

Determinants of Homocysteine levels in Ivorian rural population

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Abstract

In the present study, homocysteine (Hcy) and vitamin B status were determined in healthy subjects living in two opposite region of the Ivory Coast. 56 subjects from a coastal region (Bodou) having a fish-based diet and 56 subjects from a mountainous area (Glanle) having a vegetarian diet were included to determine on fasting blood sample Hcy, folate, vitamin B12, creatinine, lipid, inflammation and nutritional parameters. An increased prevalence of Hcy $\geq 15 \mu\text{mol/L}$ was observed, reaching 60% of all subjects. Bodou group exhibit significant higher Hcy levels than Glanle (20.1 [9.7-41.4] vs 13.6 [5.5-48.7] $\mu\text{mol/L}$, $p < 0.0001$), despite higher vitamin B12 levels (593 [163-1860] vs 234 [83-585] pg/ml , $p < 0.0001$). Although folate levels were lower in subjects from Bodou compared to Glanle (3.2 [2.0-7.3] vs 6.0 [1.9-18.2] ng/ml , $p < 0.0001$), there was no significant relationship with Hcy levels in any groups. Interestingly, there was significant higher creatinine levels in subjects from Bodou compared to Glanle and a significant positive relationship with Hcy levels was evidenced in each group. In conclusion, Hcy levels in an Ivorian population having a fish-based diet appeared significantly higher compared to subjects having a vegetarian diet. However, folate and vitamin B12 status are not emerged as the major determinants of this difference but a stronger relationship was observed with creatinine levels.

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is formed by demethylation of dietary methionine. In physiological conditions, an half of Hcy is remethylated in methionine and an half is condensated with serine to provide cystathionine and ultimately cysteine [1]. Vitamin B2 (riboflavin), vitamin B9 (folic acid) and vitamin B12 (cobalamin) act as cofactors for Hcy remethylation, while vitamin B6 (pyridoxal phosphate) is involved in the transsulfuration pathway providing cysteine. Increased Hcy levels can be associated to cardiovascular diseases [2-4] through its pro-oxidant activity leading to endothelial dysfunction, smooth muscle cell proliferation and a prothrombogenic profile [5-7]. More recently, a relationship between hyperhomocysteinemia (HHcy) and bone mineral diseases was highlighted [8,9] and could be related also to oxidative stress [10]. In addition, abnormal metabolism of Hcy is recognized as a key feature in neural tube defect occurrence [11]. In regard with Hcy involvement in such several health area, an adequate intake of vitamin B through nutrition appears of great interest [12]. Beyond physiological determinants including age, gender and renal function, plasma Hcy level is under the influence of nutritional status and genetic factors that interact with each other [13,14]. Interestingly, the methylenetetrahydrofolate reductase (MTHFR) 677C/T polymorphism that is known to interact with folate status is reported with a lower prevalence in West African than in European subjects [15]. However, studies on Hcy levels in West Africa countries are contradictory as higher Hcy levels were reported in gambia men compared to european men [16], while low Hcy levels were observed in Burkina Faso [17]. Because there is no available data on Hcy levels in Ivorian population, we studied the influence of dietary habits in a south and a west region

of Ivory Coast (fish-based or vegetarian diet, respectively) on plasma levels of Hcy, folates, vitamin B12, lipids, inflammation and nutritional parameters.

Subjects and methods

1- Subjects

112 apparently healthy subjects from 19 to 69 years old were recruited according to a random selection in Bodou region (56 subjects, 24 women and 32 men) and in Glanle region (56 subjects, 23 women and 33 men) situated respectively in the south and the west of Ivory Coast. Subjects of Bodou were from Adjoukrou ethnicity, related to the Akan group and subjects of Glanle were from Yacouba ethnicity, related to the Mande group. These 112 subjects were included in a study designed to assess the nutritional status, antioxidant capacity and oxidative stress biomarkers of rural populations living in a selenium deficient area in Ivory Coast [18]. The study protocol was approved by the Medical Sciences University of Abidjan, Ivory Coast, and was explained to the subjects before they gave their informed consent.

