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GENETIC EVALUATION OF INTERLUKIN-22 SINGLE NUCLEOTIDE POLYMORPHISM (RS1179251) WITH *PLASMODIUM FALCIPARUM* CLEARANCE AMONG CHILDREN LESS THAN 10 YEARS IN THE NORTH REGION OF CAMEROON

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ABSTRACT: *IL-22 is a pro and anti-inflammatory cytokine which induces the regeneration of hepatocytes cells during the immune response. Evidence has shown that genetic polymorphisms on IL-22 gene could affect the immune response and consequently the parasite clearance and the treatment outcome of malaria. This study aimed at determining the prevalence of the SNP rs1179251 of the Interleukin-22 gene and a possible association with P. falciparum clearance among children less than 10 years old in the North region of Cameroon. A case-control study was performed on 184 conveniently collected blood samples, spotted on Whartman No 3 filter paper from the SPAQ (Sulfadoxine-Pyrimethamine + Amodiaquine) clinical trial carried out in Garoua and Yagoua in 2015, from which DNA was extracted using Chelex-100 method. Genotyping of the IL-22 gene SNPs was performed using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP). Parasite clearance was defined as the disappearance of pre-treatment parasites without subsequent recurrence, irrespective of whether recurrence is a reinfection or a recrudescing parasite. Treatment outcomes were classified according to the WHO guidelines; patients that experienced early treatment failure (ETF), and late parasitological failure (LPF) were classified as failures (56 cases), while patients that experienced adequate clinical parasitological response (ACPR) were classified as successes (128 controls). The chi square test was used to establish the association between the SNP rs1179251 and parasite clearance. A P-value less than 0.05 was considered as statistically significant. The findings revealed that the mutant allele C was the most predominant with a frequency of 74.46%, with the ancestral allele G having a frequency of 25.54%. These results showed that carriers of the mutant allele C could be 3.7 times more likely not to clear the parasites during treatment but this was not statistically significant (P=0.07, OR= 3.72). In conclusion, no association was found between the SNP rs1179251 of IL-22 gene and Plasmodium falciparum clearance. So, in spite of the importance of IL-22 gene in immune responses, the studied polymorphism does not serve a decisive role in Plasmodium falciparum clearance.*

KEYWORDS: Interleukin-22, Gene polymorphism, Malaria, Parasite clearance.

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INTRODUCTION

Malaria is a tropical disease spread through the bite of a female anopheles mosquito infected with plasmodium. Malaria remains one of the most common vector diseases, causing high morbidity and mortality. More than a hundred species of plasmodium exist, but only five species are responsible for malaria in humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*) (Autino *et al.,* 2014). *P. falciparum* and *P. vivax* are the two main species known to cause complications in humans (Autino *et al.,* 2014). In 2020, WHO estimated 220 million new malaria cases worldwide and 409,000 deaths, with pregnant women and children under 5 being the most affected, and Cameroon being amongst the eleven highest burden countries (WHO, 2020). To fight against malaria, many interventions have been recommended by WHO, including the provision of rapid treatments based on the administration of an antimalarial drug (WHO, 2019). The effectiveness of this anti-malarial drug used to clear the parasite depends on many factors, including the intrinsic activity of the drug, age, intensity of transmission, the parasite and other host-dependent factors including the immune response (Alifrangis *et al.,* 2003; Francis *et al.,* 2006; Mawili-mboumba *et al.,* 2003). The immune response during malaria infection is characterized by excessive inflammation, and the establishment of a perfect balance between the pro and anti-inflammatory immune response is essential to ensure parasite control and host survival (Paula *et al.,* 2012). During the immune response, many cytokines are produced such as Interleukin 22 (IL-22), Tumor necrosis factor (TNF) and Interferon Gamma (IFNγ). IL-22 is ubiquitous and play a dual role as pro-inflammatory and anti-inflammatory effects in various conditions and in different tissues of the body (Asadi *et al.,* 2019). This cytokine belongs to the IL-10 super family, and it is produced and secreted by innate lymphoid cells (ILCs) and T helper 22 (Th22) cells. IL-22 stimulates through its receptors the growth and regeneration of epithelial cells, hepatocytes, and keratinocytes. It is also involved in antimicrobial control through the recruitment of pro-inflammatory cells, the secretion of antimicrobial peptides and the repair of tissues and cells (Wolk *et al.,* 2006). Protection of IL-22 against liver pathology and lethality of an experimental blood-stage malaria infection has been demonstrated (Mastelic *et al.,* 2012). The liver and blood stages of *Plasmodiums* induce a proinflammatory response in the host which, although important for the clearance of the parasite, can lead to severe immune-mediated pathology (Paula *et al*., 2012). The involvement of IL-22 in the modulation of the acute phase reactants suggests that in malaria, this cytokine may mediate early pro-inflammatory responses critical for parasite clearance. In 2015, Ali *et al.* reported the first evidence about the association between treatment response and SNPs in immune mediators in Cameroon's multi-ethnic (Ali *et al.,* 2014). Another study showed that some single nucleotide polymorphisms (SNP) in the IL-22 gene, such as (rs2227483, rs2227481), contribute to the protection against *Plasmodium falciparum* parasites and thus influence its clearance (Aljarba *et al.,* 2020). Also, Kock *et al.* in a case control study has shown that two IL-22 haplotypes were respectively associated with susceptibility and resistance to malaria (Koch *et al.,* 2005). Furthermore, studies have shown association between the SNP rs1179251 and inflammatory diseases such as gastric cancer and thyroid autoimmune disease (Qin et al., 2015; Song et al., 2017). Till date, very few studies have been conducted on IL-22 gene polymorphisms, and its association with malaria parasites clearance. Thus, this study aimed at investigating the association of the SNP rs1179251 of IL-22 and *Plasmodium falciparum* parasite clearance, to consider in perspective the development of protective immune response to malaria and improve the health care of malaria in Cameroon.

