

Interaction between HFE and haptoglobin polymorphisms and its relation with plasma glutathione levels in obese children

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Abstract: Obesity among children has emerged as a serious public health problem. The growing prevalence of childhood obesity has led to the appearance of serious complications, including a chronic systemic inflammation associated with oxidative stress. In the present study, we analysed the interaction between two genes related with iron metabolism - *HFE* and haptoglobin – and the plasmatic concentration of glutathione, as a way to evaluate the antioxidant response capacity in obesity. To achieve this, 118 obese children and 89 eutrophic children were recruited for the study. Results showed that although obese children present a significantly decreased tGSH levels, once we analysed separately children based on their haptoglobin phenotype, the decreased tGSH levels is significant only for the Hp 2 allele. Additionally, Hp 2.2 obese children carrying H63D polymorphism show significantly lower tGSH/GSSG values. Our results found an association of haptoglobin and HFE with oxidative stress in childhood obesity.

Key words: Obesity; Inflammation; Glutathione; HFE; Haptoglobin; Gene interaction.

Introduction

Obesity is a pathologic condition whose incidence increased substantially in the last four decades (1). The pathogenesis of this disease is complex and includes genetic and environmental factors, as well as their interaction (2). Obesity in childhood, as in adults, causes a wide range of serious complications such as diabetes, cardiovascular diseases, chronic inflammation and oxidative stress (3–8).

Glutathione (GSH) is considered the main non-protein thiol involved in the antioxidant cellular defence, being essential for mechanisms of cellular detoxification and protection against oxidative attack (9–13). Maintaining adequate GSH levels, turnover rates and oxidation status are important for a number of critical cell functions, and disruptions in these processes are observed in many human pathologies (14, 15). Reduced GSH levels are frequently used as a marker for oxidative stress response (14). GSH exists in reduced and oxidized forms. In normal conditions, more than 95% of total glutathione pool is in the reduced form and less than 5% exists in the oxidized form in the cell (16). Total GSH (tGSH) includes reduced GSH and oxidized GSH (GSSG - glutathione disulphide). A decrease in the tGSH/GSSG ratio is considered indicative of oxidative stress.

Haptoglobin (Hp) is an acute phase protein involved in clearing extracellular haemoglobin and regulating in-

flammation (17). Haptoglobin levels are increased during inflammation and giving that obesity is an inflammatory condition, Hp is in general amplified in the blood of obese people (18). Hp major physiological function is to capture and remove free haemoglobin (Hb) from plasma, preventing the damage caused by Hb oxidative activity. Certainly, one of the problems after intravascular haemolysis is due to the fact that free Hb is capable to pass-through the glomerular filter and damage the kidney (19). In addition, free Hb can catalyse the oxidative reactions of LDL and injure the endothelial vascular cells (20). Also, the release of iron from the heme group will promote the Fenton reaction leading to an oxidative overload (21). In fact, the increment of this protein during the inflammation condition is probably activated by a compensatory mechanism of the antioxidant response (22). There are three possible genotypes/phenotypes of the Hp protein found in humans: Hp 1.1, Hp 2.1, and Hp 2.2. The different Hp genotypes have been shown to bind Hb with different affinities, with Hp 2.2 being the weakest binder (23). Indeed, others studies have shown that the Hp oxidative protection is phenotype-dependent (24–26), with Hp 2.2 presenting a lower antioxidant response (24, 25, 27–29). An association of Hp 2.2 with elevated levels of inflammatory cytokines was observed in obese subjects (30).

Iron overload leaves a fraction of the iron free, and especially its cellular redox-active form, the labile iron pool, catalyses the generation of ROS (Reactive Oxy-

gen Species), leading to oxidative stress (31). Principal mechanisms of iron-induced free radical activation are Fenton (21) and Haber–Weiss (32) reactions. Others authors have demonstrated that the dietary iron in the context of genetic obesity, can accelerate the progression of liver disease because of several factors such as inflammation and hepatic oxidative stress (33). Obesity is characterized by increased adipose tissue iron content producing preconditions for adverse effects of iron overload (34). The hemochromatosis gene, *HFE*, on chromosome 6p, encodes the major histocompatibility complex MHC class I-like protein HFE that binds beta-2 microglobulin and regulates iron absorption by modulating the expression of hepcidin, the main controller of iron metabolism (35). Defects in this gene are related to hereditary hemochromatosis, a disease characterized by excessive iron absorption (36). Hemochromatosis is primarily associated with the homozygosity for the C282Y mutation in the *HFE*, but another variant in this gene, the c.187C>G (rs1799945), known as H63D, that is more frequently distributed worldwide, appears to be also associated with hereditary hemochromatosis (37) and iron metabolism deregulation.

