

Haptoglobin Phenotype and Gestational Diabetes

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OBJECTIVE — Haptoglobin (Hp), an Hb-binding plasma protein, exists in two major allelic variants. Hp1 has higher Hb binding and antioxidant capacity compared with Hp2. Individuals with Hp1 exhibit a lower incidence of angiopathies. Gestational diabetes mellitus (GDM) is an early manifestation of type 2 diabetes in pregnant women. It is usually confined to the time of gestation, but carries an increased risk to develop type 2 diabetes later in life.

RESEARCH DESIGN AND METHODS — From consecutive Caucasian pregnant women ($n = 250$) referred for oral glucose tolerance testing, the Hp phenotype was determined. Significance of distribution and odds ratios (ORs) associated with Hp phenotype were calculated for women with GDM ($n = 110$) and women with normal glucose tolerance ($n = 140$).

RESULTS — Frequency of GDM in Hp phenotype classes increased with the number of Hp2 alleles ($P < 0.001$). ORs for GDM in women heterozygous and homozygous for Hp2 were 2.7 (95% CI 1.06–6.84) and 4.2 (1.67–10.55), respectively.

CONCLUSIONS — Hp phenotype is an apparent risk factor for the development of GDM in our study population. This might be due to the low antioxidative potential of Hp2 compared with Hp1.

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Haptoglobin (Hp) is an acute-phase protein synthesized in the liver and, to some extent, in fat tissue (1) in response to interleukin 6–type cytokines. The Hp gene is located on the long arm of chromosome 16 (16q22.3) (2). The two major Hp alleles (*Hp1* and *Hp2*) are coding for proteins with one (*Hp1*) or two (*Hp2*) SH groups used in multimerization. The *Hp1* molecule forms only dimers with a molecular weight of 86 kDa, whereas the *Hp2* molecule forms multimers with a molecular weight between 170 and 900 kDa. A mixture of *Hp1* and *Hp2*, as present in heterozygous individuals, exhibits dimeric Hp as well as

oligomeric Hp, but the latter with a lower degree of polymerization than in *Hp2* homozygotes. The Hp phenotype is inherited in a Mendelian pattern.

Hp forms complexes with free Hb that are rapidly cleared by the liver and by macrophages. Rapid complexation of free Hb, stemming from common, low-grade intravascular hemolysis (3), is important because free Hb catalyzes the generation of reactive oxygen species, like the extremely reactive hydroxyl radical, by the so-called Fenton reaction. Reactive oxygen species promote endothelial activation and inflammation and play a crucial role in the development of endothelial

dysfunction (4), which is also observed in gestational diabetes mellitus (GDM) (5). Thus Hp functions as an antioxidant and an essential vascular endothelial protector. However, the Hb-binding capacity (6) and antioxidant capacity of *Hp1* is higher compared with *Hp2* (7–9). The increased antioxidant function of *Hp1* is thought to confer protection from angiopathies. This has been reported for coronary (10) and peripheral (11) atherosclerotic lesions, cardiac transplant vasculopathy (12), diabetic nephropathy (13), mortality in coronary heart disease (14), restenosis after peripheral and coronary angioplasty (15) or stenting (16), and cardiovascular disease in diabetic individuals (17). The overall picture appears to confirm a protective role of *Hp1* in clinical settings associated with increased oxidative stress.

GDM is a most common complication of pregnancy and carries considerable health risks for both the fetus and the mother (15). It is well established that glucose intolerance detected during pregnancy is predictive of later maternal type 2 diabetes (18,19). Furthermore, diabetic patients (especially women) carry a significantly greater cardiovascular risk than nondiabetic individuals (20), and even subtle disturbances in glucose metabolism appear to be linked with premature atherosclerosis (21,22). Women with prior GDM feature endothelial dysfunction in relation to insulin resistance and obesity. Obesity is the most prominent risk factor for GDM and type 2 diabetes in prior GDM and is linked to inflammatory processes and angiopathy, increasing the cardiovascular risk (5,23,24). As the pathogenesis of insulin resistance in type 2 diabetes is considered to be associated with endothelial oxidative stress, we expected the different antioxidative capacity of Hp phenotypes to contribute to the development of hyperglycemia at times of increased endothelial oxidative stress, such as GDM (25).