2- Anthropometric measurements and blood sampling

After 10 to 12 hours fast, anthropometric measurements were performed on each subject. Blood samples (10 ml) were collected by venipuncture in dry tubes (5 ml) and heparinized (5 ml) vacutainer tubes (Choay laboratories, Paris, France). Samples were stored in a refrigerated box and immediately transferred to the biochemistry laboratory of the Medical Sciences University of Abidjan. The supernatant was isolated by centrifugation at 4000 rpm/min for 15 minutes and aliquoted. Aliquots were stored at -80°C and carried by plane to the biochemistry

laboratory of Lapeyronie hospital, Montpellier, France. Aliquots were kept frozen at -80°C until analysis.

3- Assays

In this part of study, total plasma homocysteine (Hcy), serum vitamin B12 and folate were determined. Plasma Hcy concentrations were determined by high performance liquid chromatography (HPLC) method carried out on a Dionex apparatus equipped with an RF 1000 Dionex spectrofluorimeter (Dionex Corporation, Sunny Vale, CA, USA) as previously described [19]. We defined hyperhomocysteinemia (HHCY) as i) moderate for Hcy ranging from 15 to 30 $\mu\text{mol/L}$, ii) intermediate if $30 < \text{Hcy} < 100 \mu\text{mol/L}$, iii) severe for $\text{Hcy} \geq 100 \mu\text{mol/L}$, according to the classification of Kang et al [20].

Plasma folates and vitamin B12 levels were measured by a chemiluminescent immuno-enzymatic assay on a Access analyzer (Beckman and Coulter France SA, Villepinte, Roissy CDG, France). Folates and vitamin B12 concentrations less than 3 ng/ml (6.8 nmol/l) and 150 pg/ml (110 pmol/l) respectively served as criterion for defining low vitamin levels. In addition, the ratio Hcy/folate was calculated and a cut off at 4.4 was considered as a potential reflect of heterozygosity for cystathionine beta synthase (CBS) deficiency [21].

4-Statistical analysis

Characteristics of the population were described by using the median and the range for quantitative variables and proportions for categorical one. The distributions of continuous variables were tested with Shapiro-Wilk test and were skewed. Comparisons of quantitative variables were performed with Mann-Whitney test

and comparisons of categorical variables were performed with Chi square test. For the correlation studies, Spearman's correlation were used.

For all comparisons, significance was set at $p \leq 0.05$. Statistical analyses were performed using the SAS software, version 9.1 (SAS Institute, Cary, NC, USA).

Results

1- Anthropometric and biological data

There was no statistical difference between patients recruited at Bodou or Glanle in term of age (median 45 vs 37.5 years, respectively) and gender (57.1% were men in Bodou group vs 58.9% in Glanle group). Patients from Bodou had higher BMI than those from Glanle (Table 1). Biological parameters including lipid, nutritional and inflammation markers were reported on Table 1. As previously published, total cholesterol, creatinine and albumin levels were statistically lower in the Glanle group than in the Bodou group [18]. Seven patients at Bodou (12.5%) have creatinine levels higher than 120 $\mu\text{mol/L}$, while there was no pathological value at Glanle. Evaluation of the glomerular filtration rate (GFR) through the Modified Diet Renal Disease (MDRD) algorithm [22] highlighted normal renal function in the two groups (i.e mean $\text{GFR} \geq 60 \text{ ml/min/1.73m}^2$), despite significant difference between Bodou and Glanle ($p < 0.0001$). Four subjects at Bodou and one at Glanle have a $\text{GFR} < 60 \text{ ml/min/1.73m}^2$.

2- Frequency of hyperhomocysteinemia and low vitamin levels

HHcy ($\text{Hcy} \geq 15 \mu\text{mol/L}$) was detected in 65 participants consisting to a proportion of 58.6 %. Among these 65 patients, 8 had intermediate HHcy, while no severe HHcy ($\geq 100 \mu\text{mol/L}$) was identified.

Frequency of HHcy observed in Bodou group was significantly higher than in Glanle group (79 % vs 38 % $p<0.0001$). Higher proportion of low folate concentration (<3 ng/ml) was observed in Bodou compared to Glanle group (40 % vs 13 %, $p<0.01$, respectively). By contrast, no patient of Bodou region had vitamin B12 <150 pg/ml, while a proportion of 20 % of low vitamin B12 concentration was observed in Glanle group.