Others authors' studies demonstrated the existence of Hp gene as a modifier gene in Hemochromatosis (36, 38–40). Indeed, Hpt 2-2 phenotype was already associated with an accelerated iron overload (38).

The aim of this work was to gain insight into the role of the epistatic relationship between two iron metabolism related genes - *Hp* and *HFE* - in the antioxidant response capacity in obese children.

Materials and Methods

Subjects

In this work we studied a total group of 118 obese children (56 males and 62 females), according to Cole cohort, followed up in the Paediatric Department from Hospital Santa Maria. Their average age was 12.89 ± 2.50 years. A total of 89 eutrophic children were used as control sampling (40 males and 49 females), with an average age of 13.95 ± 0.79 years. During the experiments, some of the data did not achieved our threshold established for the quality standards and it was decided to exclude them from results, in addition given the hospital practises, it was not possible to repeat the collection of sample from the same subject. Both circumstances contributed for the situation of having a different number of subjects (n) studied for each variable. The study protocol was approved by the local Ethical Committee and an informed consent was obtained from the guardian of each patient before participation.

Quantification of plasma tGSH and GSSG concentrations

A spectrofluorimeter Shimadzu RF-5000 was used after plasma protein precipitation with 25% (v/v) metaphosphoric acid in order to achieve total plasma glutathione concentration. This method is based on that described previously by Hissin and Hilf (41) which uses GSH reaction with o-phthalaldehyde 1 mg/mL (OPT) in phosphate buffer 0.1 M-EDTA 0,005 M, pH 8 and between GSSG and OPT in NaOH 0.1 N, pH 12, in the presence of N-ethylmaleimide 0.04M to avoid GSH in-

terference in GSSG quantification. GSH and GSSG excitation and emission spectrums present maximums at 340 and 430 nm, respectively. Data were expressed as means \pm standard deviation.

Haptoglobin phenotyping

The Hp phenotype was determined by polyacrylamide gel electrophoresis (PAGE). A 10% hemoglobin solution in water was prepared from heparinized blood by first washing the blood cells five times in phosphate-buffered saline (0.1 mol/L, pH 7.2) and then lysing the cells in 9 mL of sterile water per milliliter of pellet cell volume. A supernatant of a cell lysate containing hemoglobin was aliquoted in 1 or 0.5 mL and stored at -20°C. Hp phenotyping was determined by gel electrophoresis and peroxidase staining, using a modified version of the method described previously (42). Briefly, serum (20mL) was mixed with 10 mL of the 10% hemoglobin solution and 15 mL of 40% saccharose, and the samples were left to stand for 5 minutes at room temperature to allow the formation of Hp-Hb complexes. The Hp-Hb complex was resolved by PAGE using a buffer containing 50 mmol/L Tris base and 384 mmol/L glycine. The gel was 14 mL of 40% acrylamide/bis-acrylamide in 14 mL of 3 mol/L Tris-HCl, pH 8.9 and 21 mL of bidestilated water. Three hundred and fifty microliters of N,N,N',N'-Tetramethylethylenediamine and 1 mL of ammonium persulphate (12 mg/mL) were added to the previous solution. After the completion of electrophoresis, which was performed at a constant voltage of 250 for 4 hours, the Hp-Hb complexes were visualized by soaking the gel in two freshly prepared staining solutions in a glass tray. The first staining solution contained ortho-dianisidine 5 mg/mL in 50% (vol/vol) glacial acetic acid, and the second one was made of 2% (vol/vol) hydrogen peroxide. The bands corresponding to the Hp-Hb complex were readily visible within 15 minutes and were stable for more than 48 hours.

Genomic DNA Isolation

Whole blood samples from patients and controls were stored with EDTA at -20°C. The genomic DNA was isolated through a nonenzymatic method adapted from Lahiri and Numberger (1991) method (43).

HFE genotyping

The c.187C>G polymorphism in the *HFE* gene, corresponding to the p.His63Asp variant, was screened in DNA samples by a PCR-restriction approach, as previously described (44). PCR was carried out in 25 μ L reaction volume, containing 1 μ L (\approx 90 ng) of the genomic DNA template and 1 μ L (20 pmol) of each of sense and antisense primers: 5'-ACA TGG TTA AGG CCT GTT GC-3' and 5'-CTT GCT GTG GTT GTG ATT TTC C -3' (primers from Metabion, Germany). These primers amplify a fragment with 294 bp that was then restricted by *Mbo* I (enzyme from New England Biolabs, Germany). The PCR program included a step of 94 °C, for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. An additional extension step was performed in the final at 72 °C for 5 min. To improve clarity, we will henceforward use the protein change (H or D) as a surrogate for genotype.

