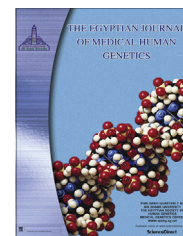




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ORIGINAL ARTICLE

# Haptoglobin phenotypes as a risk factor for coronary artery disease in type 2 diabetes mellitus: An Egyptian study



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## KEYWORDS

Type 2 DM;  
Haptoglobin polymorphism;  
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(PCR)

**Abstract Objective:** Diabetes has long been known to be an independent risk factor for cardiovascular disease. Recognition of diabetic individuals at greatest risk of developing coronary artery disease (CAD) would have important public health importance by allowing the distribution of limited resources to be directed on those who would most benefit from aggressive management. Several functional differences between haptoglobin (Hp) phenotypes have been demonstrated that appear to have important biological and clinical consequences in the development of CAD in patients with type 2 DM. The present study was conducted to demonstrate the relationship between

*Abbreviations:* Hp, haptoglobin; CAD, coronary artery disease; DM, diabetes mellitus; CRP, serum C-reactive protein; PCR, polymerase chain reaction; ECG, electro-cardiograph; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; 2hsBS, 2 hours postprandial blood glucose; HbA1c, glycated hemoglobin; TC, total cholesterol; TG, triglycerides; LDL-c, low density Lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol.

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the Hp phenotypes and the development of CAD among Egyptian patients with type 2 DM. To our knowledge this work had not been carried out in Egypt before.

**Subjects and methods:** The study included 160 subjects divided into three groups. Group I: 72 type 2 DM patients without CAD, Group II: 48 type 2DM patients with developed CAD, Group III: 40 age and gender matched apparently healthy subjects to serve as controls. All patients and controls were subjected to full history taking, complete clinical examination, and routine laboratory investigations. Serum C-reactive protein (CRP) levels and serum haptoglobin levels were measured. Polymerase chain reaction (PCR) was used for Hp phenotypes' determination.

**Results:** Analysis revealed association between Hp2-2 phenotype and the presence of CAD in type 2 DM. Hp and CRP serum levels were significantly higher in patients with CAD. Although the levels of Hp did not reach significance among patients with different Hp phenotypes yet the individual with Hp2-2 phenotype had trend toward higher level.

**Conclusion:** Hp2-2 phenotype is considered to be a major susceptibility gene for the development of CAD in type 2 DM. Awareness of this gene susceptibility should raise future research for proper treatment and prevention of CAD development in type 2 DM.

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## 1. Introduction

Regardless of efforts and advances in management, patients with type 2 diabetes Mellitus (type 2 DM) represent a significant global health problem and continue to be at high risk of cardiovascular complications. Risk factors such as hypertension, hyperlipidemia, and cigarette smoking independently increase the risk to the DM patient of developing CAD, but the effect of DM appears to be independent of conventional risk factors [1]. Identifying genes that contribute to complication development has been challenging. The susceptibility to diabetic complications is partially controlled by complex unknown genetic factors. One such genetic factor appears to be a functional allelic polymorphism in the haptoglobin (Hp) gene [2–4].

Haptoglobin (Hp) is an acute phase protein synthesized primarily in the liver, and to a lesser extent in other tissues including the lung, skin, spleen, and kidney in response to inflammatory cytokines [5]. It is a plasma  $\alpha_2$ -glycoprotein. Hp possesses an innate phenotype-dependent antioxidant, one of its main functions is to bind oxygenated, free hemoglobin with stabilization of the heme iron within hemoglobin (Hb) [6] and thereby prevents the oxidative tissue damage in areas of inflammation that may be mediated or catalyzed by free hemoglobin through the generation of highly reactive oxygen species which promotes endothelial activation and inflammation leading to endothelial dysfunction [7]. The Hp–Hb complex is rapidly removed from circulation via monocyte–macrophage cell surface scavenger receptor (CD163) mediated endocytosis by hepatic Kupfer cells [8]. Several lines of evidence have suggested the role for pro-inflammatory cytokines in regulating insulin action and glucose homeostasis. Type 2 diabetic patients exhibit higher serum levels of pro-inflammatory cytokines and acute-phase reactants [9]. Diabetes mellitus is also associated with increased oxidative stress and imbalance of antioxidant defense mechanism, that results in damage of several cellular bio molecules [10]. The role of Hp in regulation of inflammation suggests a potential role in type 2DM pathogenesis [11–12]. The ability of haptoglobin to protect against hemoglobin driven oxidative injury is abrogated when hemoglobin becomes glycated, a process that is markedly accelerated in the diabetic state. Glycohemoglobin–haptoglobin

complexes are catalytically redox active and therefore the rate at which haptoglobin–hemoglobin complexes are cleared from the serum and extravascular space is of heightened importance in the diabetic state [13].

