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Table of Content

Prevalence and associated risk factors of Vitamin D deficiency in children under five years of age, at the Diamniadio Children's Hospital in Senegal	47
Abou Ba, Najah Fatou Coly, Idrissa Basse, El Hadji Ibrahima Kane, Penda Awa Ka, Mamadou Soumboundou, Souleymane Thiam, Abdourahmane Samba, Arame Ndiaye, Idrissa Yaya Soumah, Fatou Diedhiou, Fatou Cissé, Moustapha Djité, Néné Oumou Kesso Barry, Pape Matar Kandji, Papa Madiéye Gueye, Fatou Diallo Agne, Ndéye Ramatoulaye Diagne Gueye	
Investigation into the intake of a popular polyherbal drug (Jalin Herbal Mannex Liquid) on selected biochemical indices of male wister rats	55
Kpomah, E. D. and Arhoghro, E. M.	
Physicochemical parameters of <i>Blighia sapida</i> (K.D. Koenig) oil extracted in Togo	63
Aklesso Nabede, Haziz Sina, Tiatou Souho, Mamatchi Mélila, Farid T. Bade, Adolphe Adjanooun, Batcha Ouadja, Lamine Baba-Moussa and Kou'santa Amouzou	
Effects of Quantitative Trait Loci (QTLs) of BCL11A and HBS1L-MYB genes on clinical-biological variability of sickle cell disease in a Senegalese pediatric population	71
Fatou G. Tall, El hadji M. Ndour, Moustapha Djité, Oumou K. Barry, Pape M. Kandji, Papa M. Gueye, Aynina Cissé and Philoméne Lopez Sall	

Full Length Research Paper

Prevalence and associated risk factors of Vitamin D deficiency in children under five years of age, at the Diamniadio Children's Hospital in Senegal

Abou Ba¹, Najah Fatou Coly^{2,3*}, Idrissa Basse^{2,4}, El Hadji Ibrahima Kane⁴, Penda Awa Ka⁴, Mamadou Soumboundou^{2,3}, Souleymane Thiam⁵, Abdourahmane Samba⁵, Arame Ndiaye⁵, Idrissa Yaya Soumah⁵, Fatou Diedhiou⁵, Fatou Cissé⁵, Moustapha Djité⁶, Néné Oumou Kesso Barry⁶, Pape Matar Kandji⁶, Papa Madiéye Gueye⁶, Fatou Diallo Agne⁵, Ndéye Ramatoulaye Diagne Gueye^{2,4}

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Vitamin D deficiency (VDD) is a public health problem which affects all human beings including dark-skinned subjects. In children, it can cause disabilities associated with skeletal abnormalities such as rickets or stunted growth. VDD is also associated with a significant risk of extra-skeletal, infectious, auto-immune, neoplastic, and cardiovascular diseases. The concentration of 25 (OH) D is currently considered as the best VDD indicator. Whereas VDD has been well studied in Western countries and North America, very few studies have been conducted in sub-Saharan Africa. The aim of this study was to assess the prevalence and risk factors of VDD in children aged between 0 to 59 months. This is a cross-sectional prospective study conducted from August 5, 2019, to November 30, 2020. A total of three hundred children were included in this study, two hundred of whom were malnourished and the rest with a normal P/T ratio. The variables studied were vitamin D, serum calcium, magnesium, phosphorus and iron. Ferritin, haemoglobin, protein, albumin and prealbumin were also studied. The prevalence of VDD in the general population was 30%. No significant statistical difference in vitamin D concentration values was noticed between malnourished and nourished children with $p = 0.388$. Children over 24 months of age are 2.34 times more likely to be VDD than others. Given the prevalence of VDD in the study population, it would be necessary to integrate screening and supplementation into current medical practice.

Key words: Vitamin D deficiency, malnutrition, children under 5 years of age, risk factors.

INTRODUCTION

Vitamin D deficiency is a global public health problem affecting more than one billion children and adults

worldwide. Its prevalence varies and ranks between 30 to 60% depending on the region (Mogire et al., 2020).

Pregnant women, people of colour (Black, Hispanic and people with increased skin pigmentation), children and adults suffering from obesity or who are less exposed to the sun are particularly at risk (Holick, 2017). In children, besides rickets, vitamin D deficiency can lead to respiratory disorders and muscle weakness. In addition to the precarious nutritional status that pre-disposes children to acute respiratory infections (Diop et al., 2020), there is an inverse relationship between plasma vitamin D concentration and the risk of these infections (OMS, 2009).

Patients with severe vitamin D deficiency and hypocalcaemia develop neuromuscular sensitivity, such as numbness of the limbs and even seizures that can cause mis-diagnosis (Baudin, 2014). In addition to the classic bone manifestations, vitamin D deficiency is increasingly implicated in several other extra-skeletal conditions, including cardiovascular atheromatous diseases, cancer, dysimmune pathologies and certain neurological conditions (Jan et al., 2019).

Vitamin D deficiency is an aggravating factor in much chronic pathology (Chauveau and Aparicio, 2013). Indeed, low levels of vitamin D are associated with the susceptibility and severity of acute infections and unfavourable outcomes for some chronic infections (Jan et al. 2019). The objective of this study is to evaluate the prevalence of vitamin D deficiency and associated risk factors in children up to 59 months of age.

MATERIALS AND METHODS

Type – study framework

A prospective cross-sectional study was conducted from 5 August 2019 to 30 November 2020. Recruitment was conducted at the Diamniadio Children's Hospital (HED) and biological analyses were performed at the Department of Medical Biochemistry.

Study population

After calculating the population size, according to the formula of Lorentz, patients aged between 0 to 5 years followed as outpatients or hospitalized for acute malnutrition, as well as children received for consultation with normal nutritional status were included after parental consent. Children with chronic pathology were not included in this study.

Three hundred children aged up to 59 months were recruited. 200 patients had acute malnutrition and 100 children with normal nutritional status. The average age of the children was 16.7 ± 11.8 and 16.8 ± 12.7 months respectively, with malnourished children and controls. The sex ratio was 1.02 in malnourished children and 2.3 in nourished children. The ethics committee of Cheikh Anta Diop University approved the study: Ref CER/UCAD/AD/MSN/015/2020.

Assessment of nutritional status

Children were weighed using the "Uniscale scale", with a tare function. Height was measured using the UNICEF fleece, consisting in a lying down position for patients under two years of age and standing for patients over 2 years of age.

The nutritional status of the children was determined based on the weigh to height ratio in relation to The WHO growth standards. Normal nutritional status is defined by a weigh to height ratio between -2 and +2 z-score. Moderate acute malnutrition (MAM) is retained in front of a weigh to height ratio between -2 and -3 z-score and it is classified severe acute malnutrition (SAM) for a weigh to height ratio < -3 z-score (OMS, 2009).

Sample taking

Blood samples were collected from patients on an empty stomach with bent elbow using a dry tube, a tube with ethylene diamine tetraacetic acid (EDTA) and another tube with sodium heparinate. The blood sample on EDTA tube was used immediately to determine the complete blood count (CBC). The blood sample on dry and heparinated tubes was centrifuged at 3000 rpm for 5 min. The protein was measured immediately on a portion of the serum. The remaining quantity was kept at -20°C for the measurement of vitamin D, ferritinemia, albumin and zinc. The samples were dosed over several days per group. The other parameters are dosed on the plasma and stored at minus 20°C.

Methods of analysis

Vitamin D and ferritin were measured using the Maglumi 600 automate reference 23020018, by the immunoluminometric method (CLIA) at the Medical Biochemistry Laboratory of the Faculty of Medicine, Pharmacy and Dentistry, Cheikh Anta Diop University.

Principle test

The 25-OH Vitamin D assay is a competitive chemiluminescence immunoassay. The 25-OH Vitamin D assay is a two-incubation chemi-luminescence immunoassay for the quantitative determination of total 25-OH vitamin D in human serum in the first incubation, the 25-OH vitamin D is dissociated from its binding protein by the displacing reagent, and binds to the 25-OH vitamin D antibody on the magnetic microbeads forming an antibody-antigen complex. Following a second incubation, the 25-OH Vitamin D labeled ABEI (N-aminobutyl-N-ethylisoluminol) are added. The rest unbound material is removed during a wash cycle. Subsequently, the Starter 1+2 are added to initiate a flash chemi-luminescent reaction. The resulting chemi-luminescent reaction is measured as relative light units (RLUs) which is inversely proportional to the concentration of 25-OH Vitamin D present in the sample (or calibrator/control, if applicable) (MAGLUMI" 25-OH Vitamin D (CLIA) reference 130211004M).

The CBC was determined using the Sysmex XS-1000i and other biochemical parameters (calcium, phosphorus, serum iron, proteinemia, albumin and prealbumin) with the A25 Biosystem® at the medical analysis laboratory of Children's Hospital in Diamniadio. Vitamin D status was defined as 25(OH)D < 30 nmol/L, 25(OH)

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D < 50 nmol/L and 25(OH) D between 50 and 75 nmol/L (20 to 30 µg/L).

The level of 25(OH) D greater than 75 nmol/L is normal (Holick et al., 2011). To perform the biological diagnosis of anaemia in children, Beutler study standard was used (Beutler and Waalen, 2006). A value of less than 60 g/L, 30 g/L and 150 g/L was used for hypo-prothrombinemia, hypo-albuminemia and low pre-albuminemia, respectively. Hypoferritinemia and low serum iron are retained for a value of less than 30 ng/ml and 50 µg/dl. Hypophosphoremia, hypomagnesaemia and hypocalcemia were selected for values below 25, 15 and 85 mg/L, respectively. These values were adopted after considering the pediatric reference values provided by Canadian laboratories (Adeli et al., 2017).

Data collection and statistical analysis

After collecting and entering data on Excel, the statistical analyses were performed using the Jamovi software (Version 1.6.22.0). Categorical data are described by numbers and percentages, while quantitative data are presented, based on their statistical distribution, in terms of mean and standard deviation or median and interquartile interval. Normality (Gaussian distribution) was studied by the Shapiro-Wilk test. Comparisons between groups (according to malnourished and teminus) concerning the parameters of a categorical nature were made using the Chi2 test or, if applicable, the exact Fisher test. Comparisons between groups for quantitative variables were made using the T test or the Mann Withney test. Univariate and multivariate binary logistic regression is performed for the study of risk factors. A p-value greater than 5% is considered significant.

RESULTS

Sociodemographic and clinical characteristics of patients

The average z-score of weight for height ratio is 0.09 ± 0.19 Kg/cm in malnourished patients and 0.12 ± 0.22 Kg/cm in controls. Socio-demographic characteristics are shown in Table 1. Most of the children were exclusively breastfed.

73.3% lived in the suburban city. 58% of the malnourished children had an acute form of malnutrition. The average vitamin D level is 45.2 (µg/dl) and 45.1 (µg/dl) in malnourished and nourished children, respectively. As illustrated in in Table 2, a large proportion of prealbumin, iron and hemoglobin deficiency are observed regardless of the group in Table 2. On the other hand, the percentage of hypo-phosphoremia is low. Severe vitamin D deficiency is observed in malnourished people (Table 2).

No significant differences were found between concentrations of biochemical markers at the level of the two groups as evidenced in Table 3.

Factors associated with vitamin D deficiency

For malnourished, in bivariate analysis, the age of the child, severe acute malnutrition and low serum iron were

significantly associated with vitamin D deficiency (Table 4). Certainly, the probability of vitamin D deficiency in children under 12 months aged between 13 and 24 months and between 25 and 60 months is respectively 3.13, 2.38 and 3.47 times. This association was found with SAM and low iron concentration in children. For the nourished children, only a low serum iron level is associated with vitamin D deficiency. Hypocalcaemia and hypo phosphoremia are not associated with vitamin D deficiency (Table 4). In multivariate analysis, the most predictive factor for vitamin D deficiency was age, specifically between 25 to 60 months (OR=2.34; 95% CI = [1.18 to 16.0]; p=0.027) (Table 5). Age between 7 to 12 months and between 13 to 24 months was not confirmed as risk factors with p-values of 0.098 and 0.158 respectively. The same is true for low serum iron (p = 0.107).

DISCUSSION

In the study population, vitamin D deficiency is common, both in acutely malnourished children (28.3%) and in children with good nutritional status (33%). However, severe forms are found only in malnourished children (0.5%) (Table 2).

This high prevalence of vitamin D deficiency is reported in other similar studies conducted in Africa, particularly in Tanzania (Wally, 2017) with a rate of 30%. A systematic literature review conducted in Africa highlighted a prevalence of 58.54% of vitamin D insufficiency, 34.18% of vitamin D deficiency, and 17.31% of severe vitamin D deficiency (Mogire et al., 2020).

Lower prevalence of vitamin D levels below 50 nmol/L and below 30 nmol/L of 7.8 and 0.6% respectively, were found in another study conducted in Kenya, Uganda, Burkina Faso, Gambia, and South Africa (Mogire et al., 2021). In sub-Saharan Africa, although sunshine is permanent, children are not protected against vitamin D deficiency. Moreover, dark-skinned patients are also at risk of vitamin D deficiency because photosynthesis by ultraviolet rays is hindered by melanin. The role of melanin as a protective screen against the harmful effects of ultraviolet rays is not in favour with the synthesis of vitamin D (Clemens et al., 1982). Fair skinned people synthesize up to six times more vitamin D than those with dark skin. A case-control study in Nigeria found higher vitamin D levels in albino patients (95.9 (50.1 to 177) ng/ml) than in pigmented skin (78.2 (12.1 to 250) ng/ml) (Enechukwu et al., 2019). This high prevalence of vitamin D deficiency in Africa and particularly in Senegal could be explained by the lack of vitamin D supplementation in children and the consumption of unfortified or vitamin D defective food. Foods that are rich in vitamin D are fish such as salmon, horse mackerel, tuna, liver and eggs (Cediel et al, 2018).

The high prevalence of vitamin D deficiency in children

Table 1. Socio-demographic characteristics of malnourished children and controls.

Variable	Malnourished (%)	Controls (%)	Total (%)
	n=200	n=100	N=300
Sex			
Male	101 (50.5)	70 (70)	171 (57)
Female	99 (49.5)	30 (30)	129 (43)
Age (months)			
Less than 6	23 (11.5)	19 (19)	42 (23)
7-12	70 (35)	27 (27)	97 (32)
13-24	71 (35.5)	32 (32)	103 (34)
25-60	36 (18)	22 (22)	58 (19)
Breastfeeding			
Exclusive	20 (87)	17 (89.5)	37 (12.3)
Mixed	2 (8.7)	2 (10.5)	4 (1.33)
Artificial	1 (4.3)	0	1 (0.2)
Ethnic group			
Wolof	88 (44)	48(48)	136 (45.3)
Serer	26 (13)	15 (15)	41 (13.6)
Diola	9 (4.5)	7 (7)	16 (5.3)
Peulh	56 (28)	20 (20)	76 (25)
Other	21 (10.5)	10 (10)	31 (10.3)
Address			
Urban	3 (1.5)	0	3 (1)
Suburban	132 (66)	88 (88)	220 (73.3)
Rural	65 (32.5)	12 (12)	77 (25.6)
Nutritional status			
Severe acute malnutrition	116 (58)	0	116 (38)
Moderate acute malnutrition	84 (42)	0	84 (28)
Normal	0	100 (100)	100 (33)
Malnutrition			
Oedematous	8 (4)	0	8 (2.7)
No oedematous	92 (96)	0	92 (97.3)

Source: Authors

was also reported in a study conducted in China with (Zhang et al., 2020) a prevalence of 48.1% of vitamin D deficiency (< 50 nmol/L) in pre-school children (3 to 6 years), 21.2% in infants aged between 1 to 3 years of age and 17.9% in infants under 1 year of age.