To our knowledge, the Hp phenotype has not been reported to be a risk factor for the development of GDM. The aim of the present study was to investigate a pos-

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Abbreviations: GDM, gestational diabetes mellitus; Hp, haptoglobin; OGTT, oral glucose tolerance test. A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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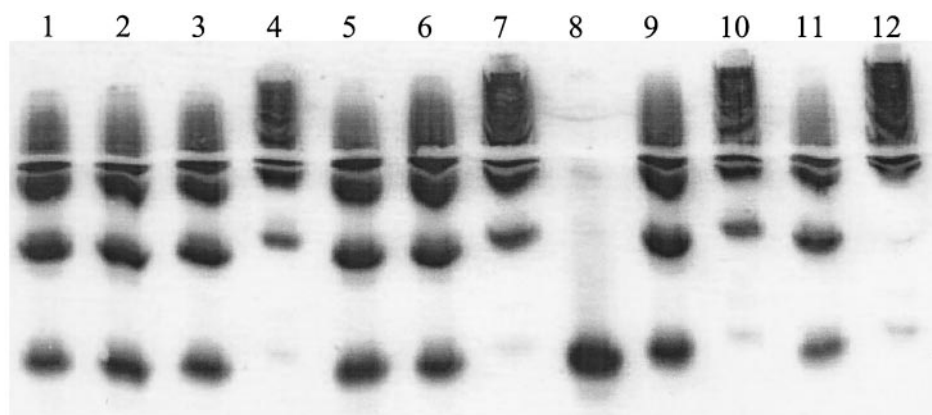


Figure 1—Electropherogram with typical Hp phenotype patterns, as observed in serum samples. SDS-PAGE of Hp. Hp phenotypes: Hp1-1 (lane 8), Hp2-1 (lanes 1–3, 5, 6, 9, and 11), and Hp2-2 (lanes 4, 7, 10, and 12).

sible association between the Hp phenotype and GDM.

RESEARCH DESIGN AND METHODS

Cross-sectional analysis was performed in 250 consecutive, pregnant Caucasian women. Women were referred from the Department of Obstetrics at the General Hospital of Vienna to the outpatient clinic of the Division of Endocrinology and Metabolism at the General Hospital of Vienna for screening for GDM between the 24th and 28th gestational weeks by oral glucose tolerance test (OGTT). In our hospital, general screening for GDM is recommended in all pregnant women, but referral is mandatory for women with a BMI >28 kg/m², age >35 years, a family history of type 2 diabetes, previous GDM, and previous obstetric complications. In case of a normal test, but development of clinical symptoms, such as glucosuria or macrosomia, the OGTT was repeated. Measurements of all serum parameters were performed in blood samples drawn at the time of the OGTT between the 24th and 28th gestational weeks. All patients were Vienna residents. One hundred twenty-five women were native Austrians, whereas the other Caucasian women came from the Balkans ($n = 56$), Turkey ($n = 51$), and Arabic countries ($n = 18$).

The aim of this study was to determine the association between Hp phenotype and GDM in previously healthy women. GDM was diagnosed according to the criteria of the 4th Workshop Conference of Gestational Diabetes (18) and adapted by the German and Austrian Diabetes Association, which suggest treatment in women with one abnormal value (gestational impaired glucose tolerance by 75-g OGTT: fasting plasma glucose

≥ 95 mg/dl, 1 h ≥ 180 , and 2 h ≥ 155) (26). All pregnant women with GDM were negative for islet cell antibodies (GAD, islet cell antibody, and insulinoma-associated protein 2). No women developed type 1 diabetes.

Aside from the Hp phenotype, no other inherited or genetic marker was tested for association with GDM.

All subjects gave informed consent for participation in the study, which was approved by the local ethics committee.

This study was performed according to the guidelines described by Cooper, Nussbaum, and Krawczak (27).

Oral glucose tolerance test

All women ingested an isocaloric diet containing 200 g of carbohydrate per day. Oral glucose tolerance tests (OGTTs) were performed after 10–12 h of overnight fasting. All women ingested 75 g of glucose solution within 2 min, and venous plasma samples were collected (Vacuette FE sodium fluoride/EDTA tubes; Greiner Bio-One, Kremsmünster, Austria) for glucose measurements at fasting and 1 and 2 h following glucose loading. Glucose measurements were done on a Hitachi Modular System (Hitachi, Tokyo, Japan) using a hexokinase method (Roche Diagnostics, Basel, Switzerland).

Hp phenotyping

Hp phenotyping was performed essentially as described previously (28). Briefly, samples were pretreated by mixing 5 μ l serum and 500 μ l SDS glycerin Tris buffer (pH 8.6), electrophoresed on a Pharmacia Phast System (Pharmacia Biotech, Uppsala, Sweden) using a 10–15% polyacrylamide gradient gel at 250 V/80 AVh, blotted on nitrocellulose, and blocked

with 3% milk powder in Tris-buffered saline. For detection, rabbit anti-human Hp IgG and, subsequently, goat anti-rabbit IgG conjugated with alkaline phosphatase (Dako, Glostrup, Denmark) were used; bands were visualized with phosphatase developing solution (Technoclon, Wien, Austria). Electropherograms (Fig. 1) were analyzed independently by two experienced investigators. Faintly stained samples were reanalyzed using the 10-fold serum volume (50 μ l).