Hp is composed of four chains: 2  $\alpha$  chains and 2  $\beta$ -chains.  $\alpha$  and  $\beta$  chains are encoded by a single gene and are synthesized as a single polypeptide chain that is proteolytically cleaved into a short  $\alpha$  chain and a long  $\beta$  chain that remain connected through a disulfide bond [14]. In humans, Hp is characterized by a genetic polymorphism which arises from differences in  $\alpha$  chains while  $\beta$  chains are identical in all Hp types. The Hp locus is located on chromosome 16 (16q22.1). Two common alleles exist for Hp, Hp1 and Hp2 that give rise to three major phenotypes. Individuals homozygous for allele Hp1 express the phenotype 1-1, those homozygous for allele Hp2, express phenotype Hp2-2, and heterozygous individuals express phenotype Hp1-2 [15–16]. A link between Hp polymorphism and a broad range of pathological conditions has been demonstrated, and such associations probably reflect functional differences among the phenotypes [17].

A key discrepancy between the alleles is that the protein product of the Hp1 allele has a more potent antioxidant compared with that produced by the Hp2 allele [18]. This functional allelic polymorphism in the haptoglobin gene, may determine susceptibility to a wide variety of vascular disorders associated with an increase in oxidative stress [4,19–20].

There exists a growing body of evidence that diabetic vascular disease develops only in those patients who are genetically susceptible. Diabetic individuals homozygous for the haptoglobin 2 allele (Hp2-2) are at significantly greater risk of developing cardiovascular disease as compared with diabetic individuals homozygous for the haptoglobin 1 allele (Hp1-1) with an intermediate risk being found in the heterozygote [2]. Hp1-1–Hb complexes are cleared much more rapidly than Hp2-2–Hb complexes by CD163 (monocyte–macrophage scavenger receptor) providing one mechanism for decreased oxidative stress and cardiovascular disease in Hp1-1 diabetic patients [13].

To our knowledge, there is no study conducted to clarify the relationship between haptoglobin polymorphisms of Egyptian type 2 DM and the development of CAD. Therefore this study is designed to investigate the distribution of Hp

phenotypes among Egyptians with type 2 DM with and without CAD compared to healthy subjects. We also investigated the relationship between different Hp phenotypes and other conventional risk factors involved in the pathogenesis of cardiovascular diseases. The serum levels of inflammatory markers namely CRP and Hp were examined.

## 2. Subjects and methods

### 2.1. Study design and subjects

From November 2011 to August 2013, 120 patients with type-2 DM who attended the Internal Medicine departments, Faculty of medicine, Cairo and Al Azhar universities were studied. They were divided into 2 groups. Group I included 72 type 2 diabetic patients (45 males and 27 females) without CAD; Group II included 48 type 2 diabetic patients with CAD (33 males and 15 females). A third group of 40 age and gender matched apparently healthy subjects (28 males and 12 females) serving as controls was included. They were recruited from volunteers of blood donation attending the above Hospitals. Control subjects were normal fasting and postprandial blood sugar, free of clinical manifestations of CAD by history, physical examination and electro-cardiographic (ECG) findings. Each patient was subjected to full physical examination. The clinical history of each patient including demographic data with tracing of the major traditional CAD risk factors (age, sex, hypertension, dyslipidemia, family history of premature cardiovascular disease, and current smoking), disease condition and treatment history was also recorded. Participants were weighed in light clothing without shoes and their heights were measured. Body Mass Index (BMI) was calculated by the Quetelet's index, as weight (kg)/height<sup>2</sup> (m). The mean of two blood pressure (BP) readings, measured on the right arm after participants had been seated for 5 min, was recorded. Hypertension was defined as systolic blood pressure  $\geq 140$  mmHg or diastolic blood pressure  $\geq 90$  mmHg or self reported use of antihypertensive medication. The diagnosis of type 2DM was based on the World Health Organization criteria [21]. CAD was defined as angina, ischemic electrocardiogram, myocardial infarction confirmed by Q-waves on electrocardiogram or hospital records, angiographic stenosis  $> 50\%$ , or revascularization.