In a study conducted in Bahrain (Isa et al., 2020), all children (93.4%) had low levels of vitamin D, 78.3% were deficient and 15.1% had vitamin D deficiency. A significantly higher proportion of girls were deficient in vitamin D compared to boys ($p < 0.001$). More primary school children and adolescents were deficient in vitamin D in comparison to pre-schoolers ($p < 0.001$). However,

its prevalence is much higher than the one in the control group. In our study, vitamin deficiency is mostly found in children with good nutritional status (33%) than in malnourished (28.3%). However, it is more severe in malnourished (24.5µg/L) than in children with a good nutritional status (26.3 µg/L). In a study conducted in Tanzania, children with marasmus were more at risk of vitamin D deficiency than children with kwashiorkor or marasmic kwashiorkor (Walli et al., 2017). In the Netherlands, a study of a multi-ethnic cohort of children aged under six years showed that malnourished are more at risk of vitamin D deficiency (<50 nmol/L) than well-

Table 2. Frequency of decrease in biological parents of malnourished children and controls.

Variable	Malnourished children (n/total and %)	Controls (n/total and %)	Total (n/total and %)
Normal vitamin D levels	136/190 (71.5)	65/98 (66.3)	201/288 (69.7)
Vitamin D deficiency	42 (22.1)	29 (29)	71/288 (24.6)
Moderate vitamin D deficiency	11 (5.7)	4 (4)	15/288 (5.2)
Severe vitamin D deficiency	1 (0.5)	0 (0)	1/288 (0.34)
Hypocalcaemia	54/195 (27.6)	18/80 (22.5)	72/275 (26)
Hypophosphatemia	9/189 (4.7)	2/79 (2.5)	11/268 (4.1)
Hypomagnesemia	63/190 (33)	33/80 (41.2)	96/270 (35.5)
Hypoprotidemia	79/196 (40.3)	28/84 (33.3)	107/280 (38.2)
Low prealbumin	129/192 (67)	62/81 (76.5)	191/273 (69.9)
Hypoalbuminemia	45/193 (23)	16/81 (19.7)	61/274 (22.2)
Anemia	174/200 (87)	84/100 (84)	258/300 (86)
Low serum iron	132/188 (70.2)	58/79 (73.4)	190/267 (71)
Low ferritinemia	71/177 (41)	27/93 (29)	98/270 (36.3)

Source: Authors

Table 3. Variation in biological parameters by nutritional status.

Variable	Malnourished		Controls		p-value
	N (%)	Median (QT)	N (%)	Median (QT)	
Protein (g/L)	196 (98%)	62.6 (54-70)	84 (84%)	63 (58-68)	0.668
Albumin (g/L)	193 (96.5%)	40 (35-46)	81 (81%)	39 (36-42)	0.314
Prealbumin (mg/L)	192 (96%)	200 (140-275)	81 (81%)	202 (164-243)	0.083
Vitamin D (ng/ml)	190 (95%)	38.6 (28.4-55.3)	98 (98%)	34.3 (17.8-50.5)	0.388
Calcium (mg/L)	195 (97.5%)	94.1 (84-103)	80 (80%)	91.0 (85-97)	0.460
Phosphorus (mg/L)	189 (94.5%)	54 (43-61.8)	79 (79%)	51 (41.5-58.9)	0.495
Hb (g/dl)	198 (99%)	10.1 (9.10-11.1)	97 (97%)	10.5 (9.50-11.3)	0.119
Serum iron (µg/dl)	188 (94%)	47.9 (20.2-60.4)	79 (79%)	43.3 (18.8-51.7)	0.550
Ferritin (ng/ml)	177 (88.5%)	42.8 (12.6-103)	93 (93%)	53.2 (23-130)	0.073

Source: Authors

nourished children (Pham et al., 2019). In Ecuador, the average vitamin D level was about the same in malnourished as in nourished children. However, vitamin D deficiency was more common in malnourished than in those with good nutritional status (22.0 vs. 12.3%).

Infants aged 6 to 12 months had higher average rates than older infants (>12 months) and malnourished were twice as likely to have vitamin D deficiency as normal children (unadjusted OR = 2.0; 95% CI 1.2 to 3.4) (Mokhtar et al., 2018).

To identify factors associated with vitamin D deficiency, sociodemographic, biological, and clinical parameters were studied (Table 4 and 5).

Thus, univariate binary logistic regression analysis showed a significant association for age groups greater than six months, acute malnutrition, and low serum iron levels. On the other hand, in multivariate binary analysis, only the age group greater than 24 months is a factor

significantly associated with the occurrence of vitamin D deficiency (Table 4).

The probability of vitamin D deficiency in children over 24 months of age is 3.47 times (95% CI: 1.45 to 8.30) (Table 4).

Therefore, age above 24 months is considered as a risk factor associated with vitamin D deficiency. This result highlights the importance of food intake, because beyond this age, children are no longer breastfed as they start dietary diversification. Breastfeeding could be a protective factor knowing that before the age of six months, most children are on exclusive breastfeeding.

The results of a study conducted in Turkey (Cihan and Korğalı, 2018) showed the association of vitamin D deficiency with the decrease in serum iron. Indeed, in vitro, there is a bi-directional relationship between the metabolism of vitamin D and that of iron. 25(OH)₂ D can influence the level of iron by reducing the level of

Table 4. Prediction of vitamin D deficiency by univariate analysis in malnourished and controls.

Variable	Univariate analysis (Malnourished/controls)		
	OR	CI95%	p-value
Gender (female)			
Male	1.38 / 0.6	8.26 - 2.32 / 0.25 - 1.74	0.219 / 0.410
Female	Reference	-	-
Age (month) (Reference Less than 6)			
7-12	3.13 / 1.70	1.47 - 6.92 / 0.50 - 5.79	0.003 / 0.389
13-24	2.38 / 2.22	1.12 - 5.03 / 0.7 - 7.06	0.023 / 0.176
25-60	3.47 / 2.58	1.45 - 8.30 / 0.72 - 9.12	0.005 / 0.143
Breastfeeding (Reference exclusive)			
Mixed	6.74e-8 / 6.08e ⁺⁷	0.000- inf / 0.000 - inf	0.995 / 0.995
Artificial	2.11 / -	0.17 - 25.35 / -	0.556 / -
Ethnicity (Reference Wolof)			
Serer	0.93 / 2.62	0.42 - 2.08 / 0.65 - 10.5	0.875 / 0.175
Diola	0.47 / 0.86	0.16 - 1.37 / 0.17 - 4.35	0.168 / 0.809
Peulh	0.93 / 1.70	0.37 - 1.29 / 0.52 - 4.56	0.246 / 0.337
Other	0.90 / 1.52	0.37 - 2.14 / 0.35 - 6.66	0.813 / 0.572
Address (Reference urban)			
Suburban	1.04 / 1.36	0.092 - 11.6 / 0.35 - 5.18	0.977 / 0.656
Rural	1.65 / -	0.14 - 19.03 / -	0.691 / -
Nutritional status (Reference undernutrition)			
SAM	3.36 / -	1.28 - 8.80 / -	0.013 / -
MAM	2.4 / -	0.91 - 6.46 / -	0.075 / -
Normal	1.97 / -	0.77 - 5.02 / -	0.155 / -
Malnutrition			
Oedematous	0.83 / -	0.16 - 4.26 / -	0.826 / -
Not oedematous	Ref	-	-
Biology (Reference normal)			
Hypocalcemia	1.20 / 0.42	0.65 - 2.20 / 0.14 - 1.24	0.553 / 0.116
Hypophosphatemia	2.49 / 7.37e ⁺⁶	0.60 - 10.24 / 0 - inf	0.205 / 0.993
Hypomagnesemia	1.33 / 0.96	0.62 - 2.82 / 0.36 - 2.56	0.466 / 0.939
Hypoprotidemia	1.20 / 0.9	0.70 - 2.05 / 0.345 - 2.35	0.497 / 0.829
Low prealbumin	0.75 / 2.25	0.42 - 1.35 / 0.75 - 6.70	0.341 / 0.145
Hypoalbuminemia	1.08 / 0.95	0.56 - 2.05 / 0.29 - 3.11	0.821 / 0.932
Anemia	0.60 / 1.10	0.23 - 1.57 / 0.45 - 2.66	0.299 / 0.834
Low serum iron	0.54 / 3.07	0.30 - 0.96 / 1.06 - 8.90	0.039 / 0.02
Low ferritinemia	0.78 / 0.739	0.45 - 1.37 / 0.288 - 1.89	0.40 / 0.52

Source: Authors

hepcidin, while low iron levels can indirectly influence vitamin D status by decreasing its activation enzymes (Mogire et al., 2021).

In malnourished, the acute form is considered a risk

factor associated with vitamin deficiency with OR = 3.36, 95% CI (1.28 to 8.80). However, further studies are needed for confirmation. The decrease in other biochemical parameters is not associated with vitamin D

Table 5. Prediction of the occurrence of vitamin D deficiency by multivariate analysis in malnourished.

Variable	Multivariate analysis			
	N (%)	OR	CI95%	p-value
Age in months (Reference less than 6)				
7 -12	70 (35)	2.88	0.82 - 10.1	0.098
13 - 24	71(35.5)	2.43	0.70 - 8.36	0.158
25 - 60	36 (18)	2.34	1.18 - 16.0	0.027
Malnutrition (Reference undernutrition)				
SAM	116 (58)	1.06	0.22 - 5.10	0.933
MAM	84 (42)	0.88	0.18 - 4.32	0.882
Normal	0	0.88	0.22 - 3.5	0.864
Seric Iron (Reference normal)				
Low serum iron	132 (70.2)	1.653	0.89 - 3.05	0.107

OR= Odd ratio, CI= Confidence interval.
Source: Authors

deficiency (Table 4), especially since no significant difference is noted in concentration between the groups (Table 3). There is no great difference between the percentages of people with a decrease in the parameters studied in the two groups. For the rest of the biological markers studied, there is no association with vitamin D deficiency. Similarly, criteria such as ethnicity, the origin of children in urban, sub-urban or rural areas and the presence of oedema in malnourished people are not associated with vitamin D deficiency.

Normal concentrations of protein, albuminemia, prealbumin, calcium, and phosphorus were found in the groups by nutritional status and whether vitamin D deficiency was present (Table 3). In malnourished, their median values are 62.6 (54 to 70) g/L for proteins, 40 (35 to 46) g/L for albumin, 200 (140 to 275) mg/L for prealbumin, 94.1 (84 to 103) mg/L for calcium and 40 (43 to 61.8) mg/L for phosphorus.

In Nebata's study 52.1% of malnourished children had normal calcium levels compared to 31.7% of well-nourished children. 2.4% of these children had normal phosphorus levels compared to 43.6% of malnourished (Nabeta et al., 2015).

These results are also found in adults, except for calcium, albumin and phosphorus showed no significant difference between vitamin D deficient and non-vitamin D deficient (Merker et al., 2019). Our results demonstrate that the prevalence of vitamin D deficiency in children increases with age, which is in line with the data in the literature (Ahmed et al., 2017; Andiran et al, 2012).

Conclusion

Vitamin D deficiency is common in both malnourished and well-nourished children but is more severe in

malnourished infants. The prevalence of deficiency in children aged between 0 to 59 month's increases with age, justifying the need for vitamin D supplementation but also the fortification of food.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Investigation into the intake of a popular polyherbal drug (Jalin Herbal Mannex Liquid) on selected biochemical indices of male wister rats

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Jalin Herbal Mannex Liquid (JHML) is a polyherbal formulation from honey, Panax ginseng, Liriosma ovate, and Lepidium mehenil, intended for enhanced sexual performance and improve sperm count in men. JHML was studied via its effect on body weight, biochemical indices and histopathology of the testes. Twenty healthy Wistar rats of 120 -150 g weight were allotted into groups A and B., of ten rats each, group A (control), were given 2 mL/kg B.W of tap water, group B were dosed with 2 mL/kg B.W of JHML for four weeks with the animals allowed access to feed and water ad-libitum. Blood were obtained through heart perforation, kidney and testes were excised, washed with normal buffered saline. Results from the investigation indicated that JHML had androgenic properties with marked significant increase ($p < 0.05$) in body weight of rats administered with JHML. Significant increase ($p < 0.05$) was observed in LH, FSH, testosterone and with a concomitant significant decrease ($p < 0.05$) in PRL. The JHML caused non-significant decrease ($p > 0.05$) on triglycerides and cholesterol but with positive significant effect ($p < 0.05$) on LDL and HDL. No significant changes ($p > 0.05$) on creatinine, urea, uric acid and serum electrolytes. Testicular sections of rats treated with JHML exhibited normal features, seminiferous epithelium and interstitial tissues with active spermatogenesis. The significant increase ($p < 0.05$) in testosterone, LH, FSH with a concomitant significant decrease ($p < 0.05$) in PRL may account for its sex invigorating potential, the non-significant changes observed in some biochemical indices of the rats showed that JHML is relatively safe at the studied dose.

Key words: Jalin Herbal Mannex Liquid, sex hormone, renal function, lipid profile, serum electrolyte.

INTRODUCTION

In recent times, the production, sales, and intake of different brands of packaged polyherbal products in Nigeria had increased astronomically, owing to their general acceptability. They are considered cheap when compared to mainstream medicines, readily available and with little or no stringent pharmacological bottleneck with regards to their prescription and use (Kpomah and

Odokwo, 2020). Polyherbal therapy or herbal combination has been in usage in many countries for centuries, however, there is a paucity of scientific evidence to buttress their therapeutic potential (Che et al., 2013). Drug combination therapy often produces a promising effect in the treatment and control of diseases over single-drug therapy. The theory of drug combination has

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been well proven in Western medicine with significant success being accomplished over the decades. In recent times, drug amalgamation recipe in contagious diseases and cancer therapy have presented fresh hopes and frontiers to patients (Risberg et al., 2011; Muhammad et al., 2016). Naturally occurring plants and plant products organized into certain recipes have been shown to have potential interaction effects. According to Kajaria et al. (2011), these effects include mutual potency heightening, mutual assistance, mutual restraint and mutual antagonism. Due to socioeconomic and traditional influences in Africa and most especially the Nigerian system of medicine, polyherbal compounds are used for the treatment of various infections (Chickenpox, *E. coli*, Diphtheria, Common cold, Giardiasis, Infectious mononucleosis, Influenza), and also in the treatment and control of metabolic and neurodegenerative diseases (Akinyemi et al., 2018; Jamshidi-Kia et al., 2018) which may sometimes arise from the use of synthetic drugs (Arhoghro et al., 2012).