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MATERIALS AND METHODS

Study Population and Setting

Blood samples from 184 participants were obtained from patients between 6 months and 10 years old suffering from uncomplicated malaria. Clinical and parasitological outcomes among children less than 10 years old were assessed in a randomized, controlled, and double-blinded clinical trial investigating the efficacy and safety of AQ plus SP in the North region of Cameroon. Patients that experienced Early Treatment Failure (ETF) and Late Parasitological Failure (LPF) were classified as failures, while patients that experienced Adequate Clinical Parasitological Response (ACPR) were classified as successes.

Ethical Consideration

This study was approved by the Cameroonian National Ethics Committee for Research in Human Health (NECRHH) under the ethical clearance document number 2015/03/567/CE/CNERSH/SP. Prior to participant enrolment, written and signed consent from each parent or guardian of a potentially eligible study participant was obtained. The potential risks and benefits as well as data privacy and confidentiality were explained to all parents/guardians' participants. Only those who signed the informed consent form were included in the study.

Determination of Participants Parasitemia

Malaria parasitemia was determined by a qualified laboratory technician before and after treatment for days (0, 1, 2, 3, 7, 14, and 28). Blood specimens were collected from patients and a thin blood smear prepared, stained with Giemsa, and examined with 100X oil immersion objective using a light microscope. The detection threshold in Giemsa-stained thick blood film was estimated to be about 50–100 parasites/µL. Parasite clearance was defined as the disappearance of pre-treatment parasites without subsequent recurrence, irrespective of whether recurrence is a reinfection or a recrudescing parasite.

Calculation of Parasite Density

Parasite density was calculated by multiplying the number of parasites counted per microscopic field, and dividing it by the number of white blood cell counts of each. If on counting 200 WBC, the number of parasites is not up to 100, the count was continued till 500 WBC and calculations made to get the exact parasitaemia of the patient. Results were reported in (p/ul). At least 10 fields were explored before confirming a slide to be negative. Two microscopists viewed the slides and a third confirmed them for quality control. Parasitaemia was calculated using the formula below:

$$\text{Parasites per µl} = \frac{\text{Number of parasites counted X 8000}}{\text{Number of WBCs counted}}$$

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DNA Extraction and Genotyping