Determination of plasma proteins

Quantitative determination of serum levels of Hp and C-reactive protein (high-sensitivity C-reactive protein assay) was made nephelometrically (Dade Behring, Wilmington, DE).

Statistics

For calculating the significance of differences of patient characteristics, the two-sided Mann-Whitney *U* test was used. Significance of association of phenotype and GDM or phenotype and insulin therapy was calculated using the χ^2 test. For estimation of odds ratios (ORs), multivariate logistic regression was used. All multivariate models fulfilled the Hosmer-Lemeshow goodness-of-fit test. Ordered differences among classes were calculated with the Jonckheere-Terpstra test. Significance of differences of the occurrence of GDM in BMI/phenotype groups were calculated using ANOVA and post hoc least significant difference testing. The Spearman correlation coefficient was calculated for Hp levels and BMI. All calculations were performed using the SPSS statistical software package (SPSS, Chicago, IL).

Table 1—Characteristics of the study population

	Normal glucose tolerance	GDM	P
n	140	110	
Age at presentation (years)	29 (26–33)	31 (27–36)	0.010
BMI (kg/m ²)	23.6 (21–27.1)	26.9 (23.4–30.3)	<0.001
Systolic blood pressure (mmHg)	110 (110–120)	120 (110–130)	0.074
Diastolic blood pressure (mmHg)	70 (65–80)	70 (68–80)	0.204
Triglycerides (mg/dl)	192 (141–247)	217 (155–283)	0.031
Total cholesterol (mg/dl)	246 (220–289)	240 (201–278)	0.051
Total-to-HDL cholesterol ratio	3.43 (3.04–4.16)	3.66 (3.17–4.47)	0.050
HDL cholesterol (mg/dl)	70 (58–83)	62 (52–71)	<0.001
LDL cholesterol (mg/dl)	133 (117–166)	129 (96–162)	0.041
HbA _{1c} (%)	5 (4.7–5.2)	5.3 (4.9–5.7)	<0.001
Fructosamine (μmol/l)	195 (185–207)	196 (188–207)	0.670
C-reactive protein (mg/dl)	0.53 (0.26–0.92)	0.6 (0.33–0.94)	0.452
Ferritin (mg/dl)	9.3 (4.9–21.4)	11.6 (5.3–21.3)	0.552
Hp (mg/dl)	90 (66–120)	107 (71–144)	0.016

Data are median (interquartile range). Significance was calculated by the two-sided Mann-Whitney U test.

RESULTS

Metabolic characteristics

GDM was present in 110 women, and 140 had normal glucose tolerance. Patients with GDM had either one ($n = 48$; 44%), two ($n = 48$; 44%), or three ($n = 14$; 13%) elevated plasma glucose values. As expected, they showed the stigmata of the metabolic syndrome (Table 1).

GDM and Hp phenotype

GDM occurred only rarely in the Hp1-1 phenotype group ($n = 8$; 7%) and was more frequent in Hp2-1 group ($n = 43$; 39%), but was most frequent in women with the Hp2-2 phenotype ($n = 59$; 54%) ($\chi^2 = 13.537$; P [2 degrees of freedom {df}] = 0.001) (Fig. 2). ORs for GDM compared with Hp1-1 were 3.02 (95% CI 1.26–7.20; $P = 0.013$) for carriers of phenotype Hp2-1 and 4.63 (1.96–10.94; $P < 0.001$) for Hp2-2. After controlling for age and BMI, both established risk factors for GDM, the respective values remained essentially the same: 2.70 (1.06–6.84; $P = 0.037$) and 4.20 (1.67–10.55; $P = 0.002$), respectively (Fig. 2). Controlling also for lipid status (log-transformed triglycerides, total cholesterol, and HDL cholesterol), which was significantly associated with the occurrence of GDM in our study population (Table 1), revealed ORs for Hp2-1 and Hp2-2 of 2.70 (1.05–6.93; $P = 0.040$) and 4.07 (1.60–10.35; $P = 0.003$), respectively. Among women with more severe impairment of glucose

tolerance (two or three elevated OGTT values), Hp2-2 is the prominent phenotype (60%) compared with 44% of women with one abnormal value. Conversely, the frequency of the protective 1-1 phenotype increases from 5 to 10%, respectively (Fig. 2). This trend was highly significant ($P = 0.0002$ for trend, Jonckheere-Terpstra Test).