The frequency of Hcy/folate ratio ≥ 4.40 was of 73 % in Bodou group compared to 16 % in Glanle group ($p<0.0001$).

3-Plasma Hcy and vitamin B levels

As reported on Table 2, subjects from Bodou had significant higher median plasma Hcy level (20.1 [9.7 - 41.4] $\mu\text{mol/L}$) than Glanle subjects (13.6 [5.5 - 48.7] $\mu\text{mol/L}$). This increase in Hcy levels was associated to lower serum folate concentration in Bodou group compared to Glanle group ($p<0.0001$). By contrast, vitamin B12 levels were higher in Bodou group compared to Glanle (593 [163-1860] vs 234 [83-585] pg/ml, respectively $p<0.0001$). As reported in Table 1, higher creatinine level was observed for subjects from Bodou compared to subjects from Glanle (98 [61-164] vs 70 [28-119] $\mu\text{mol/L}$, respectively, $p<0.0001$) without difference of Hcy/creatinine ratio between Bodou (median:0.20 [0.08-0.79]) and Glanle (median 0.21 [0.10-0.36]). When considering only patients with creatinine values <120 $\mu\text{mol/L}$, the difference of Hcy levels between Bodou ($n=49$, median Hcy:18.7 [9.7-38 $\mu\text{mol/L}$) and Glanle ($n=56$, median Hcy 13.6 [5.5 - 48.7] $\mu\text{mol/L}$) remained significant ($p<0.01$). However, comparison of Hcy levels between Bodou and Glanle performed after adjustment for creatinine was no significant.

Influence of gender characterized by significant lower Hcy levels for women compared to men was observed in each group (Table 3). There was no difference between men and women for vitamin B12 status in each group. Men from Glanle had higher folate concentrations than women (6.6 vs 4.4 ng/ml, $p<0.01$), while there is no difference related to gender at Bodou (Table 3). Men had significant higher creatinine levels than women, whatever the region studied (Bodou: 107 vs 81 $\mu\text{mol/L}$, $p<0.001$; Glanle: 73 vs 59 $\mu\text{mol/L}$, $p<0.01$).

Surprisingly, when comparing subjects with Hcy $<15 \mu\text{mol/L}$ or $\geq 15 \mu\text{mol/L}$, there was no significant difference in folate concentrations whatever the region (Table 4). By contrast, vitamin B12 levels was statistically lower when HHcy occurred in Bodou group (497 vs 821 pg/ml, $p<0.05$) while a trend toward significance was observed for Glanle group (217 vs 269 pg/ml, $p=0.08$). Interestingly, subjects with Hcy $\geq 15 \mu\text{mol/L}$ had higher creatinine and albumin levels than subjects with Hcy $<15 \mu\text{mol/L}$, either for subjects from Bodou and Glanle (Table 4).

4-Relationship between Hcy and anthropometric or biological parameters

As reported in Table 5, a significant negative relationship between Hcy and vitamin B12 levels was evidenced, while positive correlations between Hcy and albumin levels were observed. Surprisingly, there was no significant relationship between Hcy and folate levels, whatever the region studied. In addition, no relationship was observed between Hcy and CRP levels nor between Hcy and selenium levels. The stronger relationship was observed with creatinine ($r=0.512$ at Bodou, $r=0.307$ at Glanle). This positive correlation remained significant even if considering only patients with creatininemia $<120 \mu\text{mol/L}$ (Bodou: $n=49$, $r=0.422$, $p<0.01$; Glanle: $n=56$, $r=0.307$, $p<0.05$).

Discussion

This study highlighted a strong frequency of HHcy ($\geq 15 \mu\text{mol/L}$) in Ivorian population, reaching 58.6% of the studied subjects but depending of the region of origin. Indeed, the Ivorian subjects from the south coastal region (Bodou) have higher Hcy levels and higher frequency of HHcy than subjects from the west mountainous region (Glanle). Some major difference in dietary habits and genetic polymorphisms could be involved in these specific profile observed in the Ivorian population from the west and the south areas.