Genomic DNA was extracted from dried blood spots on Whartman N^{o}3 filter papers using Chelex-100 method as previously described (Plowe *et al.,* 2016)**.** IL-22 gene was amplified with a *T3 Thermocycler* (Biometra, UK) as previously described (Asadi *et al.*, 2019; Tah *et al.,* 2023). The primer used was as follows: Forward (IL–22–251F): 5'–CAGAAATTAGCCCTATATGC–3'and Reverse (IL–22– 251R): 5'–GAAAAAGGTAGGTAGGACTGATAAC–3'. Each PCR was performed in a total reaction mix of 20 µl containing 6µl of nuclease free water (NFW), 10µl of One Taq® Hot Start 2X Master Mix with standard buffer (New England Biolabs, MA, USA.), 1 µl of each primer (0.5µM) and 2 µl of DNA template. The PCR protocol was as follows: pre-denaturation (95°C for 5mins), followed by 35 cycles of denaturation (95°C for 30 secs), annealing (60°C for 30 secs), elongation (72°C for 45 secs) and a final elongation for 72°C for 7 mins to terminate all reactions. The amplicons after amplification if not immediately used were you stored at 4°C. The PCR products were then digested with *Alw NI* (Thermo Scientific, USA) at 37°C during 18 h overnight as previously described (Asadi *et al.*, 2019; Tah *et al.,* 2023). The products of digestion were run on 2% agarose gel stained with Ethidium bromide and visualized under UV light (Figure 1).

Statistical Analysis

Data from this study were transcribed from laboratory worksheet records unto Microsoft Excel, version 2016. Allelic frequencies of the IL-22 gene were obtained using the Hardy-Weinberg formula. Data were analyzed using the IBM SPSS biostatistics version 20.0 software (SPSS, Chicago, IL). Chi Square test (X^2 test) was used to establish associations between variables. Where the number of expected observations was less than 5, the Fisher's test was used. Unadjusted Odds Ratios (ORs) were calculated with 95% Confidence Intervals (CI). A $p < 0.05$ was considered significant in all comparisons.

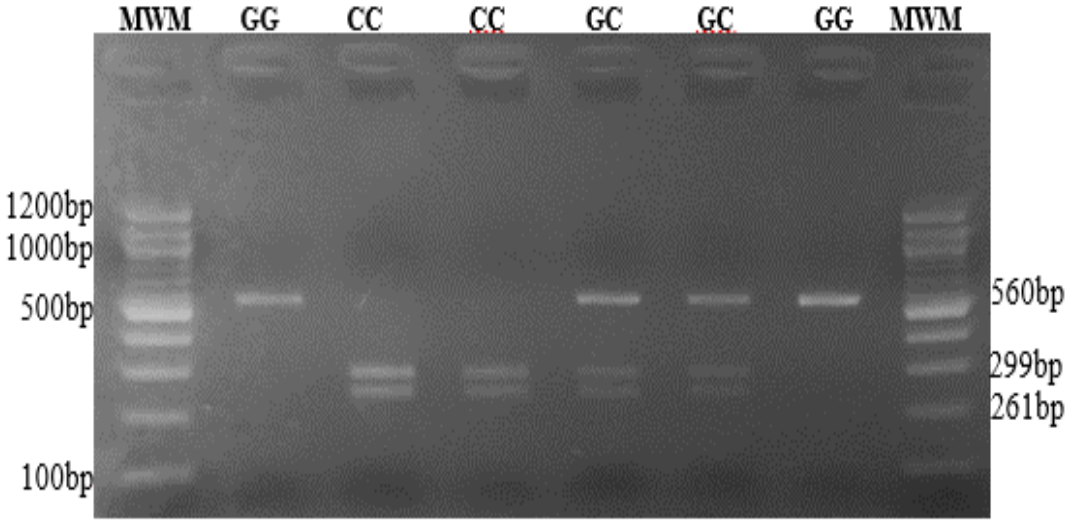

Figure 1: *Digestion patterns of IL–22 gene SNP rs1179251 with AlwNI enzyme. GG; homozygous wildtype, CC; homozygote mutant and GC; heterozygote. Both ends of the gel picture are the DNA size markers (MWM) 100bp.*

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RESULTS

Demographic Characteristics of the Study Population

Out of 184 participants included in the study, 78 children were recruited from Garoua and 106 children from Yagoua, with a mean age of 46.18 months. All participants were positive for malaria after a thick blood smear (mean parasitaemia = 7465. 80 parasites/µl) on the first day of recruitment, with a mean temperature of 38.0°C and mean weight of 14.212 kg (Table 1).

Table 1: Characteristics of Our Study Population

Characteristics	Min	Max	Mean	SD
Age (Months)	6	112	46.18	28.95
Weight (Kg)	5.8	31.8	14.212	5.39
Temperature (°C)	36.4	40.2	38.0	40.77
Parasitemia (Parasite/µl)	1000	80000	7465.80	13519.05

SD = Standard Deviation

Three main treatment outcomes were classified in this study, i.e., adequate clinical parasitological response (ACPR) with a frequency of 71.74%, early treatment failure (ETF) with a frequency of 21.74% and late parasitological failure (LPF) with a frequency of 6.52%.