Notwithstanding the medical innovations in treatment and treatment amenities for male sexual malady (Lim et al., 2005), most patients are habitually distrustful of this treatment options and these could be attributed to its sensitivity and social stigma attached to sexual feebleness in African perspective (Lim et al., 2005), these treatment protocols are often very expensive, not easily accessible to the poor and rural dwellers and are often associated with some serious side effects such as headache and heartburn, myalgia and back pain (Ojewole, 2007). Consequently many people self-medicate with unorthodox and unconventional therapies. One common and popular polyherbal used in Nigeria is Jalin Herbal Mannex Liquid (JHML) with Nafdac No A7-2077L. It is a polyherbal formulation used to augment sexual performance in men, combat weak erection, asphyxiate impotence, improvement of sperm count, eliminate waist ache, increase libido and increased sex duration for the satisfaction of both partners. It contains honey, *Lepidium mehenil*, *Panax ginseng* and *Liriosma ovate*. *Panax ginseng* commonly called ginseng has functioned as a vital component of many Chinese medicaments for centuries and currently it still fills an enviable and prominent position as the most extensively consumed herbal recipe in the world (Blumenthal, 2001). It is generally believed that ginseng does not only stimulate physical benefits but also has a positive effect on cognitive performance and well-being (Mishra and Verma, 2017). Maca (*Lepidium meyenii Walp*) is widely used as a nutritional supplement and in folk medicine to increase fertility and sexual function (Gonzales et al., 2001; Gonzales, 2012; Del Prete et al., 2018). The desiccated hypocotyls of Maca are abundant in food nutrients, such as lipids, carbohydrates, proteins, free fatty acids, essential amino acids (Canales et al., 2000; Tafuri, et al., 2019). Additionally, Maca comprises several secondary plant metabolites like alkaloids macamides, glucosinolates and macaridine (Tafuri et al., 2019), which

are vital in metabolism. *Liriosma ovate* commonly called potency wood, it is a top rated herbs in Amazonian folk medicine, which increases libido and penile stiffness. It functions as a nerve stimulating substance that amplifies the receptivity to sexual stimuli and also the physical perception and sensation of sex (Lim, 2017). It is rich in sterols like sitosterol, campesterol and lupol which activate the body's receptors for hormones like testosterone to heighten libido and enhance performance (Malo et al., 2005). Also present are volatile oils e.g. camphor that help restore sex drive and ease of arousability (Lim, 2017). Honey is a natural product formed from flower nectar by *Apis mellifera*. It is a sugary, piquant vicious liquid. It comprises of sugars, proteins, vitamins, enzymes, minerals, amino acids, scented compounds and polyphones (Alvarez-Suarez et al., 2013; Arawwawala and Hewageegana, 2017; Tafere, 2021). It is generally and extensively used as food and medicaments by many generations, cultures and traditions of the world with great application in religious functions (Tafere, 2021).

Though, herbal drugs are frequently considered safer than orthodox medicines because of their superior lenience. However, there are also documented adverse reactions that are linked with herbal medications (Komlaga et al., 2015; Mensah et al., 2019). The adverse drug reactions linked to herbal medications are largely due to the intrinsic bioactive secondary metabolites inherent in the herbal resources, poor qualities of the plant used which may be due to factors such as contamination with chemicals like pesticides, heavy metals and microorganisms, adulteration with synthetic drug with the principal objective of deceitful improvement in potency and poor quality control methods. Firenzuoli et al. (2005) however, posited that a satisfactory herbal drug must be harmless, unchanging and presented in an appropriate dosage formula and package. In many countries of the world, Nigeria has not been an exception. There are prescribed guidelines for both local and imported herbal products in Nigeria (NAFDAC, 2013), even though herbal medicine practices have not been fully incorporated into the health care delivery system. This investigation is intended at studying the efficacy, potency and biochemical effects of JHML, a popular brand of polyherbal on some vital biochemical indices of male Wistar rats.

MATERIALS AND METHODS

Experimental animals

Twenty (20) healthy adult male Wistar rats weighing 120 to 150 g were used for this study. They were obtained from the animal section of the Pharmacology Department, Niger Delta University, Amasoma, Bayelsa State, Nigeria, and were maintained under standard housing conditions. The animals were adapted for two weeks preceding initiation of experimental regimen, fed with pelletized growers' mash, exposed to clean tap water throughout the period of the study. All animal experimental protocols were

permitted by the Committee of Scientific Ethics at Niger Delta University, Amasoma, and were carried out by its guidelines for animal use.

Experimental design

The animals were allotted into groups 'A' and 'B' of ten (10) rats each in a standard plastic rat cage and were treated as follows according to their body weights for four (4) weeks. Group A: Control rats received 2 mL/kg B.W of distilled water, and group B: The treatment group received 2 mL/kg B.W of JHML.

Sample collection

Upon completion experimental duration, and 24 h after the last oral drug administration, the animals were euthanized under anesthesia in a chloroform chamber and blood were obtained through heart perforation into ordinary sample bottles. The blood samples were made to stand for 20 min for coagulation to occur, and afterwards centrifuged at 2000 rpm for 10 min and the supernatant (serum) collected and kept at 4°C prior to biochemical assay. The testes and kidneys were quickly excised and weighed, after which they were immediately taken and fixed in 10% neutral buffered formalin for histopathological examination.

Purchase of JHML

The JHML with NAFDAC registration number A7-2077L, Batch number 320 and with a production date of August 2020 and expiry date of August 2022 was purchased from Cynflac Pharmacy Limited, Yenegoa, Bayelsa State.

Assay kits/reagents

Luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and prolactin (PRL) radioimmunoassay test kits are products of BYK-Sangtic Diagnostica. Lipid parameters (Total cholesterol, total triglyceride, low-density lipoprotein) spectrophotometric test kits are products of Randox Laboratories Ltd. United Kingdom, while all other chemicals/reagents used in this investigation were of purest analytical grade.

Changes in body weight

Rats in all groups were weighed on the first day and at the completion of the treatment protocols. The percentage change in body weight was calculated using:

$$\% \text{ change in body weight} = \frac{\text{final body weight} - \text{initial body weight}}{\text{initial body weight}} \times 100\%$$

Testes/kidney as the ratio of body weight

Testes/kidneys were removed and weighed immediately. Testes/kidney ratio was calculated as a percentage using the expression below:

$$\frac{\text{weight of organ (g)}}{\text{body weight (g)}} \times 100\%$$

Hormonal assay

Serum samples were analyzed for the following hormones testosterone, follicle stimulating hormone, and luteinizing hormone by the method described by BYK-Sangtic Diagnostica. This was based on the principle of radioimmunoassay of competitive attachment between the sample serum and the standards for a constant quantity of the antisera (Tietz, 1995).

Lipid parameters

The concentrations of cholesterol and triglycerides were estimated by colourimetric method as described by Ochei and Kolhatkar (2008). High-density lipoprotein (HDL) was determined spectrophotometrically using a commercial assay kit from Biosystems S.A. Costa Brava 30, Barcelona (Spain) by adopting the methods of Grove (1979) and Tietz (1990). Low-density lipoprotein cholesterol (LDL) was also determined spectrophotometrically using a commercial assay kit from Biosystem and adopting the method described by Burstein et al. (1970).

Renal function

Urea was evaluated through the adjusted Berthelot method according to Tobacco et al. (1979). Creatinine was assayed by colourimetric kinetic method of Bartels et al. (1971). Uric acid was assessed using the enzymatic colourimetric method of Duncan et al. (1982).

Serum electrolyte

A clinical chemistry analyzer, ST-200 plus ion selective electrode system (ISE) was used for serum electrolyte (Na^+ and K^+) estimations.

Histopathological scrutiny of the testes

Histopathological inquiry of the testes for degeneration and derangement was performed using the method described by Krause (2001).

Statistical analysis

The Statistical Package for Social Sciences (SPSS, IBM, USA version 23) Computer software was used for data analysis. The results were stated as mean \pm standard deviation (S.D) with the results analyzed using a one-way analysis of variance (ANOVA). $P < 0.05$ was regarded as statistically significant.

RESULTS

The effects of administration of the JHML on the body weight and relative organ weight (testes and kidney)

The upsurge in body weight of the rats is expressed in simple percentage (descriptive statistics) as presented in Table 1. After 4 weeks protocols, both experimental groups experienced progressive increase in body weight.

Table 1. The effects of administration of the JHML on the body weight, and relative organ weight (testes, and kidney).

Treatments group	Initial body weight (g)	Final body weight (g)	Changes in body weight (%)	Weight of testes (g)	The relative weight of testes %	Weight of kidney	The relative weight of kidney %
Control (distilled water)	170.00 ± 8.15 ^a	199.60 ± 10.03 ^a	17.41 ^a	1.22 ± 0.15 ^a	0.60 ± 0.12 ^a	2.55 ± 1.71 ^a	1.28 ± 0.36 ^a
JHML (2 mL/kg body weight)	162.19 ± 5.04 ^b	194.60 ± 8.10 ^b	19.98 ^b	1.10 ± 0.05 ^b	0.57 ± 0.30 ^a	2.49 ± 2.40 ^b	1.28 ± 0.51 ^a

Data are Mean ± SD (n=10). Mean on same column with a different superscript letter(s) are significantly different, ($p < 0.05$). One way analysis of variance (ANOVA) followed by post hoc LSD.
Source: Authors

Table 2. The effects of oral route administration of JHML on the concentrations of LH, FSH testosterone, and prolactin.

Treatment groups	LH (m μ /L)	FSH (m μ /L)	Testosterone (ng/mL)	Prolactin (ng/mL)
Control (distilled water)	19.76 ± 3.21 ^a	29.33 ± 3.90 ^a	151.60 ± 7.60 ^a	6.45 ± 0.32 ^a
JHML (2 mL/kg body weight)	25.10 ± 1.89 ^b	34.20 ± 2.92 ^b	166.70 ± 6.70 ^b	4.91 ± 0.21 ^b

Data are Mean ± SD (n=10). Mean on same column with a different superscript letter(s) are significantly different, ($p < 0.05$). One way analysis of variance (ANOVA) followed by post hoc LSD.
Source: Authors

However, the findings indicated a significant increase in body and testes weight ($p < 0.05$) of the group administered with the JHML formula, but with no significant difference ($p > 0.05$) in the relative weight of testes, an indication of proportional growth. The result on kidney and relative kidney weight showed no significant difference ($p \geq 0.05$) between the control and the JHML treated group.

The effects of oral route administration of JHML on the concentrations of LH, FSH testosterone and prolactin

After 4 weeks of treatment protocols, the oral administration of JHML caused a significant increase ($p < 0.05$) in levels of all hormones investigated in this study namely LH and FSH and testosterone with a concomitant significant decrease ($p < 0.05$) in concentration of prolactin of

male albino rats as presented in Table 2.

The effects of JHML on Lipid profile

The oral administration of JHML for 4 weeks as shown in Table 3 caused a non-significant decrease ($p \geq 0.05$) in the levels of total cholesterol and triglycerides. JHML also brought about a significant decrease ($p < 0.05$) in the value of LDLc and a significant increase ($p < 0.05$) in HDLc levels.

The effects of JHML on the concentrations of creatinine, urea and uric acid activities following oral administration of JHML

The oral administration of JHML for 4 weeks as presented in Table 4 caused a non-significant change ($p \geq 0.05$) in the levels of creatinine, urea and uric acid activities of the male rats.

The effects of JHML on the concentrations of serum electrolyte activities following oral administration of JHML

JHML after 4 weeks of oral administration on the rats as presented in Table 5 caused no significant changes ($p \geq 0.05$) in levels of serum electrolytes namely sodium and potassium when compared with the control group that were administered with distilled water.

The effects of JHML on the histology of the testes

The histopathological examination of the testes after 4 weeks of oral dosing of the rats with JHML (Figures 1 and 2) showed no significant changes in histological features between the control group and the JHML treated groups, as both photomicrographs of testicular sections (H & E x

Table 3. The effects of JHML on Lipid profile.

Treatment	Tc (mg/dL)	TG (mg/dL)	LDL-c (mg/dL)	HDLc (mg/dL)
Control (distilled water)	130.90 ± 3.40 ^a	95.34 ± 3.30 ^a	112.80 ± 4.30 ^a	52.60 ± 1.10 ^a
JHML (2 mL/kg body weight)	128.40 ± 2.80 ^a	94.36 ± 3.23 ^a	107.80 ± 4.50 ^b	54.70 ± 1.20 ^b

Data are Mean ± SD (n=10). Mean on same column with a different superscript letter(s) are significantly different, (p<0.05). One way analysis of variance (ANOVA) followed by post hoc LSD.

Source: Authors

Table 4. The effects of JHML on the concentrations of creatinine, urea and uric acid activities following oral administration of JHML.

Treatments	Creatinine (µmol/l)	Urea (mol/l)	Uric Acid (mol/l)
Control (distilled water)	76.9±3.40 ^a	6.63±0.74 ^a	48.60±3.40 ^a
JHML (2 mL/kg body weight)	77.45±2.40 ^a	6.60±1.10 ^a	49.10±1.30 ^a

Data are Mean ± SD (n=10). Mean on same column with a different superscript letter(s) are significantly different, (p<0.05). One way analysis of variance (ANOVA) followed by post hoc LSD.

Source: Authors

Table 5. The effects of JHML on the concentrations of serum electrolyte activities following oral administration of JHML.

Treatments	Na ⁺ (nmol/l)	K ⁺ (nmol/l)
Control (distilled water)	129.60 ± 2.20 ^a	4.79 ± 0.46 ^a
JHML (2mL/kg body weight)	130.70 ± 2.43 ^a	4.80 ± 0.20 ^a

Data are Mean ± SD (n=10). Mean on same column with a different superscript letter(s) are significantly different, (p<0.05). One way analysis of variance (ANOVA) followed by post hoc LSD.

Source: Authors

40) showed testes exhibiting normal features of the seminiferous epithelium and interstitial tissues with active spermatogenesis.

DISCUSSION

JHML is a well-known herbal drug consumed by many for many health reasons, but mostly for aphrodisiac purposes. Kpomah et al. (2012) and Nwafor et al. (2020) described aphrodisiac as any drug, food or food products (e.g. chocolate), topical rubefacient, etc. that stimulates sexual desire, sex drive or sexual pleasure. The incidence of male sexual dysfunction is on the increase globally as it is reported by Chen et al. (2019) that 52% of men aged amid 40 and 70 years suffer from differing levels and forms of sexual dysfunction (erectile dysfunction, low libido, premature ejaculation etc.). In Nigeria, Abu et al. (2019) reported that 66.4% of men of the ages of 18 and 76 years suffer from erectile dysfunction but only 39.4% are aware of treatment and surprisingly only 26.5% of this population seek treatment.

One major reason for the low treatment-seeking

behaviour could be attributed to its sensitivity and the social stigma attached to sexual dysfunction in the African context (Lim et al., 2005), and this makes many patients to self-medicate by resorting to local herbs and sex tonics.

