Original Article

Smokers are Over-Represented in Subjects with Ahaptoglobinemia in Ghana

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Aim: Smoking has been established as a major risk factor for cardiovascular disease. It causes oxidative stress and sub-clinical inflammation, which undermine the antioxidant defense system of the body. We reasoned that natural antioxidant defense systems may be compromised in smokers. To this end, we examined whether haptoglobin (Hp), a potent antioxidant, is impacted negatively by smoking.

Methods: Study participants consisted of 121 current smokers and 105 healthy non-smokers without diabetes and without blood smear-positive *P. falciparum*. Smokers were defined as individuals who smoke at least 1 cigarette a week and are current smokers (occasional and regular). Baseline demographics, hematological indices, lipid profiles, blood pressure, lactate dehydrogenase activity and haptoglobin phenotypes were determined.

Results: Ahaptoglobinemia was found to be highly overrepresented in smokers (odds ratio (OR)=3.1, 95% confidence interval (CI)=1.5–6.5, $p=0.002$). This observation was not attributed to intravascular hemolysis. Hp2-2 phenotype was found to be under represented in smokers (OR=0.53, 95% CI=0.28–0.99, $p=0.05$). Smoking was confirmed to augment hypertension (diastolic blood pressure (DBP) and systolic blood pressure (SBP) in male smokers ($p=0.0001$). Interestingly, however, this appeared not to be related to lipid metabolism, as HDL was elevated ($p=0.0007$) while LDL was decreased ($p=0.004$) in smokers within the study population.

Conclusion: We conclude that smoking is a risk factor for ahaptoglobinemia, which will impact negatively on anti-oxidant defenses and augment pro-oxidative stress effects.

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Key words; Smoking, Ahaptoglobinemia, Antioxidant defense

Introduction

Cigarette smoking is a debilitating habitual activity and a leading cause of preventable morbidity and mortality¹. The estimated annual deaths due to smoking worldwide are 5.4 million². It is a major cause of cardiovascular diseases, obstructive pulmonary disease and cancers³, accounting for 11% of all global deaths due to cardiovascular disease⁴. In particular, athero-

sclerosis and peripheral arterial disease are firmly established to be facilitated by smoking⁵. Approximately 1.1 billion people around the world smoke and the number is estimated to increase to 1.6 billion in the year 2025^{6,7}. Given that cigarette smoking is such a major threat to global public health, understanding the mechanisms by which smoking leads to a plethora of vascular diseases is of prime importance.

Subclinical inflammation and oxidative stress form the underlying cause of the pathophysiological processes attributed to smoking⁸. Both factors lead to endothelial damage and dysfunction, which then activates atherosclerotic processes. Oxidative stress is defined as an increase in the number of reactive oxygen species (ROS), including superoxide ($\bullet\text{O}^-$), hydro-

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gen peroxide (H_2O_2), hydroxyl radical ($\bullet\text{OH}^-$) nitric oxide free radical ($\bullet\text{NO}$) and peroxynitrite (ONOO^-); and/or a reduction in the antioxidant defense system⁹). Increased ROS generation is one of the defined factors associated with smoking and cardiovascular events¹⁰. On the other hand, ROS generation due to decreased antioxidant defenses has not been clearly established. In fact, the use of antioxidant vitamins C and E as a means of reducing the ravages of cardiovascular disease in smokers has been positive^{11, 12}. In the present study, we asked whether haptoglobin (Hp), a potent antioxidant (better than vitamins C and E)¹³, is negatively impacted by smoking. Haptoglobin exists as three polymorphs in humans, namely Hp1-1, Hp2-1, and Hp2-2¹⁴. A fourth phenotype, Hp0 (ahaptoglobinemia or hypohaptoglobinemia), has been shown to have both genetic and environmental causes^{15, 16}. In Africans, the genetic cause of ahaptoglobinemia and hypohaptoglobinemia has been established to be due to two mutations (A-61C and C-101G) in the promoter region of the Hp gene¹⁵. The major environmental factor responsible for ahaptoglobinemia in sub-Saharan Africa is infection with *P. falciparum*¹⁶.

Clinically, diabetic patients with the Hp1-1 phenotype show strong resistance to diabetic retinopathy, nephropathy and cardiovascular disease¹⁷. Mechanistically, the strong antioxidant and pro-TH2-dependent properties of this phenotype^{18, 19} are suggested to play a major role in this observation. The corollary is true for the Hp2-2 phenotype, with Hp2-1 being intermediate. We reasoned that if smoking impacts negatively on Hp expression, then it could be a major facilitator for the development of cardiovascular disease in smokers. As a genetically endowed and potent antioxidant, its effect could then be explored to manage vascular damage in smokers. We show in this study for the first time that indeed smoking is associated with ahaptoglobinemia. This finding opens up a new window into the mechanism by which smoking facilitates cardiovascular events, by reducing the body's naturally potent antioxidant defense system.

