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# Increased Risk for Ischemic Stroke of Non-Cardioembolic Origin in Italian Patients Carrying the Haptoglobin 2-2 Phenotype

*Antonio Ponzetto[1]*, Giulia Fiorini[2], Emanuela Morra[1] and Alberto Marchet[3]*

[1]Department of Medical Sciences, University of Turin, Italy

[2]Department of Medical and Surgical Sciences, University of Bologna, Italy

[3]Department of Stroke, Hospital Martini, Italy

## Abstract

Free iron is toxic to cells and total body iron stores are linked to the risk of developing atherosclerosis and stroke. Haptoglobin, a physiological scavenger of hemoglobin, prevents free iron spillage into cells; however, the haptoglobin 2-2 phenotype is poorly capable of binding hemoglobin. We hypothesized that the risk of stroke may be influenced by haptoglobin phenotypes and we investigated this hypothesis on a series of patients with acute ischemic stroke of non-cardioembolic origin.

**Cases:** A 160 patients were admitted to the stroke units of two Turin hospitals due to an episode of acute ischemic stroke of non-cardioembolic origin; there were two blood donor controls for each patient.

**Methods:** Immunoblotting was used to detect the haptoglobin phenotype.

**Results:** The haptoglobin 2-2 phenotype was significantly more frequent in the stroke patients compared to the controls (69% versus 45%, respectively; p =0.003) (Figure 1).

**Conclusions:** The haptoglobin 2-2 phenotype appears to be a novel risk factor for ischemic stroke of non-cardioembolic origin in Italian patients.

**Keywords: Ischemic Stroke; Haptoglobin; Iron; Free iron; Total body iron**

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***Correspondence:***
*Antonio Ponzetto, Department of Medical Sciences, University of Turin, Corso AM Dogliotti 14, 10126 Torino, Italy, Tel: 39 011 6708483 Fax: 390116334515;*
*E-mail: antonio.ponzetto@unito.it*

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## Introduction

Stroke was ranked second cause of death after ischemic heart disease worldwide up to 2010 and third in the USA [1,2]. Ischemic Stroke (IS) doubled among people aged 18 years to 64 years so that presently it is the primary cause of long-term disability in the USA [3]. While all age groups are at risk, the highest incidence is observed in older ages with no predilection for sex [3-5]. The mortality from IS is constantly descending [3]; among those who suffered first episode at age less than 50 years, cumulative mortality risks were 2.7% after first month, 4.7% and 10.7% at 1year and 5 years respectively [6] (Table 1). Nonetheless, 68% of stroke survivors rely on external help for coping with day-to-day life. Long-term care dominates the lifetime costs for stroke patients, with an average cost of care per person per year of 30,700 Euros in Italy [7]. Given the inevitable rise in medical costs for stroke patients owing to longer life expectancies and an aging world population, prevention is the most effective means to diminish the personal, economic, and social burden.

Epidemiological studies have identified non-modifiable risk factors: age and gender, hereditary predisposition and race. Stroke incidence reportedly doubles every 8 years of age and is more prevalent among male patients aged 65 years or over [8]. In the USA, African-Americans, Asians and Hispanics have a higher rate of stroke, whilst in Europe, the highest incidence is observed in Russia and Eastern European countries [9,10].

Modifiable risk factors for stroke are hypertension, atherosclerosis, previous cardiovascular diseases and Transient Ischemic Attack (TIA) [11-14]. Effective control of blood pressure was found to diminish stroke rates by 33% to 50% compared with unsuccessfully-treated hypertensive patients [15]. Taken together, the classic risk factors account for 50% of strokes. Body iron stores have been reported as a risk factor for developing carotid atherosclerosis [16]; serum levels of ferritin are one