Body weight fluctuations serve as a sensitive indicator of the general health status of animals (Arthur et al., 2011). The results obtained showed that all rats in the two groups experienced a sizeable surge in body weight following the treatment regimen. The amplification in body weight were more significant and pronounced in the group administered with the JHML. The surge in body weight might occur owing to the androgenic properties of the plant extracts in the JHML (honey, *Panax ginseng*, *Lepidium mehenil* and *Liriosma ovate*). Androgen possesses anabolic activity (Mbongue et al, 2005; Thakur and Dixt, 2007; Yakubu et al, 2007). The steroids also block the action of other bodily chemicals that signal muscle fatigue, thereby allowing the individual to perform longer with more intensity and endurance (Singh et al., 2003). Vicissitudes in organ weight are often regarded as a positive indicator of chemically induced organ damage also had no significant change ($p > 0.05$) in the relative

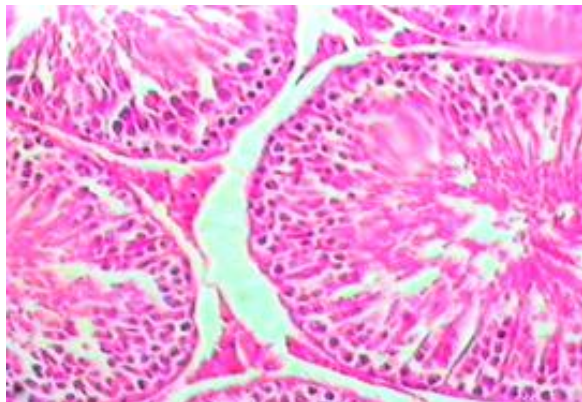


Figure 1. Photomicrographs of testicular sections of control rats (H & E x 40). Atrophied seminiferous tubules with widened interstitial space, spermatogonia, spermatids and spermatocytes.
Source: Authors

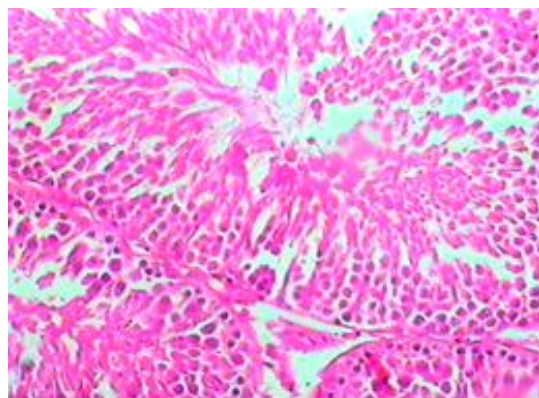


Figure 2. Photomicrographs of testicular sections of rats treated with JHML (H & E x 40). Testes exhibiting a normal feature of the seminiferous epithelium and interstitial tissue with active spermatogenesis.
Source: Authors

weight of testes, and kidneys when matched with the control group, an indication of proportional growth with no adverse or toxicity effect.

All doses of the JHML significantly increased ($P < 0.05$) testosterone, LH and FSH and significantly reduced ($P < 0.05$) prolactin levels. Testosterone is an essential steroid hormone and the most vital androgen for libido and spermatogenesis in males (Fugl-Meyer et al., 2017). A normal level of testosterone sustains the male secondary sex characteristics and normal sexual desire, which stimulates sperm maturation and protein synthesis, specifically in reproductive organs and muscles (Chen et al., 2019). Androgen insufficiency is a primary causative factor of many common clinical diseases and may lead to one form of sexual dysfunction or another and a decline in reproductive capacity in men (Chen et al., 2019). FSH

is secreted by the Sertoli cells it plays a vital part in testicular development (Algeffari et al., 2018). FSH also sustains the testosterone level in spermatogenic cells, promotes the binding of androgen binding protein (ABP) to testosterone, and regulates the number of spermatogenic cells (Chen et al., 2019). LH are also needed for upholding testosterone levels, hence an increase in LH and FSH automatically causes an increase in testosterone levels (Yakubu et al., 2007). Studies have shown that testosterone supplementation helps to improve sexual function and libido (Grahl et al., 2007; Fugl-Meyer et al., 2017), in addition to the magnification of orgasm intensity (Morales, 1996). The concentrations of testosterone, FSH and LH are often used as an indirect indices for evaluating male sexual function. The likely inability of the pituitary gland to sustain the ratio of these hormones may possibly upset several processes involved in sexual function to different levels. High concentration of prolactin in men (hyperprolactinemia) has been linked with hypogonadism, decreased sperm count and motility, erectile dysfunction and decreased libido (Paick et al., 2006; Chen et al., 2019). The positive stimulatory capability of the JHML on these sexual and reproductive hormones can be attributed to its positive stimulatory effect on sexual function.

Serum levels of lipoproteins, such as LDL-c, total cholesterol, and HDL-c are often used as a screening guide for disease diagnosis. The oral route administration of JHML caused no significant change ($p \geq 0.05$) in values of triglycerides and total cholesterol but with a significant reduction ($p < 0.05$) in LDLc, and a significant increase ($p < 0.05$) in HDLc levels. The significant increase in HDL-cholesterol levels and a reduction in LDL-cholesterol levels observed in rats administered with JHML is an indication that the polyherbal can reduce the cardiovascular risk (Chu et al., 2016; Hedayatnia et al., 2020).

Renal function is vital for homeostasis, as the kidneys play vital pleiotropic functions comprising the elimination of metabolic waste and the sustainability of water-electrolyte equilibrium as well as blood pressure (Arthur et al., 2011), thus measuring renal function is vital to ensuring safety of drug dispensing and the detection of acute kidney damages at early onset. The non-significant changes ($p \geq 0.05$) in renal function parameters (creatinine, urea, and uric acid) of the rats administered with JHML when compared with the control group is an indication of the non-adverse effect of the JHML on the kidney (Nwankpa et al., 2018; Tejchman et al., 2021).

Electrolytes are essential in maintaining homeostasis by regulating fluid equilibrium, oxygen transport, acid-base equilibrium, heart and neurological functions (Woyesa et al., 2022). However, an electrolyte imbalance may occur causing *hyper* or *hypo* level of this electrolyte in the body. Abnormalities in electrolyte balance are seen in certain metabolic diseases like diabetes mellitus

(Hasona and Elsbali, 2016; Ashraf et al., 2018; Woyesa et al., 2022). Sodium and chloride ions are the chief electrolytes in the extracellular fluid whereas potassium, magnesium and phosphate are the chief electrolytes in the intracellular fluid. Diffusion of cellular K^+ out of the cells and Na^+ into the cells is caused by trans-membrane electrical gradients. Sodium-potassium ion (Na^+-K^+) pump, which is stimulated by insulin and catecholamine hormones, reverses the movement of these electrolytes to maintain their extracellular and intracellular homeostasis (Woyesa et al., 2022). The non-significant changes ($p \geq 0.05$) in concentration of serum electrolytes namely sodium and potassium investigated in this study is an indication that the polyherbal had no adverse effect on electrolyte balance.

Histomorphological check of the control group testes (Figure 1) and the JHML administered groups (Figure 2) showed cells with seminiferous tubules containing orderly maturation of germ cells with normal spermatogenesis, an indication of no adverse or negative effect on gametogenesis and sexual function.

Conclusion

The outcome from this present investigation has shown that JHML has a positive stimulatory effect by significantly increasing ($P < 0.05$) the levels of testosterone, follicle stimulating hormone, and luteinizing hormone, while significantly decreasing ($P < 0.05$) the value of prolactin. The use of this polyherbal drug at the dose used for this study showed no statistically significant change ($p \geq 0.05$) in body weight and relative organ weight, lipid profile, renal function and serum electrolyte balance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Physicochemical parameters of *Blighia sapida* (K.D. Koenig) oil extracted in Togo

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Oils extracted from *Blighia sapida* fruits are usually consumed without any information on its chemical composition. However the bad quality of oil can be a source of toxicity. Thus, the aim of this study is to investigate the physicochemical quality of the oil extracted from *B. sapida* from Togolese flora. To reach the goal of the study, mature and immature of *B. sapida* arils were used for oil extraction by the solvent method using hexane. The physicochemical parameters including density, acid value, saponification value, ester value, iodine value and impurity content were determined according to the French Association for Standardization (AFNOR) standards. The results of the extraction yields were 41.7% (immature arils) and 48.8% (mature arils). The mature arils oil had acid value of (7.013 mg of KOH/g), saponification (195.17 mg of KOH/g), iodine (87 g I₂/100 g) and ester (188.157 mg of KOH/g) values were different for those of the immature arils' which were 30.86 mg of KOH/g (acid value), 191.68 mg of KOH/g (saponification value), 85 g I₂/100 g (iodine value) and 160.82 mg of KOH/g (ester value). The densities were 0.916 g/cm³ (mature) and 0.77 g/cm³ (immature) while the impurity content was 3.4% (mature) and 16.1% (immature). The effect of heating the oils resulted in very high values of the physicochemical parameters. The results obtained deduce that this oil meets the Codex Alimentarius standard.

Key words: *Blighia sapida* oil, physicochemical parameters, extraction, frying, Togo.

INTRODUCTION

Malnutrition remains a real public health problem and the food security appears unattainable in several developing countries (FAO, 2020). Diets pattern and lifestyles were

disrupted with increasing industrialization and rapid urbanization. This has had an impact on the health and nutritional status of populations, especially those in

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developing countries (Kennedy et al., 2004). Among the most sustainable solution approaches is the production of fruits and vegetables which contribute to the reduction of poverty and malnutrition in all its forms (CIRAD/FAO, 2021). Among the strategies adopted to overcome malnutrition and food insecurity, is the sanitary and nutritional quality control of oil, since it plays an essential role in the proper functioning of the body. Lipids provide the body essential fatty acids such as linoleic acid and alpha linolenic acid. It also contributes to the supply of fat-soluble vitamins (vitamins A, D, E and K) (Li et al., 2014). In addition, lipids contribute to the organoleptic quality of food, providing them with a smooth, creamy, melting texture, a shiny appearance and a specific flavor (Akintayo et al., 2002).

Vegetable oils are vulnerable to numerous reactions such as isomerization and oxidation of fatty acids due to their rich profile of mono and polyunsaturated fatty acids. These reactions lead to the production of trans-fatty acids. If consumed in excess, they can be associated with obesity, increased risk of cardiovascular disease, cancer and type 2 diabetes (Akintayo et al., 2002). Nevertheless, much research has been done on the chemical changes of fats during heating in relation to consumer health. The heating technique damages the integrity of lipids and proteins, thus favouring the appearance of neo-formed compounds (Prache et al., 2020).

Among the food plants of the Togolese flora, *Blighia sapida* fruits are usually consumed. This plant belongs to the Sapindaceae family and found in several regions of the world (Africa, Jamaica and Haiti). According to Dossou et al. (2014), this fruit tree has very interesting medicinal and nutritional values. Almost all of the plant's parts, such as its roots, leaves, bark, and seeds, are used in food and traditional medicine for the treatment of certain diseases (Dossou et al., 2014). Due to its high oil content (45.5%), special attention must be given to this plant species. However, despite the proven nutritional values of *B. sapida* arils, this plant can be a source of toxicity through the consumption of the fruit at an early stage of ripening (Ouattara et al., 2014). Indeed, improper consumption of *B. sapida* is reported to be toxic mainly due to the presence of hypoglycin A and B (Bowen-Forbes and Minott, 2011). The presence of these toxic compounds decreases in *B. sapida* upon its maturity (Bowen-Forbes and Minott, 2011).

In Togo, *B. sapida* stands are mostly present on fertile, deep, and well-drained soils but also on calcareous soils. Several studies have been conducted in Togo and elsewhere on the virtues of edible oils, but scientific work carried out in Togo and in the sub-region on *B. sapida* oils is almost non-existent. Nevertheless, some work has been carried out on *B. sapida*, notably on the physicochemical composition of the plant's arils in Ivory Coast (Ouattara et al., 2014). Due to its importance for the Togolese population and the harmful effects

generated, especially by the immature arils of this plant; it is important to broaden and deepen the knowledge about its oil. It is necessary for the better use and subsequent management of *B. sapida* to investigate its oil composition. Thus, the main objective of this study was to evaluate the physicochemical quality of the *Blighia sapida* oil extracted in Togo.

MATERIALS AND METHODS

Sampling and samples collections

Three towns (Figure 1) were chosen, for the collection of *B. sapida* arils, based on the representativeness of the plant in relation to its geographical distribution as previously reported by Tourey et al. (2020) and recently confirmed by Nabede et al. (2022). In these three targeted towns (prefectures of Kozah, Haho and Tône) immature and mature arils of *B. sapida* (Figure 2) were collected during the months of March to October 2021.

Sample analysis

Oil extraction

Once collected, *B. sapida* arils were sun-dried for about two weeks and then pulped using a Retsch blender type SM 2000/1430/Upm/Smfet for oil extraction. The lipid content of the pulped arils was determined according to the Association of Official Analytical Chemists (AOAC) method using hexane as solvent (AOAC, 2005). Briefly, 100 g of each dry pulped arils sample was mixed with 150 ml of hexane for about 7 h. After filtration, the solvent was evaporated (90°C for 24 h). The extract was then weighed and stored (4°C) in dark freezer for later analysis.

After extraction, the yield was determined by the ratio between the amount of oil obtained and the amount of the used plant material. This yield is given by the following formula:

$$Y (\%) = \frac{M_1}{M_0} \times 100$$

where y represents the yield, M₁=mass of the oil after evaporation, M₀=mass of the starting plant material.

Determination of the extracted oil density

The density of extracted oil was determined using the French Association for Standardization (AFNOR) standards (AFNOR, 2000). A part of the *B. sapida* arils extracted oils, peanut oil was used to make comparison. Thus, the following formula was used for its determination:

$$D = (P_3 - P_1) / (P_2 - P_1)$$

Where D: density, P₁: weight in gram of the empty pycnometer, P₂: weight in gram of the pycnometer filled with distilled water, P₃: weight in gram of the pycnometer filled with oil.