In occidental population, HHcy was associated to several clinical feature including thrombosis [2, 20] atherosclerosis [3,7], chronic heart failure [23], bone fracture [8,9], neural tube defect and pregnant complications [11,24]. No data are available on the relationship between Hcy and CV diseases in Ivory Coast. Frequency of coronary heart disease was very low as only 2.25% of hospitalization were due to myocardial infarction in the Institute of Cardiology of Abidjan [25]. A more recent study was conducted in seven countries of sub-Saharan Africa including the Ivory Coast to assess the causes and evolution of CV emergencies [26]. Severe hypertension (32.2 %), heart failure (27.5 %) and stroke (20.3%) appeared as the three major causes of hospitalization, while coronary heart disease was lower (6.1%). A high mortality rate (21.2%) was reported, in part due to a long delay between the begin of symptoms and the arrival to an hospital.

Because HHcy could be modulated through vitamin B intake, studying Hcy levels and its determinants is of interest in order to prevent complications related to HHcy. The high proportion of HHcy in Ivorian subjects is in agreement with previous report on Iranian population [27]. By contrast, low levels of Hcy were

reported for subjects from Burkina Faso, a bordering country of the Ivory Coast [17]. Another study reported higher Hcy levels for black men of Gambia, a bordering country of Senegal, compared to white british men [16], suggesting an influence of ethnicity. However, studies including African-American subjects and non-hispanic white subjects reported no difference of Hcy levels between black and white subjects [28,29], highlighting the importance of environment. Hcy levels for black Ivorian men of the coastal region determined in our study (median: 21.8 $\mu\text{mol/L}$) appeared even higher than Hcy levels reported in men of Gambia (median: 14 $\mu\text{mol/L}$) [16]. Different nutritional habits and/or genetic polymorphisms can interact with Hcy metabolism, explaining the discrepant profiles observed between regions or countries. In our study, influence of gender was observed in each region, but can not explain the higher Hcy levels in Bodou, as the sex ratio was similar between the two groups.

The nutritional habits at Glanle, located in a mountainous area, far of any town, are characterized by a vegetarian diet rich in crude palm oil. By contrast, subjects from Bodou living in front of the coast, near Abidjan, have a diet based on fish consumption. As expected, the vegetarian diet of Glanle subjects led to significant increase in folate levels, twice as high when compared to subjects from Bodou who have a fish-based diet. (See Table 2). As folate is recognized as an important nutritional factor influencing Hcy levels in healthy subjects [30], the decrease in folate levels in subjects from Bodou could be partly involved in the high levels of Hcy observed in this region. However, it should be underlined that proportion of HHcy ($\geq 15 \mu\text{mol/L}$) was much higher than the proportion of low folates ($<3 \text{ ng/ml}$) in each region. In addition, there was no significant difference in folate levels between subjects with Hcy lower or higher than $15 \mu\text{mol/L}$ (See Table 4)

neither in Bodou and Glanle regions and there was no relationship between Hcy and folates levels assessed by a Spearman correlation test. These preliminary results suggested that folate status in the Ivorian population was not the main determinant of HHcy.

By contrast to folates, vitamin B12 levels in Ivorian subjects were significantly associated with homocysteine levels either in Bodou and Glanle region (See Table 5). Vegetarian diet was usually associated to increased Hcy levels through vitamin B12 deficiency [31,32]. However, in the present study, vegetarian subjects have lower Hcy levels than non-vegetarians subjects, despite lower vitamin B12 levels. Indeed, related to the high consumption of fish, subjects from Bodou had more twice levels of vitamin B12 than subjects from Glanle. Interestingly, even with such high values at Bodou, there is a significant difference in vitamin B12 status when comparing subjects with Hcy lower or higher than 15 $\mu\text{mol/L}$ (See Table 4). However, as subjects from Bodou had both vitamin B12 and Hcy levels higher than subjects from Glanle, it is unlikely that vitamin B12 status could explain the high frequency of HHcy in subjects from Bodou.