3.2. Genotypes and Alleles Frequencies of IL-22 Gene rs1179251 SNP

Analyzing the genotyping results of IL-22 gene rs1179251 single nucleotide polymorphism revealed the distribution of GG (6, 10.71%), GC (24, 42.86%), and CC (26, 46.43%) in the treatment failures' group and GG (4, 3.13%), GC (50, 39.06%), and CC (74, 57.81%) in the controls group (Table 2).

3.3 Association between SNP rs1179251 Genotype/Allele of Participants and Plasmodium falciparum Clearance (Treatment Outcome)

Results from the association analysis showed no statistical significance between the Treatment failure group and control group, but individuals possessing the CC genotype and C allele could be exposed to treatment failure (OR=1.581, P=0.198; OR=3.720, P=0.07 respectively). The results of association analysis were presented on Table 2.

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Table 2 : Association between IL-22 rs1179251 Single Nucleotide Polymorphisms and *Plasmodium falciparum* Clearance

IL-22 SNP rs1179251	Genotypes/ Alleles	Failure (56) (ETF+LPF)	Control (128) (ACPR)	OR	95%CI	P-value
Genotypes						
	GG	6 (10.71%)	4 (3.13%)	0.269	0.073-0.993	0.070
	GC	24 (42.86%)	50 (39.06%)	0.855	0.452-1.617	0.629
	CC	26 (46.43%)	74 (57.81%)	1.581	0.841-2.973	0.198
Alleles						
	G	36 (32.14%)	58 (22.66%)	0.632	0.336-1.189	0.198
	C	76 (67.86%)	198 (77.34%)	3.720	1.007-13.747	0.07

SNP = Single Nucleotide Polymorphism, OR = Odds Ratio, level, CI = confidence interval, P-value = statistical significance level, *ETF = Early Treatment Failure, LPF = Late Parasitological Failure, ACPR = Adequate Clinical Parasitological Response.*

DISCUSSION

Cytokines are proteins that help control inflammation in the body; they play principal roles in defense against infections. Host genetic background is a crucial factor which regulates cytokine responses, and this may link with inflammation, viral and parasite clearance, or disease progression (Ali *et al.,* 2014; Asadi *et al.,* 2019). Genetic polymorphisms observed on cytokine genes can provoke a possible increase or decrease in the intensity of immune response consequently in disease progression and outcome and parasite clearance.

IL-22 can have proinflammatory roles by inducing release of chemokines and proinflammatory mediators from epithelial cells. It also stimulates the release of antimicrobial peptides from tissue (Janumyan *et al.,* 2003). IL-22 does exert protective or pathogenic effects in various other models of infection and organ damage through exerting other roles in different organs. In the liver, IL-22 has protective roles in various models of induced acute hepatitis with agents such as Concanavalin A, carbon tetrachloride and liver injury with paracetamol overdose as well as in regenerating liver tissue after partial hepatectomy (Pan *et al.,* 2004*).* IL-22 also protects the liver during infections with Dengue virus. This protection may be exerted by the induction of anti-apoptotic proteins such as Bcl-2 through the activation of STAT3 (Guabiraba *et al.,* 2013). It was discovered that IL-22 deficient mice had significantly decreased small intestinal necrosis during the infection and this was associated with reduced levels of matrix metalloproteinase 2 (MMP2), an endopeptidase that regulates the extracellular matrix which is associated with colitis (Muñoz *et al.,* 2009). This cytokine can induce the production of interleukin-18 from intestinal epithelial cells in this infection, contributing to defence against this parasite as well as increasing damaging inflammation (Muñoz *et al.,* 2015). The involvement of IL-22 in the modulation of the acute phase reactants suggests that in malaria, this cytokine may mediate early pro-inflammatory responses critical for parasite clearance.