Determination of the melting point

The evaluation of the temperature at which extracted *B. sapida* arils oils changes from solid to liquid (melting point) is based on the linear variation of the temperature along the heating plate. For this

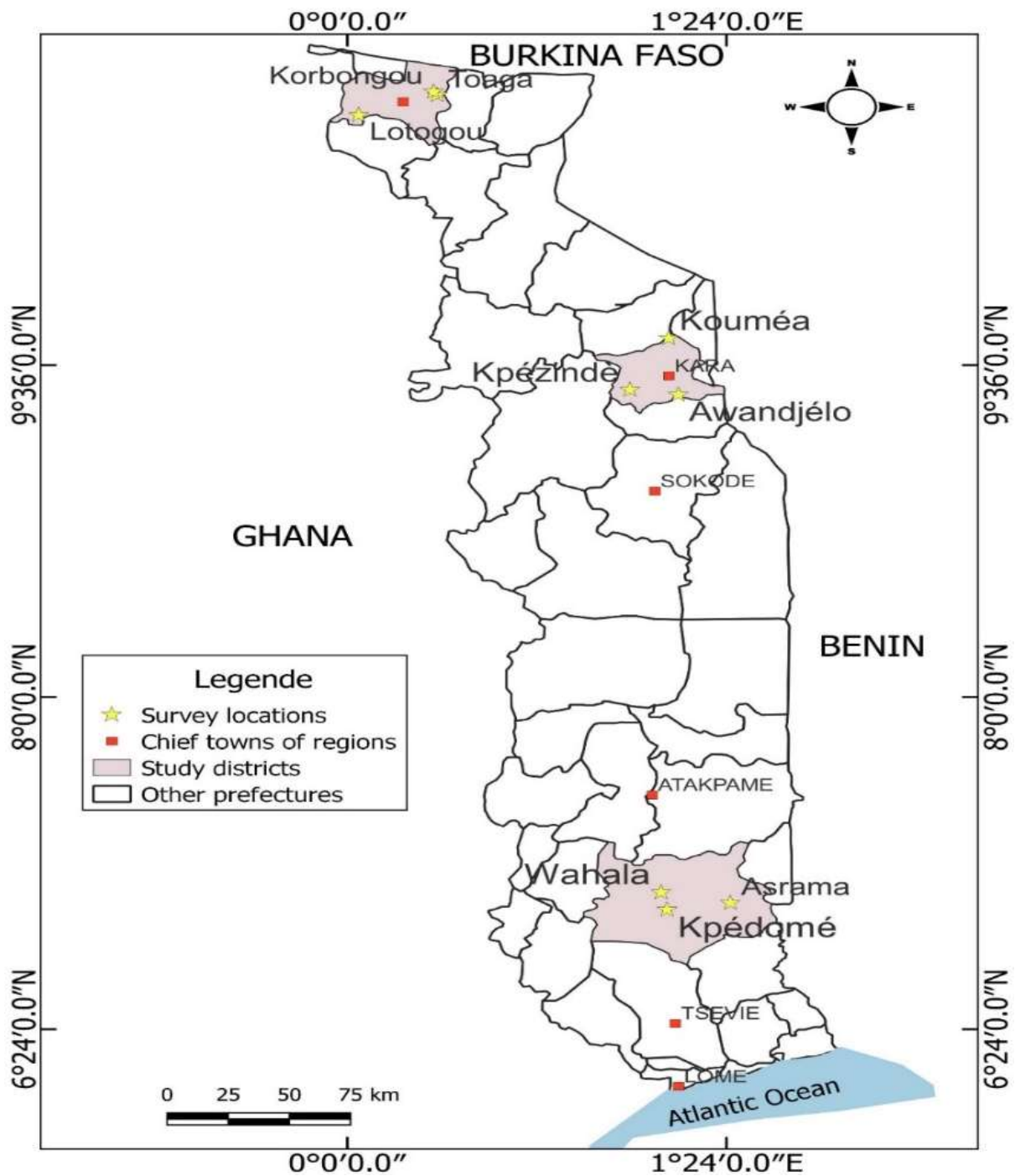


Figure 1. Map of the study area showing the samples collections places.
Source: Authors

purpose, the method described by the French Association for Standardization standard was used (AFNOR, 2000). Solid oil was deposited on the hot part of the plate after setting the plate to an initial temperature between 5 and 10°C below the expected melting point. Then, the oil paste was gently moved from the cooler region to the warmer region until the first lipid drop appeared. Finally, the index of the apparatus was adjusted to read the expected melting temperature. As it is difficult to define a single melting point for a

substance, our measurements were made in triplicate and averaged.

Determination of the hydrogen potential (pH)

The pH was determined using a pH meter. To measure it, after calibrating the pH meter with the ambient environment, 50 ml of oil to be analyzed is taken in a beaker, then the probe is immersed in the

a. *B. sapida* immature fruitb. *B. sapida* mature fruit

Figure 2. Picture of the immature and mature fruit of *B. sapida* collected for oil extraction.

Source: Authors

oil and the pH is read. In addition, between two measurements carried out in different solutions, the probe is immersed in a beaker containing distilled water and then wiped very lightly with absorbent paper. "Fried *B. sapida* oil", are mature *B. sapida* oil that was used for 15 min frying during eight cycles. Apart from the *B. sapida* arils extracted oils, peanut oil was used to make comparison.

Determination of the iodine index

For this purpose, a solution of iodine monochloride with chloroform was added to a test sample. After reaction, the excess iodine monochloride was reduced by the addition of 10% potassium iodide solution and finally the liberated iodine was titrated with a 0.1 N sodium thiosulphate solution (AFNOR, 2000). The iodine index was sought using the Wijus reagent (iodine monochloride) method. Thus, after weighing 2 g of extracted oil, 5 ml of chloroform was added. After dissolving, 25 ml of Wijus reagent was added and the resulting mixture was stoppered and shaken gently and incubated at room temperature for 1 h. Then 5 ml of the instantaneously prepared 10% potassium iodide were added. The titration was carried out with the 0.1 N sodium thiosulphate solution until the yellow color due to iodine had almost disappeared. In addition, a few drops of starch (color indicator) were added and the titration was continued until the blue-violet color disappeared. A blank test was carried out under the same conditions. Finally, the iodine index was calculated by the following formula:

$$I_i = (V_0 - V) \times 126.9 \times N/P$$

Where I_i : iodine index, V_0 : volume (ml) of $\text{Na}_2\text{S}_2\text{O}_3$ (0.1 N) required to titrate the blank, V : volume (ml) of $\text{Na}_2\text{S}_2\text{O}_3$ (0.1 N) required to

titrate the sample, P : test weight (g) of the sample, N : normality of the $\text{Na}_2\text{S}_2\text{O}_3$ (0.1 N) solution (AFNOR, 2000).

Determination of the acid index

The acid index of the *B. sapida* arils oils and peanut oil was determined according to the AFNOR NFT-60-2000 standard (AFNOR, 2000). 75 ml of 95% ethanol was added to 2 g of oil, a few drops of 1% phenolphthalein were added to neutralize the mixture, swirled vigorously and titrated with the potassium hydroxide solution (the titrated ethanolic solution is 0.1N) until a persistent pink color is obtained. This index was calculated by the following formula:

$$A_i = (56.11 \times V \times N) / P$$

Where A_i : acid index, P : mass (g) of the test sample; 56.11: molar mass expressed in g/mol of potassium hydroxide; V : volume in ml of KOH (0.1 N) required for the titration; N : normality of the potassium solution (0.1 N).

Determination of the saponification index

The saponification index of the *B. sapida* arils oils and peanut oils was determined using the AFNOR standard (AFNOR, 2000). 2 g of oil were mixed with 25 ml of 0.5 N KOH in a flask. After boiling for 1 h, the flask was cooled under tap water. Then 2 to 3 drops of phenolphthalein were added and titration with a 0.5 N HCl solution was performed until the pink color disappeared and the initial color of the mixture reappeared. A control was used according to the same procedure with a test sample of 2 ml distilled water. The saponification value was calculated using the formula:

$$S_i = (V_0 - V) \times N \times 56.11 / P$$

Where S_i : saponification index, V_0 : volume in ml of HCl used for the blank test; V : volume in ml of HCl used for the test sample; P : test sample in grams.

Determination of the ester index

The ester index (E_i) of the *B. sapida* arils oils and peanut oils was determined using the following formula (AFNOR, 2000):

$$E_i = S_i - A_i$$

Determination of the impurity levels

The dockage rate of the *B. sapida* arils oils and peanut oils, which refers to the spoilage of fats, was calculated by the following formula:

$$\% \text{ impurity} = A_i \times 100 / S_i$$

This proportion was combined with the effect of thermos-oxidation of this oil that was determined by frying the yam pieces (AOAC, 2005).

Determination of organoleptic characteristics

In order to deepen the study on *B. sapida* arils oils, some organoleptic characteristics were compared with the Codex Stan (33-1981) standard (CODEX 2001). This sensory analysis will allow the quality of this oil to be assessed.

Data analysis and processing

The data obtained were coded and inserted into the MS Excel 2016 spreadsheets and then analyzed using Minitab 17 software. The analysis of variance (ANOVA) test was used to verify the normality of the variables. Considering the effect of thermos-oxidation, both oils were subjected to analysis of variance of a two-factor mixed model (with 2 replicates): "*B. sapida* oil" and "Fried *B. sapida* oil". Results are considered significant at $P < 0.05$, highly significant at $P < 0.01$ and very highly significant at $P < 0.001$.

RESULTS

Oil extraction yield

The yields of the different extractions were 41.7 ± 0.66 and $48.8 \pm 0.5\%$ for immature and mature arils, respectively.

Physicochemical characteristics of extracted oils

The melting point results are 15.97 ± 0.3 and $16.01 \pm 0.0^\circ\text{C}$ for immature and mature *B. sapida*, respectively. These values show that there is no significant difference between these two oils ($p > 5\%$). The physicochemical characteristic of the extracted oils is summarized in Table 1. The results of this study recorded values of: 87 ± 0.7 , 85 ± 1.1 ,

89 ± 1.7 (g of $I_2/100$ g oil), respectively for oil extracted from mature arils, oil extracted from immature arils and peanut oil.

The mature *B. sapida* arils oil meets the Codex Alimentarius standard (2.2-7.26 mg KOH/g), concerning the acid value. In addition, the acid value recorded for mature *B. sapida* arils oil is not statistically different ($p > 0.05$) from that of peanut oil 6.73 ± 0.00 mg KOH/g. However, the analysis of the results of the oil of immature arils allows us to realize that this acid value is different from the Codex Alimentarius standard. The ester number deduced from the saponification number and the acid number is approximately close to international standard (186 -187.94). Peanut oil showed a significantly different effect from that set by the Codex Alimentarius standard. The pH values of the different oils are summarized in Table 2

Table 3 indicates the organoleptic characteristics of the different *B. sapida* oils used in the study. For given oil, the recorded data varies according to the organoleptic parameter. Thus, the mature *B. sapida* oil is yellow and clear with good smelling, taste and flavor. The immature oils are light yellow and viscous with fairly good smelling, bad taste and flavor. The fried oils are black and dark with good smelling, less good taste and pungent flavor (Table 3).

DISCUSSION

Different extractions yields were observed between immature ($41.7 \pm 0.66\%$) and mature arils ($48.8 \pm 0.5\%$). This difference would be due to the state of maturity of the arils because in oil seeds the percentage of oil increases with the maturity of the fruits. The recorded values in this study are close to the $44.86 \pm 0.66\%$ obtained by Akintayo et al. (2002) in the same plant species. Moreover, this proportion of oil in mature arils is also comparable to that contained in peanut which is 45.50% (FAO, 2011). This oil richness makes this plant an important potential in the oil industries.

The density, which provides information on the purity, fatty acid profile and oxidation state of an oil, was evaluated in this study. The results in Table 1 show values of 0.916 ± 0.3 g/cm³ and 0.93 ± 0.022 for mature arils and peanut oil, respectively. Indeed, these values comply with the Codex Alimentarius standard and therefore confirm a better chemical composition of these two oils. These results corroborate those of Morin and Pagès-Xatart-Parès (2012) who found similar values (0.915 and 0.964 g/cm³). On the other hand, the results revealed by the immature arils do not belong to the Codex Alimentarius standard; this therefore testifies to the importance of maturity for the quality of this vegetable oil because in the immature state water accelerates oxidation. Indeed, the melting point gives information on the structure of the fat; the lower the melting point, the

Table 1. Physicochemical characteristics of fresh *Blighia sapida* and peanut oils.

Parameter	Mature aril oil	Immature aril oil	Peanut oil	Codex standard
d ₂₀ ²⁰	0.916±0.3	0.77±0.01	0.93±0.022	0.913-0.932
Ai	7.01±0.00	30.86±0.00	6.73± 0.00	2.2-7.26
Ii	87±0.7	85±1.1	89±1.7	92-102
Si	195.17±0.04	191.6±0.04	156.83±0.04	189-195.2
Ei	188.16±0.02	160.82±0.00	150.1±0.22	186-187.94
% impurity	3.4%	16.1%	4.29%	1.16-3.71%

d₂₀²⁰: Density in g/cm³, Ai: Acid index in mg KOH/g, Ii: Iodine index in g of I₂/100g oil, Si: Saponification index in mg KOH/g, Ei: Ester index in mg KOH/g.
Source: Authors

Table 2. Table showing the pH values of the different oils.

Oil sample	pH
Mature <i>B. sapida</i> oil	7.03
Immature <i>B. sapida</i> oil	5.50
Fried <i>B. sapida</i> oil	5.71
Peanut oil	6.92

Source: Authors

Table 3. Organoleptic characteristics of the different oils used.

Organoleptic features	Types of <i>B. sapida</i> oil			Standard Codex Stan 33-1981
	Mature oil	Immature oil	Fried oil	
Color	Yellow	Light yellow	Black	Yellow to green
Smell	Good	Fairly good	Good	Good
Taste	Good	Bad	Less good	Good
Flavor	Good	Less good	Pungent	Good
Aspect	Clear	Viscous	Dark	Clear

BM: *B. sapida* mature oil; HBI: *B. sapida* oil immature; HBF: *B. sapida* oil fried.
Source: Authors

richer the fat in unsaturated fatty acids and should be recommended for consumption. Moreover, this characteristic parameter is proportional to the length of the carbon chain. Furthermore, the melting point results obtained in this study are lower than the melting point of lauric acid (44.2°C) and myristic acid (54.4°C). This difference would be due to the fact that lauric and myristic acids are saturated fatty acids whereas *B. sapida* oil would contain unsaturated fats. Nevertheless, the melting point of both oils (mature and immature *B. sapida*) is higher than that of olive oil. In addition, it should be noted that the crystalline forms significantly influence the melting point and consequently on the organoleptic properties of the lipids (Terescenco, 2018).

These values of the iodine index are slightly higher

than those obtained by Ouattara et al. (2014) (56.6 ± 2.55 g of I₂ / 100 g of oil) and Akintayo et al. (2002) (65.4 g of I₂ / 100 g of oil) but closer to the values set by the Codex Alimentarius standard. This difference would be due to the extraction conditions of the different oils and the ecological conditions in the different localities. The high-water content in the immature state would be at the origin of this result (Surmaitis and Hamilton, 2022). This result suggests that the immature oil of *B. sapida* is acidic and unfit for human consumption and that the oil from the mature arils should be preferred. Furthermore, the results for mature arils are similar to those reported by Akintayo (2001) and Ouattara et al. (2014), who found acid values of 4.91 and 2.31 mg KOH/g, respectively with *B. sapida*. Thus, the acid percentages are 2.52% (mature *B. sapida*

oil), 15.51% (immature *B. sapida* oil) and 2.82% (peanut oil) for immature *B. sapida* oil and peanut oil, respectively. These percentages are slightly higher than the Codex Alimentarius (2021) standards, whose maximum value for a refined vegetable oil is 0.6 mg KOH/g oil. Indeed, the lower the acidity, the better the oil. Furthermore, it has been shown that oil from arils harvested three days before fruit dehiscence is more acidic than oil from arils that have reached maturity on the tree (Falloon et al., 2013). This increase observed in our study would be due to the nature of the plants used and the ecological conditions. The high acidity of the immature *B. sapida* arils oil shows that a treatment of the crude oil is necessary before consumption. The refining technique could be one of the appropriate treatments.