Beside vitamin B deficiencies, renal function is an important determinant of Hcy levels [33]. In the present study, men who exhibit significant higher levels of Hcy than women had also higher levels of creatinine, whatever the region of origin. There was also a statistically significant increase in creatinine levels in subjects with Hcy $\geq 15\mu\text{mol/L}$ compared to those with Hcy $<15\mu\text{mol/L}$ either at Bodou and Glanle. In addition, a positive linear correlation between Hcy and creatinine levels was evidenced in each group (See Table 5), even if excluding patients with creatinine $\geq 120\mu\text{mol/L}$. All results of the present study were consistent with a more relevant effect of creatinine than vitamin B status on Hcy

levels. It should be underlined that median creatinine level was in the normal range either at Bodou and Glanle (98 and 70 $\mu\text{mol/L}$, respectively). Estimation of the GFR through algorithm of MDRD [22] showed a normal renal function in either subjects from Bodou (85 ml/min/1.73 m²) and from Glanle (135 ml/min/1.73 m²). However, the use of MDRD algorithm based on creatinine value is not validated in population having special diet, including vegetarian diet. Indeed, higher levels of creatinine in subjects from Bodou compared to Glanle could be related to their different dietary habits, especially to the difference in animal protein intake, as fish is a great source of creatine, the precursor of creatinine.

Another positive correlation was evidenced between Hcy and albumin levels in each group. Levels of albumin is mainly influenced by inflammation and animal protein intake. It was more likely that lower albumin levels in subjects from Glanle compared to Bodou was related to the vegetarian diet than to inflammation, in view of the similar levels of C-reactive protein between the 2 groups (See Table 1). In addition, no relationship was evidenced between Hcy and CRP levels, supporting the idea that albumin level was mainly a reflect of protein intake than inflammation in our study. So, it could be hypothetized that fish-based diet was associated to increase in methionine intake and therefore to an enhanced Hcy formation.

The observation of higher Hcy levels in a fish-based diet compared to a vegetarian diet appeared in opposition to the classical increase of Hcy in vegetarian subjects [31,32]. Beyond the high creatinine levels of subjects from Bodou that could explain their higher Hcy levels compared to the vegetarian subjects of Glanle, genetic polymorphism between subjects from the Adjoukrou and the Yacouba ethnicity could not be ruled out. Beside the most studied

polymorphism MTHFR 677C/T that was previously reported with a low prevalence in Benin and Togo [15], heterozygosity for CBS mutations can lead to moderate or intermediate HHcy. Among several mutations of CBS gene involved in CBS deficiency, the T833 mutation always co-segregated with an insertion of 68pb in the exon 8 (844ins68) that counteract the T833 mutation. However, this insertion was also associated to a decreased transcription of CBS gene [34]. Interestingly, the presence of this 844ins68 was reported with a higher frequency in black population (37.5% of heterozygotes) than in white population (13.5% of heterozygotes) [35]. Suspicion of heterozygous patients for CBS deficiency could be assessed through the ratio Hcy/folates, in agreement with previous data showing that $\text{Hcy/folates} \geq 4.4$ occurred preferentially in case of heterozygosis CBS patients [21]. In our study there was a statistical increase in frequency of $\text{Hcy/folate} \geq 4.40$ for subjects from Bodou (reaching 73%) compared to subjects from Glanle (16%), suggesting a potential genetic difference between these two groups.

A main limitation of this study was the lack of food questionnaire to analyze more precisely dietary intake. However, no diet questionnaire was validated to assess special diet, such as Ivorian diet. In addition, GFR was estimated on the basis of an algorithm with creatinine level which is directly linked to muscle metabolism, but no reference methods or alternative marker such as cystatine C was performed. Finally, blood sample was not collected in order to obtain DNA that limit us to explore genetic polymorphism. Further explorations should be conducted in a larger cohort of subjects from the Ivory Coast to assess a potential CBS polymorphism as a determinant of high frequency of HHcy.

In conclusion, multiple interactions seems to be involved as determinants of Hcy levels in the Ivorian population. Firstly genetic polymorphism, especially on the CBS gene, can not be ruled out as a possible hypothesis to explain the difference of Hcy levels between subjects from the south and the west of Ivory Coast. By contrast, vitamin B12 that was correlated to Hcy levels, can not be retained as an explanation for the high frequency of HHcy in subjects from the south area having a fish-based diet compared to subjects from the west, having a vegetarian diet. In the present study, creatinine levels, partly related to animal protein intake, appeared as a determinant of Hcy levels more relevant than vitamin B status in Ivorian rural population.

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Table 1. Anthropometric and biological characteristics (median [range]) of an Ivorian population from the South (Bodou) and the West (Glanle) regions.