Materials and methods

Subjects

The study participants were recruited consecutively from the general population in Ghana who smoked or did not smoke. A total of 232 subjects were studied, consisting of 127 current smokers and 105 healthy non-smokers without diabetes and *P. falciparum* parasitemia. The two study groups were of comparable age. The smokers were defined by the standard Office of Population Census and Surveys/Office for National

Statistics (OPCS/ONS) criteria as individuals who smoke at least 1 cigarette a week and are current smokers (occasional and regular)²⁰. The non-smokers did not smoke any cigarettes at all. The subjects were enrolled after the study objectives had been explained and their written informed consent obtained. The protocol was approved by the Ethical and Protocol Review Committee of the University of Ghana Medical School.

Procedures

Each participant was interviewed to complete a questionnaire at recruitment. The questionnaire included the standard OPCS/ONS question on smoking status: 'Which best describes you now?' with the options: never smoked, only tried smoking once, used to smoke but gave up, smoke occasionally (sometimes), and smoke regularly (at least one cigarette a week). Current smokers were asked about the number of cigarettes smoked per week and for how long. For blood pressure (BP) measurement, the subjects were made to rest for 15 minutes prior to the measurement. Other demographics collected were age, weight, height and educational background, grouped into basic (illiterate + primary) and secondary + tertiary education.

Inclusion Criteria

All subjects diagnosed as non-diabetic, defined by the ADA criteria with a fasting plasma glucose less than 6.7 mmol/L and without blood smear positive for *P. falciparum*. *P. falciparum* parasitemia was determined by Giemsa-stained thick and thin blood smears and examined by an experienced microscopist.

Exclusion Criteria

All subjects diagnosed as diabetic based on the ADA criteria, (fasting plasma glucose ≥ 7.0 mmol/L or 126 mg/dL) with a blood smear positive for *P. falciparum* and/or obese (BMI greater than 30 kg/m²) were excluded.

Blood Sample Collection

Fasting venous blood (5 mL) was collected into plain and EDTA tubes and processed for determination of serum blood sugar, lactate dehydrogenase activity (LDH), triglycerides, cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and haptoglobin phenotypes. Blood collected into EDTA tubes was used for hematological indices.

Laboratory Procedures

Fasting blood glucose, LDH, total cholesterol and triglycerides were determined with kits purchased

Table 1. Baseline demographics of smokers and non smokers

Parameter/Group	Smokers	Non-smokers	<i>p</i> value
Sex	M	M	
N	121	105	
Age, yrs	38.4 ± 12.9	42.7 ± 12.3	(0.01)
BMI, kg/m ²	22.0 ± 2.8	24.2 ± 4.9	(0.0001)
Cigarettes/day	9.2 ± 7.9		
Yrs smoked	17.5 ± 13.2		
Education (%)			
* Basic	77.4	60.7	χ^2
Secondary & Tertiary	22.6	39.4	

*Basic education consisted of illiterate subjects and those who had received primary school education. Values are the means ± SD. (N=number of subjects)

from Centronic GmbH (Kleinfeld, Germany). LDL and HDL were assayed with reagents from Biosystems (Barcelona, Spain). All assays followed the manufacturer's instructions. The analyses were performed using the Vital Scientific Microlab 300 (VSM 300) automated analyzer. Hemoglobin (Hb) was determined using Sysmex K2 hematological analyzer.

Haptoglobin Phenotyping

Haptoglobin phenotypes were determined by polyacrylamide gel electrophoresis of haemoglobin-supplemented serum followed by staining with a benzidine solution that contained o-dianisidine. The stain gives a clear visualization of the phenotypes that is unambiguous in phenotype typing.

Statistical Analysis

The results were subjected to unpaired Student's *t*-test for all variables between smokers and non smokers, except for the educational status, which was assessed by the χ^2 test. The odds ratio (OR) associated with the Hp0 phenotype and smoking was determined at the 95% confidence interval (CI) and $p < 0.05$. The phenotype distribution was assessed for agreement with Hardy Weinberg equilibrium.

Results

In general, fewer females (4.7%) smoked than males (95.3%) within comparable age limits (data not shown); as a result, only data for males are reported (**Table 1**). The smokers were found to be younger ($p = 0.01$) and leaner ($p = 0.0001$) than the non-smokers. The number of cigarettes smoked averaged 9 per day with a minimum of 1 daily, over a period of 17 years. The minimum period of smoking was 4 years.