The saponification value showed very interesting values with the two vegetable oils of *B. sapida* as they were well within the range set by the Codex Alimentarius standard (2021). Moreover, this oil with a saponification index in accordance with the official Codex Alimentarius standard (189 - 195.2 mg KOH/g oil) would be a potential source of soap manufacturing; thus, strengthening the Togolese soap industry. In addition, the saponification indices of mature and immature oils are not significantly different ($P > 5\%$) because the maturation of the arils does not influence the saponification indices of their oils. Therefore, both *B. sapida* oils can be classified as low molecular weight oils (Tsado et al., 2018). This new data reinforces the nutrition of Togolese infants because low molecular weight oils are easily digested throughout the digestive tract and are recommended for children. The present results on saponification indices are comparable to those reported by Ouattara et al. (2014).

Considering the impurity rate, this parameter was found to be interesting (3.4%) for mature aril oil within the margin set by the Codex Alimentarius standard (1.16 - 3.71%). In food industry control, an oil is only consumable if the percentage of impurities is close to 1% (FAO, 2020). In the light of these results (ester index and impurity rate), it can be deduced that *B. sapida* oils in its mature state complies with the standard and therefore can be promoted for its use as food, cosmetics and pharmaceutical potential.

These data obtained reveal approximately neutral pH values for mature *B. sapida* oil and peanut oil. On the other hand, the pH results for *B. sapida* oil from immature arils and fried oil of the said plant species revealed a high acidity. In fact, this increase in acidity would be due to the fact that in the fresh state or during frying, the water contained in the food accelerates the oxidation process (Karel, 1980), making the medium acidic by increasing the polar compounds.

Conclusion

This study showed that the density values obtained with mature arils and peanut oil are in conformity with

the food standard and confirm a better chemical composition of these two oils. At the end of this study, the acid value indicates the acidic and unsuitable state of the immature *B. sapida* oils and allows preferring the mature arils oil for our diet. In addition, the oil of this plant species, which presented a saponification index in accordance with the official Codex Alimentarius standard, would be a potential source of soap making, thus strengthening Togolese soap making.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of Quantitative Trait Loci (QTLs) of *BCL11A* and *HBS1L-MYB* genes on clinical-biological variability of sickle cell disease in a Senegalese pediatric population

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High levels of hemoglobin F (HbF) could reduce the severity of sickle cell disease (SCD) by inhibiting hemoglobin S (HbS) polymerization. HbF expression is modulated by the Quantitative Trait Loci (QTLs) located on the *HBS1L-MYB intergenic region (HMIP)* (rs28384513) and intron 2 of the *B-cell lymphoma/leukemia 11A (BCL11A)* gene (rs4671393, rs1427407). To assess the impact of QTLs of HbF on clinical and biological parameters associated with the severity of sickle cell disease in a Senegalese pediatric population, 301 children with SCD not treated with hydroxyurea were recruited. The numbers of hospitalizations and VOC were estimated over 2 years. HbF levels were determined by HPLC. Three QTLs of HbF were genotyped by High Resolution Melting (HRM), two single nucleotide polymorphisms (SNPs) of *BCL11A* and one SNP of *HMIP*. Data analysis was performed using SPSS. The mean frequency of hospitalizations was 0.84 ± 1.29 . The mean number of vaso-occlusive crises (VOC) episodes was 2.73 ± 1.98 . The mean Hb concentration was 7.76 ± 1.05 g/dl. The mean HbS was $82.28 \pm 4.78\%$ and the mean HbF was $9.49 \pm 5.12\%$. *BCL11A* (rs1427407) was associated with fewer hospitalizations. Both *BCL11A* SNPs were associated with increased HbF levels. *BCL11A* SNPs were associated with increased HbF levels, decreased HbS levels, and decreased hospitalizations (rs1427407). However, no association was noted between these SNPs and the number of VOC episodes. Thus, HbF QTLs are not the only genetic factors modulating the clinical severity of sickle cell disease, which suggests the involvement of other genetic factors such as alpha-thalassemia.

Key words: sickle cell disease, hemoglobin F, quantitative trait loci of HbF, *HBS1L-MYB intergenic region*, *B-cell lymphoma/leukemia*, vaso-occlusive crises.

INTRODUCTION

Sickle cell disease is an autosomal recessive inherited disorder characterized by the presence of mutated hemoglobin (Hb) in the red blood cells (RBCs) called hemoglobin S (HbS). The latter results from a point

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mutation in the seventh codon of the β -globin gene, GAG, which is replaced by GTG. At the protein level, this mutation will cause the replacement of glutamic acid by valine in the β chain of globin. Sickle cell disease is the most common genetic disease in the world, with nearly 7% of the world's population carrying a sickle cell or thalassaemic gene (Gueye Tall et al., 2017). It is a real public health problem in Africa where its prevalence varies from 10 to 40% of heterozygous carriers depending on the region. In Senegal, 1 person in 10, regardless of ethnicity, geographical origin, or social class, carries the sickle cell gene (Thiam et al., 2017).

Pathophysiologically in deoxygenated conditions, HbS polymerizes, leading to the sickling of red blood cells. The RBCs formed falciform, are fragile and rigid. This explains the chronic hemolytic anemia of the patients on the one hand and the occurrence of painful vaso-occlusive crises on the other. The pro-oxidant, pro-adherent and pro-inflammatory context modulates the clinical severity of the disease.

Although sickle cell disease is a monogenic disease, it is characterized by inter-individual clinical variability. This is partly related to the existence of genetic factors. Indeed, various modifier genes have been identified or suspected to have a direct or indirect influence on the clinical severity of the disease (Dahmani et al. 2016; Gueye Tall et al., 2017). HbS polymerization is the main driver of sickle cell disease pathophysiology and high levels of fetal hemoglobin may reduce the severity of sickle cell disease due to its ability to inhibit HbS polymerization and also reduce the mean concentration of corpuscular HbS (Steinberg and Sebastian, 2021). HbF is therefore, the main genetic modulator of the hematological and clinical features of sickle cell disease (Makani et al., 2011).

Many studies in sickle cell disease patients have demonstrated that genetic variations at loci influencing HbF levels modify the clinical course of sickle cell disease. These advantages and recent information on HbF regulation have prompted new efforts to induce high HbF levels in sickle cell patients (Dahmani et al. 2016; Gueye Tall et al., 2017).

Previous genetic association studies have shown that high HbF levels are associated with Quantitative Trait Loci (QTL). The action of these QTLs consists of modulation of HbF expression that has an attenuating effect on the clinical severity of sickle cell disease. These loci mainly include the *Xmn1-HBG2* polymorphism, the *HBS1L-MYB* (*HMIP*) inter-gene region, and intron 2 of the *BCL11A* gene. These QTLs are thus associated with persistent HbF levels and account for 20 to 50% of inter-individual variation in HbF.

Although several works on possible associations between QTLs and HbF levels have been reported in other literature, data on this important aspect of sickle cell

disease have not been sufficiently studied in West Africa in general and Senegal in particular. Since sickle cell disease is marked by painful, life-threatening attacks, much more extensive studies are needed on patient to provide additional information on the regulation of the genes that code for HbF.

Given these findings, this study aims to assess the frequency and influence of three SNPs found at the *BCL11A* (rs4671393 G>A; rs1427407 T \geq G) and *HMIP* (rs28384513 A>C) loci on HbF levels and to study the effect of these three SNPs on the clinical severity of sickle cell disease.

MATERIALS AND METHODS

Clinical follow-up of the cohort

The study population consisted of 301 children and adolescents with homozygous sickle cell disease SS (169 boys and 132 girls; median age of 9.1 years). These children were all followed at the Albert Royer Children's Hospital in Dakar, Senegal. Parents and guardians were interviewed; patients' medical records were reviewed to define their clinical characteristics over the past two years. These medical files were therefore consulted to find the age of diagnosis of the disease, the number of vaso-occlusive crises (VOC), and the number of hospitalizations for reasons other than a VOC during the last 2 years. All painful VOC events were observed by a clinician during an acute outpatient visit or hospital admission. Non-specific complications of sickle cell disease such as fever or pneumonia were not taken into account and the malaria crisis was systematically excluded. In addition, the patients did not benefit from neonatal screening or treatment with hydroxyurea. They have also not been transfused during the last three months preceding the study.

The study was approved by the Ethics Committee of Cheikh Anta Diop University in Dakar. All patients' parents and legal caregivers gave informed consent for the genetic diagnosis of sickle cell disease, including modifier genes.

Hematological and biochemical parameters

Hematological parameters consisting of the hemogram and reticulocytes (RET), were measured using a Sysmex XT 4000i device (System Corporation, Tokyo, Japan). Biochemical parameters were determined using the Mindray BA88 analyzer (Mahwah, NJ). These were lactate dehydrogenase (LDH), total and direct bilirubin (BIL), and C-reactive protein (CRP). The different hemoglobin fractions were also quantified by high-performance liquid chromatography using the short program VARIANT II β -thalassemia (Biorad, Hercules, CA).

Genetic analysis

DNA was extracted from peripheral blood stained with EDTA using the QIAmpVR DSP DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany) in Dakar. Genotypic analyses were performed in Lyon, France. Differential diagnosis between β^S/β^S , β^S/β^C (HBB: c.19G>A), $\beta^S/\beta^{\text{thal}}$, or β^S/β^X genotypes was performed with a dedicated amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method (Gilman et al., 1985). For

patients suspected to carry β^S/β^X or $\beta^S/\beta^{\text{thal}}$ by ARMS-PCR, a direct Sanger sequencing of the HBB gene was performed on the ABI PRISMVR 3130xl (Applied Biosystems, Foster City, CA, USA) apparatus to confirm the diagnosis and to identify the β -thal mutation or the hemoglobin (Hb) variant at the molecular level. Genotyping of the 2 SNPs in intron 2 of the *BCL11A* gene (rs4671393 G>A; rs1427407 T>G) and the SNP in the *HMIP* region (rs28384513 A> C), was performed using the HRM technique on the Light Cycler R 480 (Roche Diagnostics, Meylan, France) (Lederberg, 1999).

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 22 (SPSS Inc., Chicago, IL). The significance level was defined at $p < .05$. Continuous variables were reported as mean \pm standard deviation (SD) and categorical variables as number (N) or frequency. The normality test of the distribution of biological parameters was calculated using the Kolmogorov-Smirnov test. The comparison of means is done with the one-sample analysis of variance test (one factor ANOVA) between the three genotypes of each QTL and the post hoc test (Bonferroni goodness-of-fit test) between two genotypes of each QTL.

For each genetic variant, the allelic frequency was calculated and the balanced distribution of the three possible genotypes in the population (homozygous wild type, heterozygous and homozygous mutant) was checked by the Hardy Weinberg equilibrium.

RESULTS

Clinical and biological characteristics of the cohort

The mean number of VOC episodes over 2 years was two with extremes ranging from 0 to 12 episodes, while the frequency of hospitalizations ranged from 0 to 10 with a mean of 0.84. All these characteristics are reported in Table 1.

The blood count parameters studied were hemoglobin, leukocytes, reticulocytes, and platelets. For Hb, we noted that its concentration varied between 5.50 to 12.3 with a mean of 7.76 g/dl. The mean white blood cell count (WBC) and reticulocyte values were 14.30 and 325.03 G/L respectively. The mean platelet count was approximately 449.56 G/L. The HbS level, with a mean of 82.28%, ranged from 62.60 to 91.80%. HbF levels ranged from 1.10 to 26.80% with a median level of 9.49%. The hemolysis parameters were LDH and bilirubin (B). The mean enzyme activity of LDH was 940.43 with extremes ranging from 148 to 3318 IU/L. As for total bilirubin (TB), the values varied between 7.21 and 106.90 with a mean concentration of 43.98. All these characteristics are reported in Table 2.

Molecular characteristics of the cohort

The prevalence and allelic frequencies of the QTLs are

reported in Table 3. The SNP of the *HMIP* gene was less frequent in our study population with an allele frequency of 0.18. The two polymorphisms of the *BCL11A* gene had similar allele frequencies. Indeed, the allele frequency of rs1427407 T>G was 0.27 and that of rs4671393 G>A was 0.28. Furthermore, all three polymorphisms studied were homogeneously distributed according to the Hardy-Weinberg equilibrium ($p > 0.05$).

Effects of QTLs on clinical parameters

The results of the association studies between clinical parameters (number of hospitalizations and number of VOC episodes over 2 years) and QTLs are reported in Figures 1 to 3. VOC episodes were not associated with the two SNPs of the *BCL11A* gene (rs1427407 T>G, rs4671393 G>A) (Figure 1). However, the rs1427407 polymorphism of the *BCL11A* gene was significantly associated with hospitalizations ($p = 0.03$) (Figure 2). The SNP rs28384513 of the *HMIP* gene was not associated with either VOC episodes or hospitalizations (Figure 3).

Effects of QTLs on biological parameters

Variations of HbF level as a function of QTL

HbF level was associated with the rs467139 and rs1427407 of *BCL11A* with p-values of 0.001 and 0.004 respectively (Figure 4).

For the *HMIP* QTL (rs28384513) no statistically significant association was found between this polymorphism and the HbF level (Figure 5).

Variations of HbS level as a function of QTL

The HbS level was associated with the QTLs *BCL11A*, rs1427407, and rs4671393 with p-values of 0.006 and 0.014 respectively. For the SNP rs28384513 of the *HMIP* gene, no statistically significant association was noted (Figure 6).

DISCUSSION

Clinical and biological characteristics of the cohort

In this study, the mean number of VOC episodes for the last two years was equal at 2.73 ± 1.98 . For hospitalizations, the mean frequency was 0.84 ± 1.29 . These results were different from those reported in Cameroon by Wonkam et al. (2014). Indeed, these authors found an average of 2 episodes of VOC and 2.3

Table 1. Means of clinical variables.

Clinical parameter	Mean (n) ± SD	Max-Min
Number of VOCs over 2 years	2.73 ± 1.98	0.00 - 12.00
Number of hospitalizations over 2 years	0.84 ± 1.29	0.00 - 0.00

VOC: Vaso-occlusive crises. The mean number of VOC episodes over 2 years was 2 with extremes ranging from 0 to 12 episodes, while the frequency of hospitalizations ranged from 0 to 10 with a mean of 0.84.

Source: Authors

Table 2. Means of biological variables.

Biological parameter	Mean ± SD	Min-Max
Lactate dehydrogenase (LDH) (UI/L)	940.43 ± 499.48	148.00 - 3318.00
Total bilirubin (mg/L)	43.98 ± 24.11	7.21 - 106.90
Direct bilirubin (mg/L)	23.02 ± 15.11	2.07 - 68.30
Indirect bilirubin (mg/L)	20.96 ± 13.18	0.20 - 73.51
CRP (mg/L)	4.55 ± 5.43	0.08 - 34.6
White blood cells (G/L)	14.30 ± 4.34	4.21 - 28.4
Reticulocytes (G/L)	325.03 ± 159.9	28.12 - 954.00
Reticulocytes (%)	12.00 ± 5.59	0.60 - 36.5
Platelets (G/L)	449.56 ± 135.87	135.00 - 945.00
Hemoglobin (g/L)	7.76 ± 1.05	5.50 - 12.3
HbS level (%)	82.28 ± 4.78	62.60 - 91.80
HbF (%)	9.49 ± 5.12	1.10 - 26.80

CRP: C-reactive protein; HbS: hemoglobin S; HbF: hemoglobin F.