The work is carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study was approved by the human research ethics committee of the hospital, and informed consent was obtained from each patient. Exclusion criteria included patients who had a diagnosis of diabetic nephropathy, abnormal liver functions, CAD due to any cause other than DM, chronic inflammatory diseases, acute infections, hematologic disorders, acutely ill patients, malignancy, and pregnant women.

### 2.2. Blood Sampling

After overnight fasting blood samples were collected from patients and controls in both heparinized vacutainer and a plain vacutainer to obtain plasma and serum respectively. After centrifugation at 3000 rpm for 15 min, aliquots of plasma and serum were stored at  $-80$  °C until analysis. Total cholesterol (TC), triglycerides (TG), low density Lipoprotein

cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), fasting (FBS) and postprandial blood glucose (2hsBS), and HbA1c were determined by Integra-400 (Roche Diagnostics, Mannheim, Germany).

### 2.3. Assessment of serum haptoglobin (Hp) and C-reactive protein (CRP)

CRP was done by Eleceyes (Roche-Germany). Serum concentrations of haptoglobin were done by a latex immunoturbidimetric method using Turbi Quick analyzer (vital Diagnostics, Italy).

### 2.4. Determination of Hp polymorphism by PCR

Genomic DNA was extracted from whole peripheral blood samples using the QIAamp DNA blood isolation Kit supplied by (Qiagen, Germany). PCR was done to analyze the Hp1 and Hp2 alleles by a method described previously by Koch et al. [22].

Two different PCR primers (synthesized by Applied Biosystems) were used for haptoglobin genotyping and subtyping [23]:

Primer A: 5'-GAGGGGAGCTTGCCTTCCATTG-3'; was used for amplification of a 1500-bp Hp 1 allele-specific sequence.

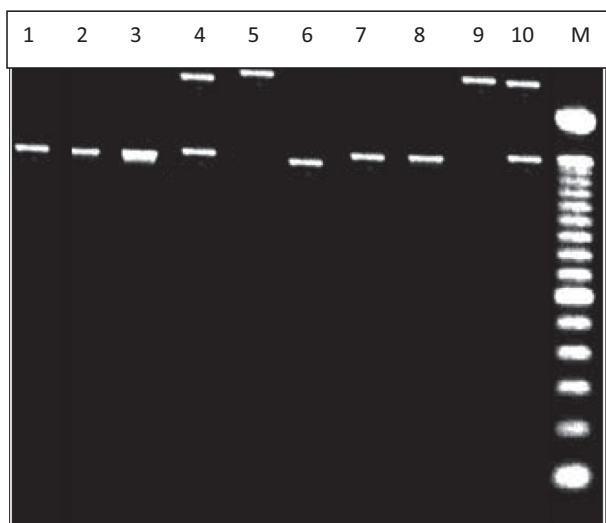
Primer B: 5'-GAGATTTTTGAGCCCTGGCTGGT-3'; was used for amplification of a 3481-bp Hp 2 allele-specific sequence.

The 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, and 0.2 mM dNTPs were added to 0.4  $\mu$ M of each primer, and 2 units of Qiagen taq DNA polymerase to a final volume of 50  $\mu$ L containing 10–20 ng of genomic DNA. The PCR was performed under the following conditions: an initial incubation at 95 °C for 2 min, 35 cycles of incubation at 95 °C for 1 min, 69 °C for 40 s, and 72 °C for 90 s, then at 72 °C for 7 min for final extension. The thermocyclers used were GeneAmp PCR systems 9700 and the 9600 mode (Applied Biosystems).

PCR products were digested using the DraI restriction enzyme (Invitrogen), applied to electrophoresis in 1.5% agarose gel, stained with ethidium bromide solution and identified by transillumination using ultraviolet light. The haptoglobin genotypes and subtypes were confirmed according to the size of the fragments obtained (Fig. 1).

### 2.5. Statistical analysis

Data were statistically analyzed using SPSS (Statistical Package for Social Science) computer program for statistical analysis, (version 15; Inc., Chicago, IL). The qualitative data were expressed as frequency and percent, and quantitative data were shown as mean  $\pm$  SD. Student's (*t*) test was used for comparison between two groups having quantitative normally distributed data, and One way analysis of Variance (ANOVA) test was used for comparison between three or more groups having quantitative normally distributed data and post hoc test was performed. Chi-square ( $\chi^2$ ) test was used to compare the qualitative variables. The allele frequencies were calculated for a two-allele system and differences in haptoglobin genotype and subtype frequencies between groups were compared using ( $\chi^2$ ) test. *P*-value was considered statistically significant when it is less than 0.05.