The Hp phenotype–dependent occurrence of GDM in lean women compared with normal and obese women is presented in Fig. 2 (ANOVA, $P < 0.0001$). Occurrence of GDM increased significantly for Hp2-1 and Hp2-2 with BMIs >22 kg/m². BMI did not significantly influence the occurrence of GDM in Hp1-1 women (Fig. 2) (least significant difference as post hoc analysis).

Insulin therapy at any time during pregnancy had to be administered in 58 of 110 women with GDM and was not associated with Hp phenotypes (Hp1-1, 6 of 8 [75%]; Hp2-1, 21 of 43 [48%]; and Hp2-2, 31 of 59 [53%]; $\chi^2 = 1.85$; P [2 df] = 0.40).

The overall distribution of Hp phenotypes in our study population was 16% (Hp1-1), 40% (Hp2-1), and 44% (Hp2-2). Allelic frequencies were 0.36 for the Hp1 allele and 0.64 for the Hp2 allele. Though the overall allelic distribution was not in agreement with the Hardy-Weinberg distribution ($n = 250$; $\chi^2 = 4.352$; P [1 df] = 0.0370), Hardy-Weinberg distribution was found in each of the four subgroups with different geo-

graphic origins (data not shown). Distribution of Hp phenotypes ($\chi^2 = 3.061$; P [6 df] = 0.801) or GDM ($\chi^2 = 5.051$; P [3 df] = 0.168) did not differ among these groups. In all subgroups of Caucasian women, GDM rates showed that the Hp2 dose-dependent increase was lowest in those with the Hp1-1 phenotype and highest in those with the Hp2-2 phenotype. In multivariate logistic regression models (geographic origin; geographic origin and Hp phenotype; and geographic origin, Hp phenotype, BMI, and age) the OR for geographic origin was not significant.

High-sensitivity C-reactive protein and Hp levels

Hp serum levels were higher in subjects with GDM than in those with normal glucose tolerance ($P = 0.016$) (Table 1). In the whole study population, Hp levels correlated significantly with BMI (Spearman correlation: $r = 0.32$; $P < 0.001$), and the OR for GDM was 1 in a multivariate logistic regression model including BMI as a risk factor (OR [Hp level] 1.00; 95% CI 0.99–1.01; $P = 0.473$; OR [BMI] 1.10; 1.05–1.16; $P < 0.001$).

Serum levels of the inflammation marker high-sensitivity C-reactive protein did not differ significantly between Hp phenotype groups (data not shown) and were slightly higher in the GDM group (Table 1).

CONCLUSIONS — This study shows an increased risk for women missing Hp1 to develop impaired glucose tolerance during pregnancy. This observation holds true after controlling for age and BMI, the most prominent risk factors for GDM. We could also observe a gene dosage effect for Hp2, with ORs for GDM of 2.62 in the presence of one Hp2 allele and of 4.27 in the presence of two Hp2 alleles. Notably, there also appears to be a significant association between the degree of glucose intolerance and Hp phenotype. Hp2-2 was the prominent phenotype among women with more severe impaired glucose tolerance. Hp2-2 is less frequent in women with only one abnormal OGTT value, but still more frequent when compared with women with normal OGTT values (Fig. 2). From these observations, we expect a significant role for the Hp phenotype in the pathogenesis of GDM.

Oxidative stress appears to play a crucial role in the development of impaired

A Association of Haptoglobin phenotype with Impaired Glucose Tolerance			
OGTT, number of elevated plasma glucose measurements	Hp-phenotype 1-1 n (%)	Hp-phenotype 2-1 n (%)	Hp-phenotype 2-2 n (%)
0 (NGT)	32 (23%)	57 (41%)	51 (36%)
1,2 or 3 (GDM)	8 (7%)	43 (39%)	59 (54%)
1	5 (10%)	22 (46%)	21 (44%)
2	2 (4%)	15 (31%)	31 (65%)
3	1 (7%)	6 (43%)	7 (50%)

B Haptoglobin phenotype as an independent risk factor for GDM			
risk-factor	Odds Ratio	CI (95%)	Significance
Hp2-1	2.696	1.063-6.837	0.037
Hp2-2	4.2	1.672-10.551	0.002
BMI (kg/m ²)	1.103	1.045-1.163	<0.001
Age (y ⁻¹)	1.044	0.988-1.103	0.123