Table 1. Comparisons of tGSH (μmol/L), tGSH/GSSG, Hp phenotypes and *HFE* genotypes between controls and obese children.

	Control	Obese	<i>p</i> -value
tGSH (μmol/L) ^a	48.78±13.95 (51)	33.06±14.63 (91)	<0.001
tGSH/GSSG ^b	8.31; 4.74 – 24.93 (51)	7.84; 2.49 – 16.79 (91)	0.173
Hp phenotype^c			
1.1	5.30 (8)	7.95 (12)	
2.1	13.91 (21)	37.75 (57)	0.510
2.2	9.93 (15)	25.16 (38)	
H63D^c			
HH	13.99 (20)	49.65 (71)	0.504
HD/DD	6.29 (9)	30.07 (43)	

^a Mean ± standard deviation (n). ^b Median; minimum – maximum (n). ^c% (n).

Table 2. Comparisons of tGSH (μmol/L) and tGSH/GSSG between Hp phenotypes, in controls and obese children.

Population	Parameter	Hp 1.1	Hp 2.1	Hp 2.2	<i>p</i> -value
Control	tGSH (μmol/L) ^a	56.46 ± 16.66 (6)	42.10 ± 14.11 (13)	46.98 ± 12.92 (14)	0.135
	tGSH/GSSG ^b	9.76; 6.77 – 11.53 (6)	7.61; 4.74 – 10.03 (13)	8.43; 5.11 – 24.93 (14)	0.226
Obese	tGSH (μmol/L) ^a	43.80 ± 13.99 (10)	32.49 ± 13.64 (47)	28.89 ± 13.94 (31)	0.015
	tGSH/GSSG ^a	9.42 ± 3.44 (10)	8.18 ± 2.74 (47)	7.16 ± 2.78 (31)	0.074

^a Mean ± standard deviation (n). ^b Median; minimum – maximum (n).

Statistical analysis

Differences between the two groups of children were tested by the χ^2 for discrete variables and T test (parametric test)/ Mann-Whitney test (non-parametric test) for continuous variables. The comparisons within the groups were performed using T test/ANOVA parametric tests or Mann-Whitney/ Kruskal-Wallis non-parametric tests. All tests were performed with SPSS 24.0 software. Statistical significance was defined as a *p*-value < 0.05.

Results

Plasma tGSH levels were determined in 91 obese children and compared to those obtained in 51 eutrophic children. Although tGSH/GSSG ratio does not present significant differences (*p*=0.173), tGSH levels are significantly decreased in obese children (*p* < 0.001) (Table 1).

Forty-four eutrophic and 107 obese children were analysed for the Hp phenotype and 29 eutrophic and 114 obese children for H63D genotype. No significant difference was found between the two populations regarding Hp phenotypes or H63D genotypes (*p*=0.510 and *p*=0.504, respectively; Table 1). However, after analysing the 33 eutrophic and 88 obese children that presented values for both tGSH or tGSH/GSSG and Hp phenotype, obese children with Hp 2 allele present significantly decreased of tGSH levels (*p*=0.015; Table 2). These children also have a trend to present lower tGSH/GSSG ratio (*p*=0.074). On the other hand, there is no significant difference between the haptoglobin genotype

and tGSH levels or tGSH/GSSG ratio in eutrophic children (*p*=0.135 and *p*=0.226, respectively; Table 2).

Regarding *HFE* gene, were analysed the 16 eutrophic and 86 obese children that presented values for the three variables - tGSH or tGSH/GSSG and Hp phenotype. No significant difference between H63D genotypes and tGSH levels or tGSH/GSSG ratio in obese (*p*=0.337 and *p*=0.272 respectively; Supplementary table 1) or eutrophic children (*p*=0.337 and *p*=0.376, respectively; online resource 3) was observed. However, we found that obese children carrying the G allele (H63D variant) and the Hp 2.2 genotype show a lower tGSH/GSSG value (*p*=0.037) and a trend to decreased tGSH levels (*p*=0.079). No significant differences were found in eutrophic children. These results are shown in Table 3.

Discussion

Obesity is now among the most widespread medical problems affecting children in developed countries. Childhood obesity represents one of the greatest health challenges by increasing the child's risk of numerous medical conditions, such as hypertension and diabetes in children and increases the risk of early cardiovascular disease in adults.

Aiming to study how the haptoglobin phenotype and the H63D polymorphism in the *HFE* gene could mediate the antioxidant response capacity to the inflammatory process in obese children we determined the tGSH and tGSH/GSSG levels in a group of well-characterized children. Diverse studies have shown that oxidative

Table 3. Comparisons of tGSH (μmol/L) and tGSH/GSSG between the presence of Hp 2.2-HD/DD and the absence of Hp 2.2-HD/DD, in controls and obese children.