**Table 1:** Haptoglobin haplotype frequency distribution in 320 controls and 160 patients with ischemic stroke.

| Haptoglobin haplotypes | Controls N=320 (%) | Patients N=160 (%) | χ2 |
|---|---|---|---|
| Hp 1-1 | 44 / 320 (13.75) | 18 / 160 (11.25) | NS |
| Hp 2-1 | 136 / 320 (42.50) | 44 / 160 (27.50) | $\chi^2$=4436; p=0.03 |
| Hp 2-2 | 140 / 320 (43.75) | 98 / 160 (61.25%) | $\chi^2$=3918; p=0.04 |
| Haptoglobin haplotypes (Hp 1-1, Hp 2-1, Hp 2-2) | controls (320) | patients (160) | $\chi^2$=13504; p=0.005 |

The χ2 test was used to calculate the statistical difference.

**Table 2:** Baseline Characteristics of the stroke patients and controls.

| | Number | Mean Age (years) | Hypertension | Obesity | Smokers |
|---|---|---|---|---|---|
| Controls Male | 168 | 51±20 | 23.81% (40/168) | 17.86% (30/168) | 39.88% (67/168) |
| Controls Female | 152 | 42±19 | 19.74% (30/152) | 18.42% (28/152) | 33.55% (51/152) |
| Patients Male | 120 | 53±20 | 83.33% (100/120) | 51.67% (62/120) | 89.16% (107/120) |
| Patients Female | 40 | 51±20 | 25% (10/40) | 12.5% (5/40) | 12.5% (5/40) |

Results are shown as means ±SD.

of the strongest indicators of carotid artery diseases in both sexes, especially in persons under 60 years of age [16]. Lipid peroxidation is considered the major pathogenic factor for developing carotid atherosclerosis because of its possible link with concentrations of tissue iron, one of the strongest oxidants *in vivo* [17-19]. Free iron is toxic for cells, particularly for those in the Central Nervous System (CNS) [20,21]. Iron-induced atherosclerosis resulted from the Kuopio Ischemic Heart Disease study, Kiechl, and Van der A. [22,19]. In postmenopausal women; the latter group found serum ferritin to be a risk factor for stroke in these patients [23]. The evidence shows that iron may cause lipid peroxidation, which directly induces Oxidation of Low Density Lipoproteins (ox-LDL), a major mechanism for atherogenesis [24,25]. The total amount of body iron stores and of free circulating iron in particular, depends on genetic traits, including Haptoglobin (Hp) levels. The Hp 2-2 phenotype is far less able to bind Hemoglobin (Hb), resulting in free iron being available [26]. Hp is a plasma protein responsible for the removal of free Hb from the circulation [27]. After hemolysis, stable Hp-Hb complexes are delivered to the hepatic parenchymal cells by receptor-mediated endocytosis [28,29]. Hp is characterized by a genetic polymorphism with three structurally different phenotypes (Hp 1-1, Hp 2-1 and Hp 2-2), Hp 1-1 is a small molecule (86 kDa) of well-defined structure, whereas Hp 2-1 is characterized by heteropolymers (86 kDa to 300 kDa), and Hp 2-2 forms large macromolecular complexes (170 kDa to1000 kDa) [26]. The hemoglobin binding capacity depends on the genetic Hp type, the amount of Hp, and the number of polymers [30,31]. Hp type 1-1 has the greatest hemoglobin-binding capacity, while type 2-2 is almost unable to carry Hb as the Hb binding sites are occupied due to the polymerization process [32].

Individuals bearing the Hp 2-2 phenotype are more likely to develop essential and refractory hypertension, Obstructive Sleep Apnea Syndrome (OSAS), and peripheral arterial occlusive disease [33-35].

We decided to investigate whether the Hp phenotype could also be involved in the pathogenesis or severity of non-cardioembolic IS.

## Materials and Methods

### Patients and controls

The patient group consisted of 160 subjects (120 males and 40 females, mean age 52 ± 20 years old) admitted to the Stroke Units of the Ospedale Martini and the Ospedale San Giovanni Battista (Molinette), Torino, in one year.