**Figure 1** The amplification products of Hp phenotypes: lanes 1, 2, 3, 6, 7 and 8 representing Hp1-1 (1500 bp), lanes 4 and 10 representing Hp2-1 (3841/1500 bp) and lanes 5 and 9 for Hp2-2 (3841 bp) and M representing MW size ladder band.

### 3. Results

#### 3.1. Patients' risk factors

This study comprised 120 patients with type 2 DM of them 48 patients had documented CAD. As shown in [Table 1](#) significantly higher percentage of those patients with CAD suffered from hypertension, dyslipidemia and also had significantly higher family history of CAD.

#### 3.2. Demographic and laboratory data

[Table 2](#) summarizes the main demographic and laboratory data of the studied groups. There were no significant differences between groups in terms of age, total cholesterol and triglyceride levels. Diabetic groups showed significantly higher systolic and diastolic blood pressures (SBP and DBP) compared to controls. Also diabetic groups showed significantly higher FBS, 2hsBS, HbA1c and LDL-c while HDL-c was significantly lower compared to the controls.

#### 3.3. Inflammatory markers

As regards the studied inflammatory markers, serum Hp level was significantly higher in diabetic patients with CAD (mean  $\pm$  SD,  $149.3 \pm 8.4$ ) compared to controls (mean  $\pm$  SD,  $78.2 \pm 11.2$ ) and diabetic patients without CAD (mean  $\pm$  SD,  $126.1 \pm 10.5$ ) ( $P < 0.001$ ,  $P < 0.05$  respectively). In the latter group, its level was significantly higher than controls ( $P < 0.01$ ) ([Fig. 2](#)). Also serum CRP level was significantly higher in diabetic patients with CAD (mean  $\pm$  SD,  $1.3 \pm 0.7$ ) compared to controls (mean  $\pm$  SD,  $0.43 \pm 0.07$ ) and diabetic patients without CAD (mean  $\pm$  SD,  $0.9 \pm 0.2$ ) ( $P < 0.01$ ,  $P < 0.05$ , respectively). In the latter group, its level was significantly higher than controls ( $P < 0.05$ ) as shown in [Table 2](#).

#### 3.4. Distribution of the Hp phenotypes among the studied groups

[Table 3](#) shows that 42.5% of the Egyptian type 2 DM were of Hp2-2 phenotype compared to 15% of the healthy controls. Conversely 16.7% of patients with type 2 DM had Hp1-1 phenotype compared to 40% of the healthy controls. The frequency of Hp2-1 was 40.8% and 45% for diabetic individuals and controls respectively. Among the diabetic patients, those with CAD had higher frequency of Hp2-2 (62.5%) compared to controls and diabetic patients without evidence of CAD (15% and 29.2%) ( $P < 0.001$ ;  $P < 0.01$ , respectively). Significant number of diabetic patients without evidence of CAD had higher Hp2-2 compared to controls ( $P < 0.05$ ). A high frequency of Hp1 Allele was found among the controls (0.625) while Hp2 allele was more frequent among diabetic patients with CAD (0.750) as shown in [Table 4](#).

#### 3.5. The relationship between various CVD risk factors and haptoglobin phenotypes in patient groups

As shown in [Table 5](#) diabetic patient with Hp2-2 phenotype had significantly higher BMI compared to Hp1-1. Also Hp2-2 phenotype had significant higher HbA1c and CRP serum levels and were more hypertensive and dyslipidemic compared to other phenotypes. Hp serum level did not differ significantly between phenotypes however Hp2-2 individual showed tendency toward higher levels.