Table 2. Clinical variables, lipid profiles and hematological markers in the subjects

Parameter/Group	Smokers	Non-smokers	<i>p</i>
Sex	M	M	
N	121	105	
† SBP, mmHg	138.4 ± 24.0	124.7 ± 25.9	0.0001
DBP, mmHg	87.9 ± 14.7	74.6 ± 14.7	0.0001
Pulse, bpm	75.6 ± 10.7	72.4 ± 10.6	NS
FPG, mmol/L	4.4 ± 0.8	4.6 ± 0.8	NS
TC, mmol/L	4.5 ± 1.1	4.8 ± 1.2	NS
TG, mmol/L	0.7 ± 0.4	0.8 ± 0.4	0.06
LDL, mmol/L	2.9 ± 1.2	3.4 ± 1.4	0.004
HDL, mmol/L	1.3 ± 0.7	1.0 ± 0.6	0.0007
Hb, g/dL	13.4 ± 1.9	13.2 ± 1.9	NS
MCV, fl	85.7 ± 8.8	84.3 ± 8.7	NS
LDH, U/l	723.4 ± 496.9	750.9 ± 467.8	NS

† SBP=systolic blood pressure, DBP=diastolic blood pressure, FPG=fasting plasma glucose, TC=total cholesterol, TG=triglycerides, LDH=lactate dehydrogenase. Values are the mean ± SD. (N=number of subjects).

In male smokers, a significant proportion (3:1) had received only a basic education. The odds of a male with at least a secondary education smoking was very low: OR=0.47, 95% CI=0.25–0.86, $p = 0.02$, $\chi^2 = 4.7$, $p = 0.03$).

On the clinical indices, significant differences between smokers and non-smokers were seen for diastolic blood pressure (DBP), systolic blood pressure (SBP), LDL and HDL (**Table 2**). DBP and SBP were significantly higher in smokers ($p = 0.0001$, respectively). Serum LDL was lower in smokers ($p = 0.004$) while HDL was elevated ($p = 0.0007$). TG was marginally decreased in smokers ($p = 0.06$). Serum LDH levels and Hb and MCV levels did not differ between the groups.

Smoking was strongly associated with ahaptoglobinemia (OR=3.1, 95% CI=1.5–6.4, $p = 0.002$ (**Table 3**)). The Hp1 allele frequencies in non smokers and smokers were 0.45 and 0.54, respectively. The difference was not significant ($p = 0.07$). Subjects with Hp2-2 phenotype were underrepresented in smokers (OR=0.53, 95% CI=0.28–0.99, $p = 0.05$). When the clinical indices were categorized by Hp phenotypes in smokers and non-smokers (**Table 4**), it emerged that Hp0 subjects who smoked had lower LDH levels than those with other phenotypes who smoked.

Discussion

The present study is the first to show that smok-

Table 3. Odds for association of smoking with ahaptoglobinemia

Subject category/ Hp phenotype	Hp0	Hp1-1	Hp2-1	Hp2-1M	Hp2-2
Smokers	35	26	27	11	22
Non-smokers	12	24	31	7	31
OR (95%CI)	3.1 (1.5-6.5)	0.92 (0.49-1.73)	0.69 (0.38-1.25)	1.4 (0.52-3.75)	0.53 (0.28-0.99)
P	0.002	0.92	0.22	0.67	0.05

Table 4. Clinical variables, lipid profiles and hematological markers in subjects with Hp0 and other Hp phenotypes for smokers and non-smokers

Parameter	Smokers Hp0 <i>n</i> = 35	Non-smokers Hp0 <i>n</i> = 12	<i>p</i>	Smokers Other Hp [§] <i>n</i> = 86	Non-smokers Other Hp <i>n</i> = 93	<i>p</i>
SBP, mmHg	133.5 ± 27.5	116.7 ± 17.0	0.0001	130.9 ± 23.2	121.2 ± 12.9	0.0002
DBP, mmHg	83.4 ± 14.4	66.8 ± 8.9	0.0001	81.0 ± 15.6	72.7 ± 8.9	0.0001
Pulse, bpm	73.7 ± 14.3	71.0 ± 8.6	0.0001	74.2 ± 11.2	72.8 ± 9.2	NS
FPG, mmol/L	4.5 ± 0.9	4.6 ± 0.7	NS	4.3 ± 0.7	4.8 ± 0.7	0.0001
TC, mmol/L	4.5 ± 1.2	4.9 ± 0.7	0.003	4.5 ± 1.1	5.2 ± 1.2	0.0001
TG, mmol/L	0.8 ± 0.4	0.9 ± 0.7	NS	0.8 ± 0.4	0.9 ± 0.6	NS
LDL, mmol/L	2.7 ± 1.3	3.8 ± 0.7	0.0001	2.8 ± 1.2	4.0 ± 1.3	0.0001
HDL, mmol/L	1.4 ± 0.6	0.7 ± 0.3	0.0001	1.3 ± 0.6	0.8 ± 0.5	0.0001
Hg, g/dL	13.3 ± 1.4	12.6 ± 2.0	0.0001	13.0 ± 2.0	13.3 ± 2.0	NS
MCV, fL	86.5 ± 10.2	84.6 ± 5.8	0.09	85.7 ± 8.6	82.3 ± 8.5	0.003
LDH, U/l	680 ± 356.9	858.5 ± 447.8	0.001	680.4 ± 355.8	770.3 ± 479.3	NS

