been made on the basis of the observation alone. Rather, the alleles serve as risk markers and any possible causal relation is inferred based upon the biochemical and physiological contentions raised in the discussion of our reports.

The case control study of Moczulski et al is considerably larger (5X) than our study and therefore it is certainly possible that we have obtained a false positive due to a relatively small patient number. However, it is also important to note that the study population inclusion and exclusion criterion used in our respective studies were quite different. In contrast to Moczulski et al we have taken consecutive patients with Type I (insulin-dependent) diabetes mellitus early in the course of their diabetic renal disease. Patients were excluded from our studies if they had an abnormal serum creatine or hypertension. The study population of Moczulski et al could have a considerable selection bias (i.e. more patients with 1–1 in the group with diabetic nephropathy) due to the inclusion of patients with more advanced disease. This is the result of the fact that haptoglobin 1–1 confers a survival benefit, prominently due to a reduced incidence of cardiovascular disease in diabetic patients. This potential bias might only be eliminated in a population-based longitudinal study.

The importance of the haptoglobin phenotype in predicting diabetic nephropathy could indeed differ in different geographic locations or ethnic groups. There are numerous examples of polymorphic genetic loci being predictive of diabetic microvascular complications in one population but not in another.

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