**Table 1** Risk factors of the studied groups.

Parameters	Controls (N = 40)	DM (N = 72)	DM with CAD (N = 48)	P-value
Gender male (%)	28 (70)	45 (62.5)	33 (68.8)	NS
BMI (kg/m <sup>2</sup> )	27.9 $\pm$ 3.1	30.2 $\pm$ 3.2	32.2 $\pm$ 4.6	NS
Disease duration (years)	–	5.5 $\pm$ 2.1	6.13 $\pm$ 1.9	
DM	–	3.9 $\pm$ 1.3	–	NS
CAD				
Family history of DM N (%)	8 (20)	50 (69.4)	32 (66.7)	$P^1 < 0.01$ ; $P^2 < 0.01$
Family history CAD N (%)	6 (15)	19 (26.4)	27 (56.3)	$P^2 < 0.01$ ; $P^3 < 0.05$
Current smokers N (%)	13 (32.5)	26 (36.1)	20 (41.7)	NS
Hypertension N (%)	–	21 (29.2)	36 (75)	$P^3 < 0.01$
Dyslipidemia N (%)	–	20 (27.8)	23 (47.9)	$P^3 < 0.05$

$P^1$  = DM versus controls;  $P^2$  = DM with CAD versus controls;  $P^3$  = DM versus DM with CAD.

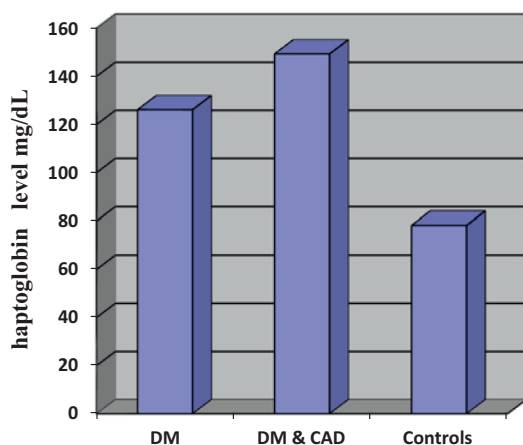
P values  $< 0.05$  and  $< 0.01$  are statistically significant. NS, none significant; BMI, body mass index; DM, diabetes mellitus; CAD, coronary artery disease.



**Table 2** Demographic and laboratory data between patient and control groups.

Parameters	Controls ( <i>N</i> = 40)	DM ( <i>N</i> = 72)	DM with CAD ( <i>N</i> = 48)	<i>P</i> -value
	M ± SD	M ± SD	M ± SD	
Age (years)	45.2 ± 3.1	49.3 ± 5.8	54.6 ± 8.9	NS
SBP (mmHg)	112.8 ± 5.2	139.5 ± 12.3	142.3 ± 9.3	$P^1 < 0.05$ ; $P^2 < 0.05$
DBP (mmHg)	72.3 ± 9.5	89.3 ± 10.4	92.3 ± 14.1	$P^1 < 0.05$ ; $P^2 < 0.05$
FBS (mg/dL)	78 ± 4.5	149.3 ± 11.6	153.2 ± 16.4	$P^1 < 0.01$ ; $P^2 < 0.01$
2hsBS (mg/dL)	99.6 ± 3.7	215.8 ± 26.1	224.8 ± 28.3	$P^1 < 0.001$ ; $P^2 < 0.001$
HbA1c (%)	5.3 ± 0.4	8.6 ± 2.5	9.23 ± 2.8	$P^1 < 0.05$ ; $P^2 < 0.05$
Cholesterol (mg/dL)	178.4 ± 9.2	189 ± 9.2	194.5 ± 11.7	NS
Triglycerides (mg/dL)	152 ± 27.7	151.4 ± 31.5	160 ± 23.7	NS
HDL-c (mg/dL)	42.3 ± 4.2	31.4 ± 4.9	28.3 ± 6.2	$P^1 < 0.05$ ; $P^2 < 0.05$
LDL-c (mg/dL)	108.2 ± 3.5	139 ± 11.7	144.6 ± 10.3	$P^1 < 0.05$ ; $P^2 < 0.05$
Haptoglobin (mg/dL)	78.2 ± 11.2	126.1 ± 10.5	149.3 ± 8.4	$P^1 < 0.01$ ; $P^2 < 0.001$ ; $P^3 < 0.05$
CRP (mg/dL)	0.43 ± 0.07	0.9 ± 0.2	1.3 ± 0.7	$P^1 < 0.05$ ; $P^2 < 0.01$ ; $P^3 < 0.05$

$P^1$  = DM versus controls;  $P^2$  = DM with CAD versus controls;  $P^3$  = DM versus DM with CAD. *P* values <0.05 and <0.01 are statistically significant. NS, none significant; DM, diabetes mellitus; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; 2hsBS, 2 hours postprandial blood glucose; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; CRP, serum C-reactive protein.