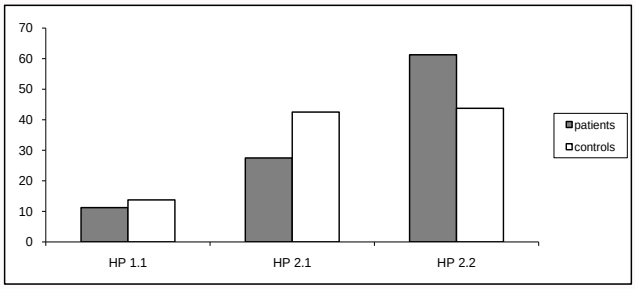


**Figure 1:** Haptoglobin (HP) haplotype frequencies as distributed in 320 controls and 160 patients with ischemic stroke.
$\chi^2$ = 13504; p =0.005

Diagnosis of ischemic stroke was based on the criteria of the World Health Organization Report of the WHO Task Force on stroke and other cerebrovascular disorders [36]. All patients underwent diagnostic imaging for CNS and cardiac pathologies, including Computed Tomography (CT) and/or Magnetic Resonance Imaging (MRI) scans, duplex ultrasound for carotid artery diseases and transthoracic echocardiography.

Exclusion criteria were: previous or present cardiac arrhythmias; known blood clotting abnormalities; cancer with or without radiation therapy to the head and neck region. A further exclusion criterion was being of non-Italian descent, but no such individual presented to either stroke unit for acute stroke of non-cardioembolic origin during the study period.

The control group consisted of 320 blood donors (168 males and 152 females, mean age 46 ± 20 years) referred from the Blood Bank of the San Giovanni Battista Hospital. Cases and controls resided in Turin, Northwestern Italy (Table 2, 3).

### Haptoglobin determination

Blood samples from patients were collected, immediately centrifuged, and serum was stored at -70°C. A volume of 5 µl of plasma diluted in saline solution at 1:50 was mixed with 2 µl of 1 M phosphate buffer pH 7.0 and 10 µl of 9 M urea, and immediately frozen at –70°C for 2 hours. Before electrophoresis, samples were mixed with bromophenol blue and 10 µl of × 5 sample buffer (10% SDS and 2.5 mL 0.5 M TRIS-HCl, pH 6.8 and 2 mL glycerol), then boiled for 5 min [37]. Samples were run on an 8% acrylamide/bis-acrylamide gel (30% w/v GIBCO Life Technologies, Gaithersburg,

**Table 3:** Risk factor analysis in 320 controls and 160 patients with ischemic stroke.

| Markers | Controls N =320 (%) | Patients N =160 (%) | P value |
|---|---|---|---|
| Smokers | 118 (36.38) | 112 (70) | 0.01 |
| High blood pressure | 70 (21.87) | 110 (68.75) | <0.001 |
| Obesity | 58 (18.13) | 67 (41.87) | 0.015 |

**Table 4:** Analysis of four blood markers in 320 controls and 160 patients with ischemic stroke.

| Markers | Controls N =320 | Patients N =160 | P value |
|---|---|---|---|
| Blood glucose level | 85 ± 16 mg/dL | 124 ± 33 mg/dL | <0.003 |
| Total cholesterol | 179.0 ± 36.1 mg/dL | 236.7 ± 68.7 mg/dL | <0.05 |
| White blood cells | 7.23 ± 3.63 K/µL | 8.5 ± 4.39 K/µL | NS |
| Hemoglobin | 12.67 ± 2.30 g/dL | 16.9 ± 1.0 g/dL | 0.05 |