C Occurrence of GDM in relation to BMI and Hp-phenotype			
	Hp-phenotype 1-1 mean +/- SE (n)	Hp-phenotype 2-1 mean +/- SE (n)	Hp-phenotype 2-2 mean +/- SE (n)
BMI<22kg/m ²	0.18+/-0.12 (11)	0.25+/-0.1 (20)	0.26+/-0.09 (23)
BMI>22kg/m ²	0.24+/-0.09 (25)	0.51+/-0.06 (67) *	0.64+/-0.06 (74) †
All	0.22+/-0.07 (36)	0.45+/-0.05 (87)	0.55+/-0.05 (97)

Figure 2—Hp phenotype and GDM (n = 250). A: Association of the Hp phenotype with impaired glucose tolerance. Distribution of Hp phenotypes (Hp1-1, Hp2-1, and Hp2-2) in women, categorized by the number (zero, one, two, or three) of elevated glucose measurements in an OGTT. The Hp phenotype distribution in women with normal glucose tolerance (NGT) and GDM (one, two, or three elevated glucose measurements) was significant ($\chi^2 = 13.537$; P [2 df] = 0.001). Among women with more severe impairment of glucose tolerance (two or three elevated OGTT values), Hp2-2 is more prominent than in women with only one abnormal value. Conversely, the frequency of the protective 1-1 phenotype increases (P = 0.0002 for trend). The percentage of the respective Hp phenotype in each OGTT group is given in parentheses. B: Hp phenotype as an independent risk factor for GDM. The ORs to develop GDM for carriers of the Hp2-1 and Hp2-2 phenotypes, compared with carriers of the Hp1-1 phenotype, show a Hp2 dose-dependent increase. Controlling for BMI and age at presentation, both established risk factors for GDM, demonstrated BMI to be associated with a significant 1.1-fold risk increment per 1 BMI unit (1 kg/m²). The OR for age is calculated for the increment of 1 year of age and, though higher than 1, did not turn out to be significant in our sample. C: Occurrence of GDM in relation to BMI and Hp phenotype. Hp1-1-related protection against GDM was prominent at a BMI >22 kg/m². ANOVA for this relation was highly significant (P < 0.0001). The occurrence of GDM increased significantly with Hp2-1 and Hp2-2 phenotypes only when BMI was >22 kg/m². *P < 0.04; †P < 0.003, compared with each group with BMI <22 kg/m² and with the Hp1-1 group with BMI >22 kg/m².

glucose tolerance (29). Free Hb-driven oxidation is more effectively inhibited by Hp1 compared with Hp2, which is less efficient as a physiologic antioxidant, in vitro (7,8) and in vivo (9). A lower antioxidative potential in carriers of Hp2 may therefore contribute to the development of GDM. The graded risk observed in Hp phenotypes also parallels reported Hb-binding capacities (6).

Risk for type 2 diabetes increases at a BMI >22 kg/m² (30), and Hp phenotype influenced the occurrence of GDM only in the presence of an elevated BMI (Fig. 2). Because BMI correlates positively with oxidative stress (31), this finding supports the suggestion that differences in the antioxidative function of Hp phenotypes contribute to the development of GDM. Elevated serum levels of Hp found in GDM (Table 1) may reflect the higher BMI present in women with GDM, leading to increased expression of Hp in fat tissue (1). In fact, we observed a significant cor-

relation between BMI and Hp levels, and no significant risk could be attributed to Hp levels in a multivariate risk model for GDM that included BMI.

All patients were Vienna residents, and only Caucasian patients were included in this study. The mix of Caucasians (due to recent migration) present in the current Vienna population explains the observed impairment of Hardy-Weinberg law in our study population. Nevertheless, phenotype frequencies were similar to those observed in another Caucasian population from neighboring Switzerland (19% Hp1-1, 48% Hp2-1, and 33% Hp2-2; n = 4,004; P > 0.10; allelic frequencies of 0.43 for Hp1 and 0.57 for Hp2) (32). In our study population, similar distribution of Hp phenotypes and GDM ruled out statistical bias due to coincident clustering in subgroups from different geographic backgrounds. Also, GDM rates in Hp phenotypes of

each subgroup reflected findings in the entire study population.

As shown by Kanakoudi et al. (33), neonatal Hp is present at only very low levels (below the level of detection) and therefore may also be absent in neonates. Erroneous detection of the Hp phenotype of the infant in maternal blood samples drawn between gestational weeks 24 and 28 therefore appears very unlikely.

Hp2 is a risk factor for the development of GDM in our study population. This was independent of age, BMI, and lipid profile. Further prospective studies in other populations will establish the role of the Hp phenotype as a risk factor for GDM.

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