**Figure 2** The serum levels of haptoglobin among the three studied groups.

#### 4. Discussion

Cardiovascular disease continues to disproportionately affect individuals with diabetes worldwide despite significant advances in prevention efforts and medical care for these patients. It has therefore become apparent that genetic predisposition plays a key role in the development of vascular complications in diabetes, and a functional polymorphism in the Hp gene has been identified as a potential determinant of vascular diabetes complication risk [24].

Early detection of the disease and timely interventions can reduce the morbidity and mortality associated with it. Earlier studies strongly linked DM and diabetic microvascular complication namely nephropathy and retinopathy to Hp polymorphism [4,19–20], raising current interests in research to elucidate the role of Hp phenotypes in DM and its cardiovascular complications. This study was carried out to provide data on Hp phenotypes and type 2 DM in Egyptians with a

**Table 3** Haptoglobin phenotypes in patient and control groups.

Parameters	Controls ( <i>N</i> = 40)	Total patients ( <i>N</i> = 120)	DM ( <i>N</i> = 72)	DM with CAD ( <i>N</i> = 48)	<i>P</i> -value
Hp1-1	16 (40%)	20 (16.7%)	14 (19.4%)	6 (12.5%)	$P^1 < 0.05$ ; $P^2 < 0.01$ ; $P^3 < 0.05$
Hp2-1	18 (45%)	49 (40.8)	37 (51.4%)	12 (25%)	$P^1$ NS; $P^2 < 0.01$ ; $P^3 < 0.01$
Hp2-2	6 (15%)	51 (42.5)	21 (29.2%)	30 (62.5%)	$P^1 < 0.05$ ; $P^2 < 0.001$ ; $P^3 < 0.01$

$P^1$  = DM versus controls;  $P^2$  = DM with CAD versus controls;  $P^3$  = DM versus DM with CAD. *P* values <0.05 and <0.01 are statistically significant. NS, none significant; DM, diabetes mellitus; CAD, coronary artery disease.

**Table 4** Hp allele frequencies in patient and control groups.

Parameters	Controls ( <i>N</i> = 40)	Total patients ( <i>N</i> = 120)	DM ( <i>N</i> = 72)	DM with CAD ( <i>N</i> = 48)
Hp*1 allele frequency	0.625	0.375	0.458	0.250
Hp*2 allele frequency	0.375	0.625	0.542	0.750

Hp, haptoglobin; DM, diabetes mellitus; CAD, coronary artery disease.

**Table 5** Relations between risk Factors and haptoglobin phenotypes in patient groups.

Parameters	Total patients (N = 120)	Haptoglobin phenotypes			P-value
		Hp 1-1 (N = 20)	Hp 2-1 (N = 49)	Hp 2-2 (N = 51)	
		M ± SD	M ± SD	M ± SD	
Age (years)	51.9 ± 7.4	42.6 ± 3.1	47.9 ± 4.3	56.4 ± 5.3	NS
BMI (kg/m <sup>2</sup> )	31.2 ± 3.9	27.9 ± 1.8	30.43 ± 2.5	33.21 ± 2.1	P <sup>1</sup> < 0.05
Disease duration (years)	5.8 ± 2.1	4.9 ± 1.3	6.16 ± 1.8	6.42 ± 1.2	NS
FBS (mg/dL)	156.3 ± 14.5	145.4 ± 11.2	153.7 ± 10.6	159.3 ± 12.1	NS
HbA1c (%)	8.9 ± 2.7	7.2 ± 1.6	9.78 ± 1.7	10.62 ± 1.9	P <sup>1</sup> < 0.05; P <sup>2</sup> < 0.05
Haptoglobin (mg/dL)	137.8 ± 9.5	129.6 ± 7.6	135.4 ± 12.4	139.1 ± 18.3	NS
CRP (mg/dL)	0.9 ± 0.4	0.68 ± 0.15	0.74 ± 0.03	1.21 ± 0.8	P <sup>1</sup> < 0.05; P <sup>2</sup> < 0.05
Smoking N (%)	46 (38.3)	7 (35)	19 (38.8)	20(39.2)	NS
Hypertension N (%)	57 (47.5)	4 (20)	22(44.9)	31 (60.8)	P <sup>1</sup> < 0.05; P <sup>2</sup> < 0.05; P <sup>3</sup> < 0.01
Dyslipidemia N (%)	43 (35.8)	4 (20)	14 (28.8)	25 (49)	P <sup>1</sup> < 0.05; P <sup>2</sup> < 0.05; P <sup>3</sup> < 0.01

P<sup>1</sup> = Hp2-2 versus Hp1-1; P<sup>2</sup> = Hp2-2 versus Hp2-1; P<sup>3</sup> = Hp1-1 versus Hp2-1 P values <0.05 and <0.01 are statistically significant. NS, none significant; BMI, body mass index; FBS, fasting blood sugar; HbA1c, glycated hemoglobin; CRP, serum C-reactive protein.

view to determining whether the various Hp phenotypes have any association with the development of CAD in diabetes.