In this study, we found the mutant allele C and the CC genotype to be the most predominant with a frequency of (74.46%, 54.35% respectively). These results are in line with the study

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carried out in 2017 by Marquet *et al.* in Nigeria and Mali among children suffering from cerebral malaria and that carried out in Cameroon in 2023 by Tah *et al.* (Marquet *et al.,* 2017; Tah *et al.,* 2023). These similarities could be attributed to the ethnicity and lifestyle of these populations that can promote the development of mutations. IL-22 gene is highly polymorphic and single nucleotide polymorphisms (SNPs) on IL-22 gene have been associated with several diseases. SNP in introns can introduce novel splice sites, activate novel promoters or introduce/eliminate enhancer activity. Genetic polymorphisms on IL-22 gene can provoke a possible increase or decrease in the intensity of immune responses and consequently in disease progression and outcome. The SNP rs1179251 corresponds to a mutation by substitution of guanine by cytosine in intron 4 (Akbari *et al.,* 2018). Evidence has shown associations between these SNPs and other diseases such as gastric cancer and COVID-19, but also a predisposition to autoimmune thyroid disease (Qin *et al*., 2015; Song *et al*., 2017; Tah *et al*., 2023). In a study conducted in Saudi Arabia by Aljarba *et al.*, they found that some SNP at the level of the IL-22 gene such as (rs2227483, rs2227481) could contribute to the protection against *P. falciparum* and thus influence its clearance (Aljarba *et al.,* 2020).

The SNP rs1179251 on IL-22 gene results from a switch from the ancestral G to the derived mutation C on intron 4 (Akbari *et al.,* 2018). This switch could introduce novel splice sites, activate novel promoters or introduce/eliminate enhancer activity and therefore affect the gene expression of IL-22. Ali *et al*. demonstrated that some SNPs on the IL-22 gene could be associated with parasite clearance and therefore limit the mortality risk of patients under anti malaria treatment (Ali *et al.*, 2014). Results from this study showed no association between the SNP of IL-22 rs1179251 and *Plasmodium falciparum* clearance. These findings are similar to that of a study carried out in 2023 by Tah *et al.* on COVID-19 host susceptibility in Yaounde, Cameroon (Tah *et al.,* 2023). In 2019, Asadi *et al.* also reported no statistical association between SNP rs1179251 in the IL-22 gene and chronic hepatitis B infection (Asadi *et al.,* 2019). Marquet *et al.* also found similar results with the IL-22 rs1179251 and the development of cerebral malaria amongst Nigeria and Malian children, but found a significant difference in rs1012356, rs2227476, and rs2227473 between children with cerebral malaria and healthy controls. They claimed that SNP rs2227473 is placed in a vital production regulator position of IL-22, which also seems that individuals with T allele of rs2227473 express higher levels of IL-22 than those without this allele (Marquet *et al.,* 2017).

In our study, the observed IL-22 SNP may result in an increase in the expression of this protein in the liver, resulting in hepatoprotective effects. In addition, a more efficient mechanism of clearance via modulation of acute phase reactants by IL-22 may be possible. Another possibility could be that IL-22 may simply be a marker that exists in linkage disequilibrium with other neighboring SNPs or genes. In 2017, Song *et al.* demonstrated the association between IL-22 gene and predisposition to autoimmune thyroid disease (Song *et al.,* 2017). Also, in 2014 Ali *et al.* found independent association of SNP rs2227491 in IL-22 with clearance of amodiaquine and sulphadoxine/pyrimethamine-resistant parasites in children under five years in Cameroon (Ali *et al.,* 2014). Interestingly, the influence of some of the genetic factors may have a geographic distribution (Tah *et al.,* 2023; Yamamoto-Furusho *et al.,* 2016), which implies that ethnic differences may be a plausible explanation for the lack of an association in this study.

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CONCLUSION

IL-22 is a ubiquitous pro- and anti-inflammatory cytokine product of innate lymphoid cells and adaptive immune cells. Despite the fact that IL-22 induces the regeneration of hepatocytes during malaria infection and has a pro-inflammatory effect during the immune response against the parasite, no statistically significant association was found between rs1179251 single nucleotide polymorphism in IL-22 gene and *Plasmodium falciparum* parasites clearance.

Authors' Contributions

Study design, WFM, WONT, CFT. Methodology, AMN, WONT, JPKC, CFT, CNNT, WFM. Sample collection, RAN, IMA, JPKC, WFM. Data analysis, WONT, CFT. Molecular analysis, CFT, WONT, MCVF. Writing of original manuscript, CFT, WONT. All authors contributed to the revision of the manuscript and approved the final version of the manuscript prior to submission.

Conflicts of Interest

The authors declare no conflict of interest.

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