[§]Other Hp phenotypes were Hp1-1, Hp2-1, Hp2-1M and Hp2-2, [†]SBP=systolic blood pressure, DBP=diastolic blood pressure, FPG=fasting plasma glucose, TC=total cholesterol, TG=triglycerides, LDH=lactate dehydrogenase. Values are the means ± SD. (N=number of subjects)

ing is associated with ahaptoglobinemia. Ahaptoglobinemia has been established to be due to environmental and genetic factors in all populations^{15, 16, 21, 22}. The environmental factors precipitate hemolysis either intravascularly or extravascularly, leading to depletion of haptoglobin as it mops up released hemoglobin. In African populations, the major environmental factor associated with ahaptoglobinemia is malaria parasitemia¹⁶. This cause was ruled out in the present study as subjects with malaria parasitemia were excluded. In addition, markers of intravascular hemolysis, which includes elevated LDH, decreased Hb concentration (<11.0 g/dL) and in some cases elevated MCV²³ were not seen in smokers or non-smokers or subjects who smoked but were ahaptoglobinemic. Cumulatively, this suggests that intravascular hemolysis is an unlikely cause of the ahaptoglobinemia seen in smokers. A causal relationship between tobacco smoking and several cancers is well established^{24, 25}. Genetic analysis of these cancers, which include lung, pancreatic, head and neck, upper respiratory tract, lower respiratory tract and liver, has revealed that cancers arise from

smoke carcinogens that induce the sequential accumulation of multiple genetic and epigenetic alterations that eventually lead to overt cancer²⁶. We have previously reported that C-101G and A-61C mutations in the promoter of the Hp gene are respectively associated with hypohaptoglobinemia and ahaptoglobinemia in Africa¹⁵. The A-61C mutation is also responsible for decreased expression of the Hp gene in the Hp2-1M phenotype^{15, 27}. Could it be that smoking accelerates the generation of these mutations or causes new mutations that affect Hp gene expression? These questions are currently being investigated. The reduced numbers of subjects with the Hp2-2 phenotype is interesting. Although it is not clear to us why this is so, one scenario is possible. The Hp2-2 phenotype has been associated with a strong risk for the development of cardiovascular disease in diabetes, due to its weak antioxidant property compared to the Hp1-I phenotype^{17, 28}. It may be that individuals who smoke and have the Hp2-2 genotype are less able to survive cardiovascular insults from smoking.

Haptoglobin is an acute phase protein secreted in

response to inflammation, trauma, injury or malignancy. It is a potent antioxidant in the blood²⁹, reported to be 20 times more potent than vitamin C and 5 times more than probucol¹³. Recently there has been increased interest in the search for antioxidants that may effectively reduce cardiovascular events. This is based on the antioxidant hypothesis that inhibiting oxidative stress with antioxidants may lead to decreases in cardiovascular diseases. Although the use of the antioxidant vitamins C and E has not yielded the desired impact on other risk factors associated with cardiovascular disease, smokers have been seen to respond positively following treatment with vitamins C and E^{11, 12}. This suggests that antioxidant mechanisms are deficient in smokers. The present study provides evidence that ahaptoglobinemia may be another means by which smoking sustains subclinical inflammation and oxidative stress.

We observed, as already established, that the smokers were leaner than their non-smoking counterparts. As expected, blood pressure was elevated in smokers in the population studied. In contrast with some reports, LDL cholesterol was lower in smokers while HDL cholesterol was elevated³⁰. Clearly, the effect of smoking on lipid metabolism differs among populations.

We conclude that smoking is a risk factor for ahaptoglobinemia in the Ghanaian population, underlying the fact that a reduced antioxidant defense mechanism in smokers contributes to oxidative stress. In addition, the role of smoking in lipid metabolism needs further studies in different populations.

Competing Interest Disclosure

The authors declare that they have no competing interests.

Authors' Contributions

Contribution: EAT and KA performed the experiments under the supervision of IKQ, NAA and AGA. AGA also provided vital equipment for the analysis of samples. IKQ and AGA designed the study, while IKQ analyzed the results and wrote the paper. All authors read and approved the final manuscript.

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