In this study we demonstrated a relationship between DM and Hp2-2 phenotype. The frequency of the three major Hp phenotypes in Egyptian diabetic patients was 16.7% for Hp1-1, 40.8% for Hp2-1 and 42.5% for Hp2-2.

Several studies on Hp phenotypes, diabetes and its complications from different parts of the world have been reported, however, conflicting data as regards the association between Hp phenotypes and predisposition to DM exist. A study on Jordanian suggested that type II diabetes mellitus is Hp phenotype-independent [25], Hp2-1 was the predominant phenotype among Saudi type 2 diabetics as well as healthy subjects [26], while a population study by Stern et al., [27] indicated that diabetes is associated with the Hp1-1 phenotypes. In agreement to our results Hp2-2 was the culprit for the development of type 2 diabetes in hypertensive overweight Ghanaians [28]. Ethnic and racial differences as well as dramatic changes in the life style which accelerate the onset of type 2 diabetes may override genetic predisposition and would be the cause of these discrepancies between the study populations. However it may possibly underscore the need for identification of genetic factors that contribute to differences in susceptibility to and the pathology of the disease [26,28].

This study demonstrated an increased risk of CAD among diabetic patients carrying the Hp2-2 genotype compared with those carrying the other haptoglobin genotypes. The frequency of Hp2-2 phenotype was greater in patients with CAD than in those without documented CAD or the controls (P value <0.01 and 0.001, respectively). We also found that Hp2 allele frequencies among patients with and without CAD and controls were 0.750, 0.542 and 0.375, respectively.

In fact, individuals with the Hp2-2 phenotype appear to have reduced clearance of the macrophage–Hp–Hb complex, which affects iron deposition, oxidative stress, and active macrophage accumulation [29]. These changes would be consistent with an increased risk of atherosclerotic cardiovascular diseases.

A number of studies have shown increased cardiovascular disease risk among diabetic individuals carrying the Hp2-2 genotype. In a case-control analysis of the Strong Heart Study, the Hp2-2 compared with the Hp1-1 or 2-1 phenotypes was an independent predictor of cardiovascular disease among American Indians with type 2 diabetes, whereas no association was

observed in the non diabetic population [2]. Also an almost two-fold risk of the incidence of a major adverse cardiac event within 1 year after percutaneous transluminal coronary angioplasty among type 2 diabetic patients having Hp2-2 phenotype compared with those of Hp1-1 phenotype had been observed in the Munich Stent Study [3]. Increased risk of Hp 2-2 phenotype with peripheral arterial occlusive disease has been reported as well [30]. Surprisingly a recent study on Chinese did not find any significant relationship between Hp phenotypes and CAD [31].

Our results prove that CAD among Egyptian diabetic patients illustrates the least association with Hp1-1 phenotype (frequency 12.5%). This finding is consistent with that of other investigators who attributed the phenomena to the protective function of Hp1-1 [2,32]. In agreement with our finding, Suleiman et al., [33] analyzed the Hp phenotypes in diabetic patients with acute myocardial infarction and demonstrated that the Hp1-1 phenotype was associated with smaller infarct size and lower mortality rates at 30 days.

Understanding functional differences between the Hp1 and Hp2 allelic protein products, particularly in diabetes, may provide insight into why Hp2-2 diabetic individuals have more CAD and how this increased burden of disease might be reduced [34]. *First:* gross differences in the size of the haptoglobin protein in individuals with the different phenotypes could account for discrepancy in the oxidative protection afforded by the different types of haptoglobin. Hp1-1 is markedly smaller than Hp2-2 and may, thus, be better able to sieve into the extravascular compartment and prevent hemoglobin-mediated tissue damage at sites of vascular injury [35]. *Second:* the Hp1 protein is more efficient in preventing oxidation by hemoglobin and in preventing heme release from the Hp–hemoglobin complex [13,18]. *Third:* the Hp1 allele is more efficient in promoting the uptake of the Hp–hemoglobin complex by the CD163 macrophage receptor [13,32,36]. These macrophages have been described in atherosclerotic lesions and they were suggested to exhibit an antiatherogenic phenotype when examined in vivo [37]. Also it has demonstrated that Hp1-1–Hb complexes result in a greater production of the anti-inflammatory cytokine IL-10 as compared with Hp2-2–Hb complexes [38].