Plus-minus values are means ± SD

MD, USA) using the buffer system of Laemmli for 45 minutes at 200 volts, then transferred onto a nitrocellulose membrane (Hybond ECL, Amersham, Cleveland, OH, USA) for 80 minutes at 50 volts, according to the Western blotting procedure [38-40]. After overnight saturation in 10% PBS-BSA (phosphate buffered saline-bovine serum albumin), membranes were washed with PBS-Tween 0.1% and incubated with the primary antibody (mouse anti-human haptoglobin Abs, Sigma-Aldrich, Milan, Italy), at a dilution of 1:4000 (in PBS-Tween), for 90 minutes. Before exposure to a 1:1000 PBS-Tween dilution of peroxidase-conjugated anti-mouse IgG from sheep (Amersham), membrane strips were washed for 30 minutes with four changes of PBS-Tween buffer. The Hp bands were visualized with a chemiluminescence method using ECL reagents (Amersham). The maximum light emission was 428 nm detected by a short exposure to blue-light sensitive autoradiography film (Hyperfilm ECL, Amersham). Protein concentrations were determined with BCA reagents (Pierce, Rockford, IL, USA) using BSA as the standard.

### Laboratory blood chemistry

Standard laboratory methods were used to measure serum concentration of glucose, total cholesterol, white blood cells and hemoglobin.

### Statistical analysis

Data are reported as means ± Standard Deviation (SD). The distribution of the Hp phenotype in cases and controls were compared using the chi-square test ($\chi^2$) (Table 4).

## Results

### Hp phenotype frequency

The incidence of Hp 2-2 was 61.25% in the ischemic stroke group and 43.75% in the control group (p =0.04) ($\chi^2$ =3918); the incidence of Hp 1-1 and 2-1 in the ischemic stroke group was 11.25% and 27.50%, respectively, and 13.75% and 42.50%, respectively, in the control group (Table 1). The higher frequency of the Hp 2-2 phenotype in patients with acute ischemic stroke suggests that it may genetically favor cardiovascular diseases (Table 1).

## Discussion

This study report- for the first time in an Italian population- a correlation between the Hp 2-2 phenotype and susceptibility to acute ischemic stroke of non-cardioembolic origin. The increased frequency of the Hp 2-2 phenotype in stroke patients suggests that this genetic trait may represent a marker for increased susceptibility to ischemic stroke of non-cardioembolic origin. The $\chi^2$ test showed a significant difference (p =0.04) between Hp 2-2 phenotype distributions in patients and controls ($\chi^2$ =3918). In a study on different European populations, the reported distribution of Hp phenotypes 1-1, 2-1 and 2-2 was 16%, 48%, and 36%, respectively [41]. The distribution in our controls was slightly different: 13.75%, 42.50%, and 43.75%, respectively. This discrepancy in phenotype distribution weakened the support for the apparent association between the Hp 2-2 phenotype and susceptibility for ischemic stroke.

A highly significant increase in the incidence of the Hp 2-2 phenotype was reported for a group of patients at high risk for myocardial infarction compared to a control group [42]. Ijäs et al just reported a 2.4 higher risk of dying due to cardiovascular disease for patients carrying the Hp2 allele, after their first ischemic stroke [43].

The Hp 2-2 phenotype appears to be a genetic risk factor for developing coronary atherosclerosis and peripheral arterial occlusive disease. A higher susceptibility to cardiovascular disease in OSAS was also correlated with the Hp 2-2 phenotype.

The median age of the population of Turin was 46.1 years in 2016 much higher than that of the general population in the USA (37.9 years in 2015) [44]. Since the ageing Turin population is at increased risk of IS, methods that can ascertain risk factors for it are important.

Around 40% of Italians aged 35 years to 64 years have high blood pressure; in this age group, the mortality rate for ischemic stroke is 40 in 100,000 people [45]. This means that approximately 1 in 1000 deaths at age 35 years to 64 years are stroke-related [45]. It is therefore essential that stroke-preventive programs target high-risk individuals. Risk factor analysis (Table 3) suggests that elevated triglyceride levels and hypercholesterolemia are seldom relevant in the pathogenesis of ischemic stroke [46]. We therefore propose a method for identifying people at risk of ischemic stroke, namely those with an Hp 2-2 phenotype, alongside hypertension and other known risk factors. Further work is needed to determine whether a more aggressive treatment can benefit those patients with an increased risk of stroke.

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