Source: Authors

Table 3. Prevalences and allelic frequencies of QTLs.

	Numbers (n)	Prevalences (%)	Allelic frequencies	p*
BCL11A (rs1427407 T>G)				
Wild	158	52,5		
Heterozygous	120	39,9		
Mute	23	7,6	0,27	>0,1
BCL11A (rs4671393 G>A)				
Sauvages	151	50,2		
Heterozygous	126	41,9		
Mute	24	8,0	0,28	>0,1
HMIP (rs28384513 A>C)				
Wild	197	65,4		
Heterozygous	96	31,9		
Mute	8	2,7	0,18	>0,1

* The Hardy-Weinberg equilibrium was verified by the chi-square test.

Source: Authors

hospitalizations per year in their study population. These differences would be because the Benin and Cameroon haplotypes which are in the majority in the Cameroonian population correspond to more severe phenotypes than

the Senegal haplotype which would be in the majority of our study population (Gueye Tall et al., 2017). In addition to clinical parameters, hematological and biochemical parameters were also studied. Thus, the blood count

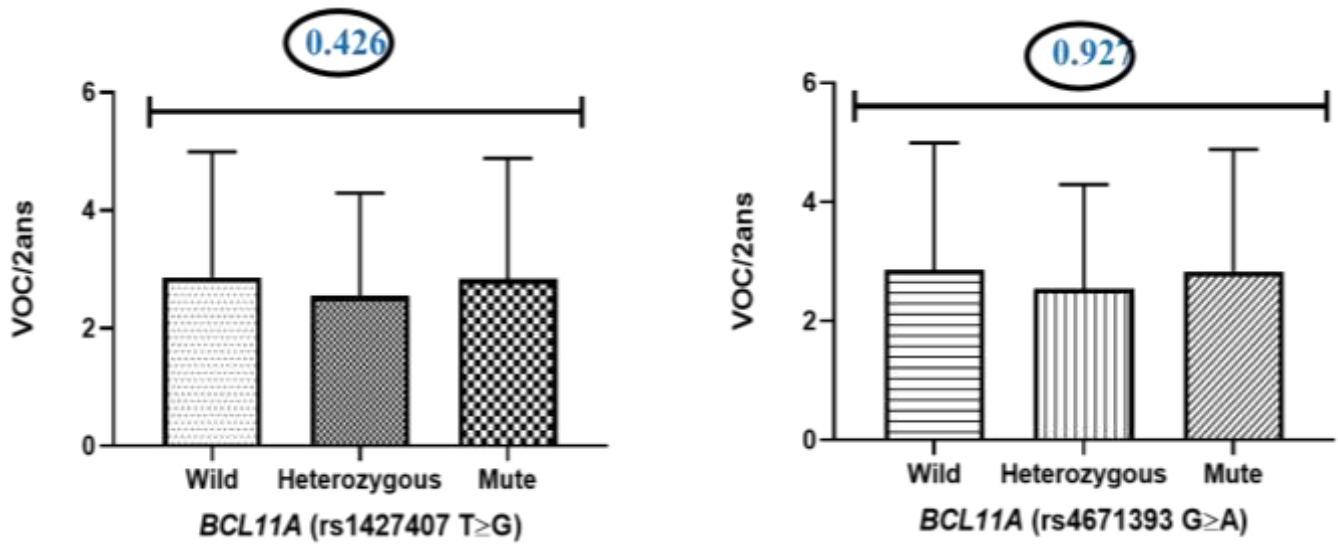


Figure 1. Association between *BCL11A* gene SNPs and vaso-occlusive crises. VOC: Vaso-occlusive crises. VOC episodes were not associated with the two SNPs of the *BCL11A* gene (rs1427407 T>G, rs4671393 G>A). Source: Authors

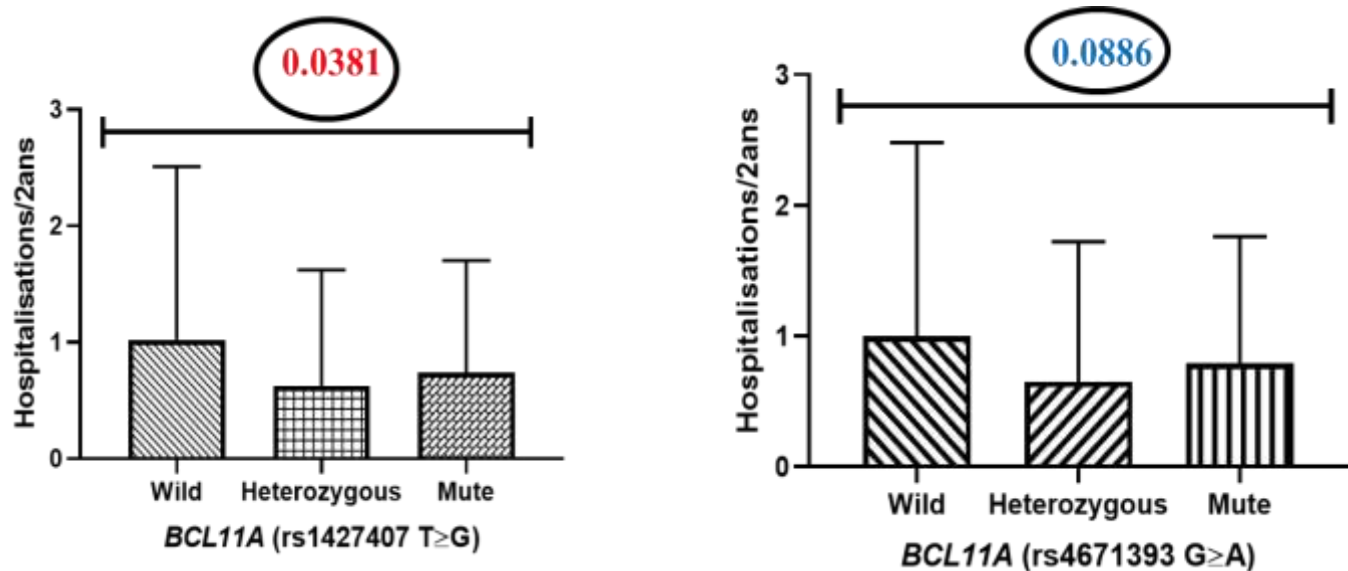


Figure 2. Association between *BCL11A* gene SNPs and frequency of hospitalization. The rs1427407 polymorphism of the *BCL11A* gene was significantly associated with hospitalizations ($p = 0.0381$). Source: Authors

performed systematically revealed anemia in all children, with a mean total Hb concentration of 7.76 ± 1.05 g/dL. This result confirms the idea that anemia in sickle cell disease is constant (Dahmani et al., 2017). The low mean total Hb concentration in our cohort was comparable to that already reported in Senegal by Thiam et al. (2017),

which was 8.6 g/dL. Many other studies confirmed the anemia and low Hb concentrations in sickle cell disease. This is the case of the study conducted by Muszlak et al. (2015) on the island of Mayotte. Indeed, these authors reported a mean Hb concentration of 7.83 ± 0.91 g/dL (Muszlak et al., 2015). Mean total Hb levels of 7.98 g/dL

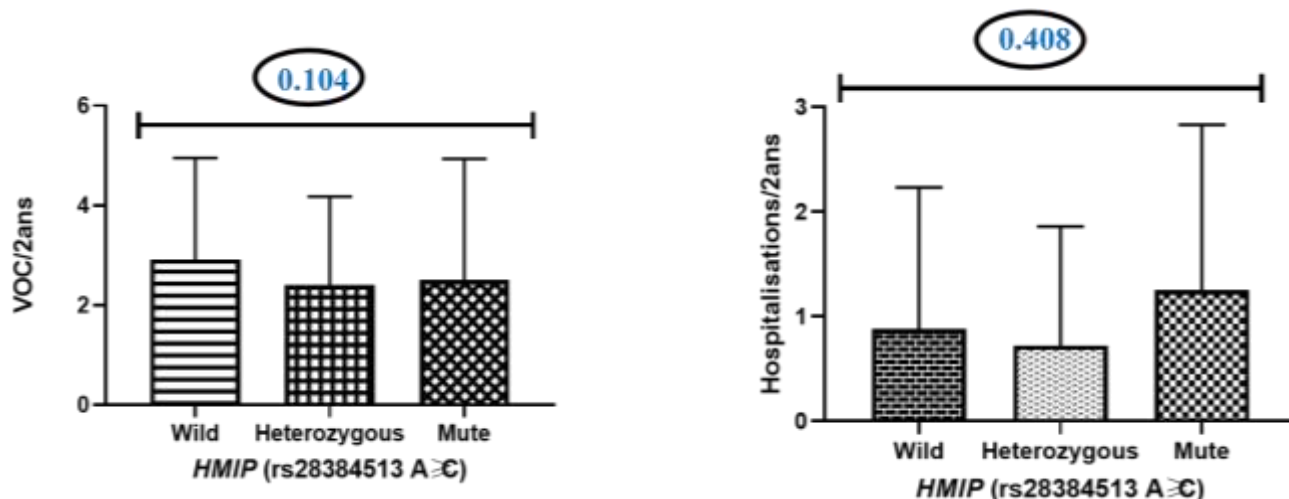


Figure 3. Association between *HMIP* gene polymorphism and vaso-occlusive crisis and hospitalization. VOC: vaso-occlusive crises. The SNP rs28384513 of the *HMIP* gene was not associated with either VOC episodes or hospitalizations. Source: Authors

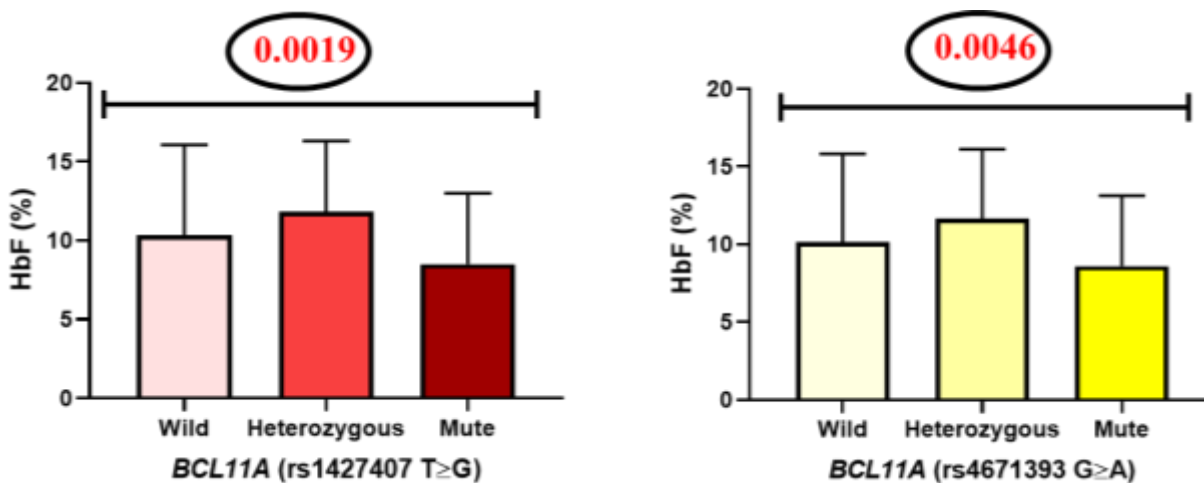


Figure 4. Mean HbF levels as a function of *BCL11A* SNPs. HbF: Hemoglobin F. HbF level was associated with the rs467139 and rs1427407 of *BCL11A* with p-values of 0.0019 and 0.0046 respectively. Source: Authors

(Belisário et al., 2010) and 8.1 g/dL (Bernaudin et al., 2018), which are similar to ours, have been reported respectively in studies carried out in Brazil and France, precisely at the pediatric center for sickle cell disease. The mean reticulocyte count in our series was 325.03 ± 159.9 G/L, suggesting that most patients in our series had regenerative anemia.

Indeed, during sickle cell disease, erythropoiesis is stimulated to compensate for the destruction of red blood cells linked to hemolytic crises. Our results are similar to those reported in the study of Dahmani et al. (2017) who

reported a mean reticulocyte value equal to 363 ± 195.5 G/L (Dahmani et al., 2018). We also noted that in our patients, the mean leukocyte count was 14.30 ± 4.34 G/L, indicating a tendency to hyper-leukocytosis. Indeed, hyper leukocytosis is physiological in sickle cell disease and can be explained by hyperactivity of the bone marrow and inflammatory phenomena (Chies and Nardi, 2001). The hypothesis of any inflammation would be improbable, especially as our patients were all in the stationary phase at the moment of inclusion. This hyper-leukocytosis could therefore be the consequence of bone

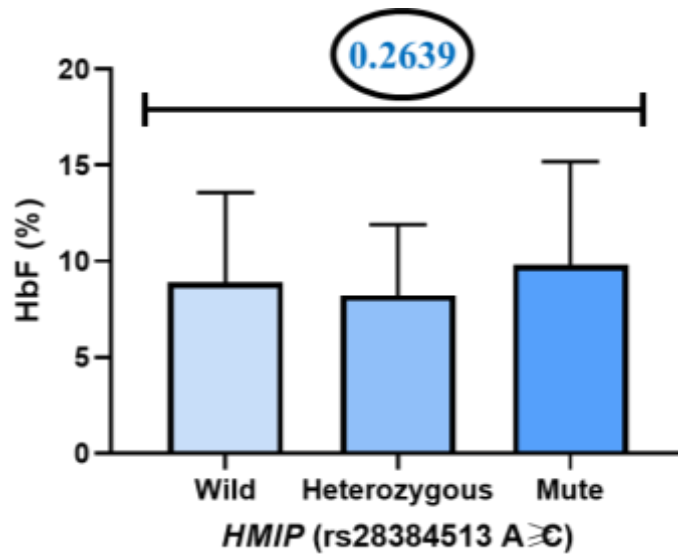


Figure 5. Mean HbF levels as a function of *HMIP* gene SNP. HbF: Hemoglobin F. No statistically significant association was found between the *HMIP* polymorphism (rs28384513) and the HbF level. Source: Authors