	Bodou (n=56)	Glanle (n=56)	P-value
Age (years)	45 [19- 68]	37.5 [19- 68]	0.08
Sex ratio (F/M)	24/32	23/33	0.85
BMI (kg/m ²)	23.9 [19.1-33.81]	22.6 [18.7-28.3]	0.0049
Total cholesterol (mmol/l)	4.10 [1.93-7.77]	3.19 [1.62-5.43]	<0.0001
Triglycerides (mmol/l)	0.98 [0.37-3.39]	1.05 [0.38-3.10]	0.3563
CRP (g/l)	1.59 [0.32-122.80]	1.78 [0.23-69.60]	0.5354
Albumin (g/l)	44 [18-53]	34.0 [15-48]	<0.0001
Creatinine (μmol/L)	98 [61-164]	70 [28-119]	<0.0001
Estimated GFR *	85 [50-131]	135 [56-363]	<0.0001

* Glomerular filtration rate estimated through algorithm [22]

Table 2: Influence of the origin region on plasma total homocysteine and vitamin B concentrations (median [range]) in subjects from the south (Bodou) and the west (Glanle) of Ivory Coast.

	Bodou (n=56)	Glanle (n=56)	P-value
Hcy (μmol/L)	20.1 [9.7-41.4]	13.6 [5.5-48.7]	<0.0001
Folates (ng/ml)	3.2 [2.0-7.3]	6.0 [1.9-18.2]	<0.0001
Vitamin B12 (pg/ml)	593 [163-1860]	234 [83-585]	<0.0001
Hcy/Folate	5.8 [2.1-18.8]	2.4 [0.6-18.3]	<0.0001

Table 3. Influence of gender on plasma median homocysteine, vitamin B, creatinine and albumin levels in subjects from Bodou and Glanle regions.

	Bodou		Glanle	
	Women n=24	Men n=32	Women n=23	Men n=33
Hcy ($\mu\text{mol/L}$)	16.7 [10.7-25.5]	21.8* [9.7-41.4]	11.4 [5.5-22.9]	15.7* [5.5-48.7]
Folate (ng/ml)	3.2 [2.2-7.3]	3.4 [2.0-6.4]	4.4 [1.9-10.1]	6.6* [2.6-12]
B12 (pg/ml)	694 [291-1860]	501 [163-1620]	237 [83-560]	231 [99-585]
Creatinine ($\mu\text{mol/L}$)	81 [61-113]	107* [74-164]	59 [36-119]	73* [28-104]
Albumine (g/l)	42 [18-53]	47* [27-51]	30 [15-46]	34 [21-48]

*p<0.01 : comparison of men vs women in each group

Table 4. Variation of biological parameters (median [range]) according to the level of plasma homocysteine in subjects from a south (Bodou) and a west (Glanle) region of Ivory Coast

Hcy (μmol/L)	Bodou		Glanle	
	< 15 (n=12)	≥ 15 (n=44)	< 15 (n=35)	≥ 15 (n=21)
Folate (ng/ml)	3.4 [2.2-5.2]	3.2 [2.0-7.3]	6.0 [1.9-13.7]	5.9 [2.2-18.2]
B12 (pg/ml)	821 [442-1860]	497* [163-1500]	269 [83-585]	217 [99-390]
Hcy/Folate	4.1 [2.1-6.1]	7.0* [2.4-18.8]	2.0 [0.6-7.5]	3.6* [1.5-18.3]
Creatinine (μmol/L)	79 [61-99]	102* [71-164]	65 [36-92]	74* [28-119]
Albumine (g/l)	43 [18-48]	46* [27-53]	32 [19-45]	35* [15-48]

* $p \leq 0.05$: comparison between subjects with Hcy <15 μmol/L and Hcy >15 μmol/L in each group

Table 5. Relationship between plasma total homocysteine and biological variables at Bodou and Glanle

	Bodou (n=56)		Glanle (n=56)	
	r	p	r	p
Age	0.116	NS	0.170	NS
Folates	-0.181	NS	-0.025	NS
Vitamin B12	-0.316	<0.05	-0.263	0.05
Creatinine	0.512	<0.0001	0.307	<0.05
Albumin	0.322	<0.05	0.299	<0.05
CRP	0.12	NS	-0.21	NS
Selenium	0.219	NS	0.093	NS