In the current study, we have exposed significantly higher serum concentrations of inflammatory markers in terms of CRP and Hp in type 2 diabetic patients with and without associated CAD compared to controls. We also notice a significant

difference in their levels in the two diabetic groups with the highest levels in those with associated CAD. Individuals with specific Hp phenotypes may respond differently to biological insults such as inflammation and vessel wall degeneration. The synthesis of Hp may reflect a regulatory response to these pathologic processes [39]. Our data as regards the significant plasma Hp concentrations in diabetics with CAD support this explanation. In agreement with these results Lee et al., [31] showed that plasma Hp concentrations were significantly increased in patients with CAD compared with patients who do not have CAD, and that plasma Hp concentrations were independently associated with CAD.

Although the levels of Hp did not reach significance among diabetic individuals with different Hp phenotypes yet we found Hp phenotype 2-2 individuals had trend toward higher levels. Several studies have shown that the different alleles lead to different levels of circulating Hp protein [19,40]. However Mohieldein et al., [26] showed that serum Hp concentrations among their diabetic and control subjects were free of phenotypic-dependence.

Different methods may overestimate or underestimate Hp concentration in different phenotype groups. Unfortunately we measured the total serum Hp level rather than phenotype specific protein which may explain differences of Hp concentration between different phenotypes and that mentioned in other published studies [19,40].

The pathogenesis of human hypertension is considered complex and multifactorial and results from the combined effect of predisposing genetic and environmental risk factors [41]. Recently Boretti et al. [42] reported that cell-free plasma Hb has potent oxidative properties that are associated with hypertensive effects. They also found that increasing the levels of haptoglobin in the circulation reduced this oxidative stress and limited both the hypertensive effects and the renal insufficiency associated with plasma hemoglobinemia.

It had been documented that hypertensive patients carrying Hp2-2 are more likely to accumulate atherosclerotic lesions of the coronary or peripheral arteries, despite comparable lipid levels, smoking habits and BMI [43]. Hp binds to ApoA1 in the same location as lecithin-cholesterol acyltransferase (LCAT), subsequently decreasing LCAT activity and therefore limiting high density lipoprotein (HDL) maturation. This inhibits reverse cholesterol transport causing HDL to become proatherogenic [44]. In addition, the tethering of Hb to HDL via Hp–ApoA1 allows the oxidation of HDL and its acquisition of proatherogenic and proinflammatory properties [45].

The current data suggest an association between functionally distinct phenotypes of haptoglobin gene and various CAD risk factors. Hypertension and dyslipidemia were more prevalent in diabetic individuals with Hp2-2 phenotype as well as higher levels of BMI and HbA1c were observed. There have been conflicting reports regarding the role of Hp polymorphism in hypertension. In agreement with our finding an association between Hp2-2 phenotype and higher occurrence of refractory hypertension was found [46]. A Brazilian study showed that individuals of the Hp1-1 phenotype had an approximately 3-fold increase in essential hypertension [47] however a more recent one revealed an approximately four fold increase in refractory hypertension in type 2 diabetics with Hp type 2-1 [48]. These conflicting reports may be due to the cross sectional nature of the studies and indicate that additional prospective studies need to be carried out.

## 5. Conclusion and recommendation

The present study conducted in Egyptian populations with type 2 DM shows that: (i) there is increased frequency of Hp2-2 phenotype among type 2 DM patients. (ii) Serum levels of inflammatory markers in terms of CRP and Hp are significantly higher in type 2 diabetic patients, those with CAD demonstrated the highest levels. (iii) Higher levels of BMI, HbA1c as well as hypertension and dyslipidemia are more prevalent in diabetic individuals with Hp2-2 phenotype. (iv) Hp phenotype 2-2 is deemed to be a susceptibility gene for the development of CAD in type 2 DM. Whether to be part of the CAD risk stratification algorithm or avenue of new preventive and therapeutic options for type 2DM need further wide scale multicenter studies free of bias.

## Conflict of interest

The authors declare no conflict of interest. There is no financial or personal relationship with other people or organizations that could inappropriately influence their work.

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