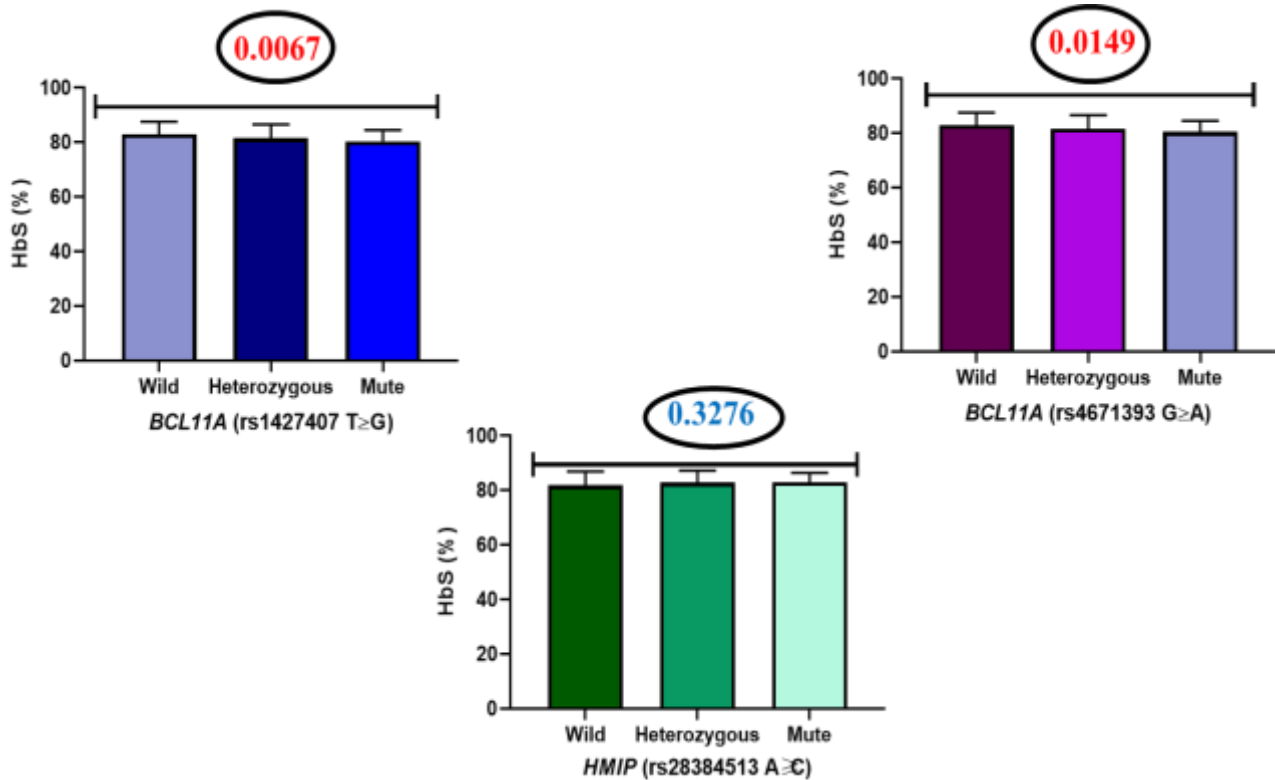


Figure 6. HbS level as a function of QTLs (*BCL11A*, *HMIP*). HbS: hemoglobin S. The HbS level was associated with the QTLs *BCL11A*, rs1427407, and rs4671393 with p-values of 0.006 and 0.014 respectively. For the SNP rs28384513 of the *HMIP* gene, no statistically significant association was noted. Source: Authors

marrow hyper production. However, the absence of a leukocyte formula does not allow us to exclude cases of false hyper-leukocytosis secondary to erythroblastosis. Indeed, in acute hemolysis, the strong regeneration of the bone marrow is responsible for an erythroblastosis at the origin of a false hyper-leukocytosis, since the erythroblasts, because of their nucleus, are counted as leucocytes by the automates. This uncorrected leukocytosis is often very important in homozygous patients. The mean platelet count was 449.56 ± 135.87 G/L. The thrombocytosis observed could be explained by the occurrence of functional or organic asplenia or could be the consequence of hyposplenism (Tchokoteu, 2004). The strong regeneration could also lead to a stimulation of thrombopoiesis. This tendency to thrombocytosis in patients with sickle cell disease was described by Mounkaila et al. (2015) with a mean platelet count of about 554 ± 25 G/L (Boutchi, 2015).

Concerning CRP, we noted an average plasma concentration of 4.55 ± 5.43 mg/L, attesting that our patients were all in the stationary phase at the time of inclusion, i.e., absence of any fever, vaso-occlusive crisis, and inflammation. Indeed, an increase in CRP (> 6.00 mg/L) would be in favor of an inflammatory reaction. Our result was lower than that of the study by Doupa et al. (2017) which reported a mean CRP value of 12.87 ± 21.9 mg/L (Doupa et al., 2017).

The results of the hemolysis parameters of our study revealed an average catalytic concentration of LDH of 940.43 ± 499.48 IU/L, reflecting the context of chronic hemolysis in our patients. However, according to the literature, LDH activity is considered a marker of severity when its value is higher than 1,000 IU/L as is the case in VOC (Mattioni et al., 2016). As for bilirubinemia, it was increased with a mean concentration of 43.98 ± 24.11 mg/L in our patients. This particular indirect increased hyperbilirubinemia (unconjugated) which, in our cohort, varied between 0.20 and 73.51 with a mean concentration of 20.96 ± 13.18 is a known biological sign associated with hemolysis in patients with SS sickle cell disease. According to the literature, there exists generally an increase in markers of hemolysis such as free bilirubin, AST, reticulocytes, and the appearance of free hemoglobin in the plasma in the case of VOC (Mattioni et al., 2016). However, our patients were all in the stationary phase at the time of inclusion, that is, in the absence of any vaso-occlusive crisis. This increase in hemolysis markers could therefore be the consequence of chronic hemolysis.

In this study, the mean hemoglobin S level was $82.28 \pm 4.78\%$. SS homozygous subjects have major sickle cell syndrome. Their total hemoglobin concentration is between 6 and 10 g/dl and the red blood cells contain mainly hemoglobin S, with an increased hemoglobin F fraction (in the range of 5 to 20%) and a low hemoglobin

A2 fraction. Hemoglobin A is absent (Jeanne, 2010). Hemoglobin S and hemoglobin F would therefore be the parameters that can be considered sensitive and found in significant proportions in cases of homozygous sickle cell disease SS. High concentrations of hemoglobin S are found in particular when the sickle cell trait is associated with β -thalassemia. This is the reason why a differential genotypic diagnosis was performed for the selection of SS homozygous subjects in our cohort. This result is corroborate the study by Somerville et al. (2001), which reported a rate of between 80 and 95% (Somerville et al., 2001).

The mean HbF level was $9.49 \pm 5.12\%$. Elevated HbF levels are found in newborns, but also in β -thalassemia major, in HbF persistence syndrome, and in sickle cell patients treated with hydroxyurea, which induces an increase in hemoglobin F. Thus, the differential genotypic diagnosis and the absence of treatment with hydroxyurea would make it possible to exclude these hypotheses. The high level of HbF would be the consequence of compensation of HbA, absent in the SS homozygous subject. Studies in Senegal have shown a mean hemoglobin F level of $6.8 \pm 5.9\%$ in children (Diagne et al., 2000) and $8.2 \pm 5.8\%$ in adults of the same group (Diop et al., 1999). This difference in our results could be explained by the age of the patient. In healthy subjects, HbF is the major constituent expressed during fetal life and is progressively substituted during the perinatal period by HbA. Substitution is normally complete by 6 months of age, with HbA becoming the major component, and HbF usually represents less than 1% of total Hb (Labie, 2000). In sickle cell disease, since HbA is not expressed, residual HbF continues to be synthesized during adult life and highly variable levels have been observed. It is now accepted that these quantitative variations are related to polymorphisms and this is the general objective of this study.

Molecular characteristics of the cohort

The results of our study revealed variable allele frequencies in the Senegalese population according to the type of QTLs. These frequencies were 0.276 for rs1427407 (*BCL11A*), 0.289 for rs4671393 (*BCL11A*) and 0.186 for rs28384513 (*HMIP*). Other authors have also evaluated the frequencies of these polymorphisms in different population groups of our cohort according to the haplotypes frequently found. For example, Bernaudin et al found that rs1427407 had a lower allele frequency (about 0.22) in children who were homozygous for the Bantu haplotype compared to those who were homozygous for the Benin haplotype (about 0.40) (Bernaudin et al., 2018). Pule et al. (2015) found an intermediate allele frequency for this same SNP (0.26) in

a very large cohort ($n = 541$) of patients of Beninese and Cameroonian origin. The allele frequency of rs28384513 was about 0.20 in Tanzanian (Makani et al., 2011) and Cameroon cohort (Wonkam et al., 2018), with a majority of Benin and Cameroon haplotypes, while it was significantly higher in Mayotte (Muszlak et al., 2015), with a majority of Bantu haplotype subjects (0.55) suggesting that the Bantu haplotype would be associated with rs28384513 of *HMIP*.

Effects of QTLs of HbF on clinical parameters

The association between genotype and phenotype in sickle cell disease is interesting to investigate as it is not well described in the literature. Although several studies have demonstrated the influence of the two loci *BCL11A* and *HMIP* on HbF levels (Lettre et al., 2008, Creary et al., 2009, Mtatiro et al., 2014) few authors have focused on their direct effects on the severity of sickle cell disease.

Only the SNP rs1427407 of the *BCL11A* gene was associated with a decrease in the frequency of hospitalizations ($p = 0.035$). Indeed, the presence of the mutation seems to reduce the number of hospitalizations since patients carrying the wild type had a mean number of hospitalizations of 1.02 ± 1.49 whereas these means were respectively 0.62 ± 1.00 and 0.74 ± 0.96 for mutated heterozygotes and mutated homozygotes. These results differ from those reported by other authors. Indeed, Muszlak et al. (2015) showed that the rs4671393 polymorphism of the *BCL11A* gene was associated with a lower number of hospitalizations in a group of 82 children with sickle cell disease on the island of Mayotte (Muszlak et al., 2015). Similarly Wonkam et al. (2018) reported that in a Cameroonian cohort rs4671393, as well as rs11886868 of the *BCL11A* gene and two other SNPs located on the *HMIP* gene (rs28384513 and rs9494142), were associated with a low frequency of hospitalizations related to painful events (Wonkam et al., 2018). This difference between our results would suggest a possible implication of haplotypes on the effect of these polymorphisms on the frequency of hospitalizations.

About VOCs, our results revealed that none of the polymorphisms of interest studied were associated with VOC episodes. These results were superposable with those of a study by Wonkam et al. (2014), whose population genotyping included all three SNPs in our study (Wonkam et al., 2014). Lettre et al. (2008) found a significant link between the association of 5 SNPs (*BCL11A* rs4671393, *HMIP* rs28384513, rs9399137 and rs4895441, and *XmnI* rs7482144) and reduced VOC in a cohort of sickle cell disease patients (Lettre et al., 2008). These SNPs are also associated with a less severe clinical phenotype in another hemoglobin disorder, beta-thalassemia (Danjou et al., 2015). This results show a

stronger gene-phenotypic correlation when several SNPs are associated (Lettre et al., 2008).

Effects of QTLs of HbF on biological parameters

Variations of HbF level according to QTL

The *BCL11A* SNP rs4671393 was associated with increased HbF levels ($p=0.004$) and carriers of the genotype including this polymorphism in the homozygous state had significantly higher HbF levels (11.66 ± 4.46). This polymorphism is located in an intron of the *BCL11A* gene and is involved in lymphoid malignancies and is expressed in erythrocyte precursors (Liu et al., 2003). This could explain its impact on HbF expression. Our results are consistent with those reported in Brazilian and African-American patients (Lettre et al., 2008) but also in Tanzanian, British, Afro-Caribbean, and West African patients (Makani et al., 2011), all with homozygous SS sickle cell disease.

Similarly, the SNP rs1427407 still in the *BCL11A* gene was also associated with increased HbF levels ($p = 0.001$) and it was the mutated homozygotes that had significantly higher HbF levels (11.84 ± 4.47). This polymorphism is commonly found in patients with sickle cell disease (Bhanushali et al., 2015).

The SNP rs28384513 of the *HMIP-1* gene was not correlated with increased HbF levels ($p = 0.268$) and instead, mutated homozygotes had significantly lower HbF levels (8.23 ± 3.68). This result is corroborated by that of a study in North Brazilians, where of three genotyped SNPs (rs28384513, block 1; rs4895441 and rs9399137, block 2), only the rs4895441 polymorphism was significantly associated with increased HbF levels (Cardoso et al., 2014). According to a Cameroonian study, all the *HMIP* SNPs explain 8.3% of the variations in HbF levels in sickle cell patients (Wonkam et al., 2014). It should also be noted, that of the common alleles of the three haplotype blocks of this gene region associated with HbF expression, block 2, 24 kb in size, accounts for the majority of the variability in HbF levels. As a reminder, *HMIP* polymorphisms are divided into three blocks of imbalance, called blocks 1, 2, and 3 of the HBS1L-MYB intergenic polymorphism. The discrepancy noted between our different results could also be explained by the ethnic-geographical origin. Indeed, *HMIP-2* is characterized by eleven SNPs, which all showed a strong association with HbF levels in European patients, but only some of these SNPs showed a significant association in patients of African origin (Thein et al., 2009). The association of rs28384513 with HbF levels was first described in a sample of Northern European descendants (Thein et al., 2009). Subsequent studies have shown that this SNP was associated with

elevated HbF levels in sickle cell patients from other African-American, Brazilian, and Tanzanian populations as well as from African-British populations (Creary et al., 2009). In summary, we note that while polymorphisms in the *HBS1L-MYB* intergenic region are associated with HbF in sickle cell disease patients of African origin, they are much less associated with HbF in European and Chinese patients due to their much lower allelic frequency (Bernaudin et al., 2018).

Variations of the HbS level according to the QTL

In our series, the study of HbS levels according to QTL type showed statistically significant associations only with rs1427407 and rs4671393 of the *BCL11A* gene. The *BCL11A* SNP rs4671393 was indeed associated with lower HbS levels ($p = 0.014$) and carriers of genotypes containing this mutated allele in the homozygous state had significantly lower HbS levels (80.58 ± 4.02) (Figure 6) The SNP rs1427407 in the *BCL11A* gene was also associated with lower HbS levels ($p=0.006$) and it was again the genotypes containing the mutation in the homozygous state that had significantly lower HbS levels (80.46 ± 4.06) (Figure 6). In homozygous SS sickle cell disease, HbA is not expressed and HbF and HbS are the two types of hemoglobin synthesized during adult life. Thus when the fraction of one increases, the fraction of the other decreases. To this end, any QTL that would induce an increase in HbF will be associated in the same way and with the same statistical strength with a reduction in HbS.

Variations of other biological parameters according to QTLs

In our study, no significant difference was found between the QTLs and the means of the other biological parameters (LDH, BT, BD, BI, CRP, and Hb, leukocytes, platelets, and reticulocytes). These results could be explained by the young age of our study population (pediatric population; median age 9 years) and the relatively high levels of fetal hemoglobin (HbF).

Conclusion

The two SNPs of *BCL11A* would induce an increase in the HbF level and a decrease in the HbS level, but no association was noted between these SNPs and the number of VOC episodes. However, the rs1427407 polymorphism of the *BCL11A* gene was associated with a decrease in the frequency of hospitalizations. On the other hand, no variation in clinical parameters was

associated with the presence of the rs28384513 SNP of the *HMIP* gene. Thus it follows from these results that HbF-QTLs are not the only genetic factors modulating the severity of sickle cell disease, which suggests the involvement of other factors. These markers interact with other parameters such as alpha thalassemia or G6PD deficiency, which must be explored to explain the inter-individual variability of the sickle cell disease clinic. Furthermore, the absence of association between the phenotypes studied and the SNP of *HMIP* would not exclude the involvement of this inter-gene region, rich in polymorphisms, in the clinical heterogeneity of this condition.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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