Population	Parameter	Hp 2.2 - HD/DD - absence	Hp 2.2 - HD/DD – present	<i>p</i> -value
Control	tGSH (μmol/L) ^a	46.18 ± 12.80 (13)	50.30 ± 14.37 (3)	0.629
	tGSH/GSSG ^b	8.42; 5.11 – 24.93 (13)	7.53; 7.51 – 10.29 (3)	1.000
Obese	tGSH (μmol/L) ^a	33.60 ± 14.14 (74)	25.71 ± 15.07 (12)	0.079
	tGSH/GSSG ^b	8.23; 3.70 – 14.26 (74)	6.41; 2.49 – 16.79 (12)	0.037

^a Mean ± standard deviation (n). ^b Median; minimum – maximum (n).

stress is increased in obese adult (45, 46) and children (47–49) and may be associated with the development of co-morbidities in this disease. In this study, we evidenced a depletion of tGSH in obese children, which is consistent with some previous studies that relate GSH to obesity (50–53). In fact, glutathione plays a crucial role in the defence against free radicals. Its depletion may lead to a cascade of harmful reactions ultimately culminating in oxidative stress in obese children.

The fact that we did not observe significant differences between Hp phenotypes among obese children and controls, allows us to conclude that Hp phenotype is not a predisposing factor to childhood obesity. However, it is verified that obese children with Hp 2.1 and Hp 2.2 present significantly decreased tGSH levels and also have a trend to present lower tGSH/GSSG ratio. Previous studies have shown a relation between Hp 2 or Hp 2.2 and oxidative stress in other diseases (28, 54, 55). It also has been reported that individuals with the haptoglobin 2.2 present increased iron concentrations (38, 39, 56). As a matter of fact, Hp alleles differ in their ability to clear free Hb from the plasma where Hp 1-Hb complexes are cleared more efficiently from the plasma than Hp 2-Hb complexes (26), thus subjects with Hp 2.2 are more susceptible to oxidative stress. Our results suggest that obese children with Hp 1.1 may be better protected against oxidative lesions resulting from the inflammatory process and are in accordance with Hp 1 higher binding affinity for hemoglobin and the consequent decreased oxidative reactions catalyzed by the Hb iron.

We did not observe significant differences between *HFE* among obese children and controls. Also, there is no significant difference concerning the *HFE* genotypes and levels of total plasma GSH and ratio tGSH/GSSG in obese children. These results lead us to suggest that this genotype is not a predisposing factor for the disease and that the H63D polymorphism in the *HFE* gene alone is not associated with the tGSH and the tGSH/GSSG ratio. However, the association between the H63D variant and the Hp 2.2 genotype in obese children is related with lower levels of tGSH/GSSG, as well as with a trend to present a decrease in the tGSH level. The H63D variant in *HFE* gene, is mainly investigated as a modulator in several diseases (57–61). It appears to be associated with a milder iron overload (62, 63). Thus, giving that H63D affects the structure of the *HFE* protein, we propose that carriers of this mutation will be more susceptible to iron overload which may promote oxidative stress by increasing the steady-state concentration of intermediate oxygen radicals.

The results of this study suggest that the association between the H63D variant, possibly responsible for the increased accumulation of plasma iron, and the phenotype of haptoglobin 2.2, with lower binding affinity to the heme group, seems to lead to a decrease in the tGSH/GSSG ratio and tGSH. Due to the excessive accumulation of iron in plasma and the lower antioxidant capacity of the Hp 2.2 phenotype, the Fenton reaction is more likely to occur, leading to increased ROS generation and consequently oxidant overload.

Our findings indicate an epistatic association between haptoglobin and *HFE* genes in the process of oxidative stress in childhood obesity. Indeed, genetic inter-

actions between different *loci* have been thought to be of major importance in complex phenotypes. However, further studies investigating the role of other genetic variants and other markers for oxidative stress will be of high importance. In addition, functional studies are required to understand the functional association between the genetic variants, oxidative stress and the development of obesity. These investigations may provide new insights into the mechanisms underlying this pathologic condition whose incidence increased substantially in the last decades.

Interest conflict

The authors declare that they have no conflict of interest.

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Author's contribution

Aguiar L made the statistical analysis with support from Ferreira J. Aguiar L also wrote the manuscript with the support from Inácio A. Marinho C conceived the presented idea and performed the haptoglobin analysis. Martins R made the HFE molecular analysis supervised by Faustino P. Alho I made de GSH laboratory analysis. Levy P was the contact to the Hospital. Bicho M contributed to the interpretation of the results. Inácio A supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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