

## Correlation of C-reactive protein levels, gene polymorphism and platelets count in Dengue infection

Sana Shamshad<sup>1</sup>, Sara Khan<sup>2</sup>, Ghazala Kaukab Raja<sup>3</sup>, Muhammad Sheeraz Ahmad<sup>4</sup>, Muhammad Javaid Asad<sup>5</sup>, Tayyaba Zainab<sup>6</sup>

### Abstract

**Objective:** To determine the correlation of polymorphism in C-reactive protein gene with variation in serum levels in dengue patients.

**Methods:** The cross-sectional study was conducted at Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan, from October 2017 to October 2018, and comprised blood samples from dengue patients which were used to measure the serum levels of C-reactive protein. Deoxyribonucleic acid extraction followed by tetra amplification-refractory mutation system polymerase chain reaction was used to analyse the genotype variation T>G for single nucleotide polymorphism rs199953854 using allele-specific primers. Correlation of serum C-reactive protein levels with the C-reactive protein polymorphism in dengue patients was explored. Data was analysed using SPSS 21.

**Results:** Of the 200 patients, 108(54%) had very high C-reactive protein levels, 48(24%) had levels slightly higher than the upper limit, 14(7%) had low and 30(15%) had normal levels. Also, 162(81%) patients had low platelets count. Amplification of only T alleles was noted.

**Conclusion:** C-reactive protein levels were found to be increased with suppressed platelets count in dengue patients. Single nucleotide polymorphism rs199953854 appeared to have no polymorphism.

**Keywords:** C-reactive protein, Dengue infection, rs199953854, CRP polymorphism. (JPMA 71: 429; 2021)

**DOI:** <https://doi.org/10.47391/JPMA.326>

### Introduction

The host-defense mechanism is integral in terms of preventing the dysfunctions of physiological processes. In our ever-changing environment, the body responds to the aberrant stimuli, such as infections, trauma and injury, by releasing proteins in plasma, such as acute phase proteins (APP). The APP is a class of protein which is released and regulated in response to any stressful encounter or change in the homeostasis of the body consisting of a large group of plasma proteins. C-reactive protein (CRP), an important member of APP proteins, is one of the blood test markers used as a diagnostic tool in a clinical setting to evaluate the inflammatory response as it is increased in a wide array of pathologies, such as viral and bacterial infections, inflammation, diabetes mellitus (DM), cardiovascular disease (CVD), cancers and numerous pathophysiological insults. CRP serves as a predictor of inflammatory response in preventing microorganisms and their toxins to cause damage, triggering complement system, trapping free haemoglobins, and plays a role in regulating the host's immune response.<sup>1</sup> During an inflammatory condition or infection, CRP is released by hepatocytes in response to the release of cytokines, like interleukin-1 (IL-1), IL-6 and tumour necrosis factor alpha (TNF $\alpha$ ) by antigen presenting

cells, such as macrophages, lymphocytes and monocytes.<sup>2</sup> Researchers initially discovered CRP and showed its ability to react with the cell wall component referred to as fraction 'C' of *Strep pneumococci*, which, upon immunological analysis, showed strong precipitation.<sup>3</sup> Thereafter, it was shown that CRP serum level predominantly increases during the infections, tissue injuries and various pathologies. This makes CRP a potent clinical marker in various inflammatory processes.<sup>4</sup> Some studies showed that increased CRP level may provide protection against invading microorganisms and can limit the tissue damage by recognising the phosphorylcholine moieties and can opsonise microbial pathogen.<sup>5</sup> CRP is encoded by the CRP gene, which is present on chromosome 1 at q arm with the cytogenetic band at position 23.2. The size of the gene is 2kb, or 2,321 bases. The production of CRP is solely regulated at the transcriptional level in the presence of specific transcriptional factors, including CCAAT/enhancer-binding protein (C/EBP) family members C/EBP beta ( $\beta$ ) and C/EBP gamma ( $\gamma$ ).<sup>6,7</sup> CRP is a polymorphic gene which is known to have polymorphism in multiple regions, like promoter, exonic, intronic and three prime untranslated regions (3'UTRs). Recent studies among diverse population have shown a causal correlation between CRP gene single nucleotide polymorphisms (SNPs) and susceptibility of different diseases, such as Diabetes Mellitus (DM), cancer, cirrhosis, atherosclerosis, cardiac diseases, and systemic lupus erythematosus (SLE).<sup>8,9</sup> However, a link between serum CRP levels and SNPs in viral infection, such as

<sup>1,3-6</sup>National Center of Industrial Biotechnology, (PMAS) Arid Agriculture University, Rawalpindi, Pakistan; <sup>2</sup>Rawalpindi Medical University, Rawalpindi, Pakistan.

**Correspondence:** Tayyaba Zainab. e-mail: [tayyaba.zainab@uaar.edu.pk](mailto:tayyaba.zainab@uaar.edu.pk)

dengue, has not been studied.

Dengue virus is an epidemic infection in Pakistan, which was first reported in 1994. Pakistan has been experiencing an epidemic of dengue fever that has, according to the World Health Organisation Regional Office for the Eastern Mediterranean (WHO-EMRO), caused 16,580 confirmed cases and 257 deaths in Lahore and nearly 5,000 cases and 60 deaths in the rest of the country.<sup>10</sup> Dengue is a single-stranded ribonucleic acid (RNA) virus belonging to the flavivirus family and is transmitted with *Aedes aegypti* mosquito bite. There are two types of dengue virus infection: dengue haemorrhage syndrome (DHS), and dengue fever (DF). Circulating viral strains can be broken down into distinct genotypes, classified as serotypes DEN-1, DEN-2, DEN-3, and DEN-4. Epidemics occur in late summer in temperate regions, but the virus is enzootic and occurs throughout the year in many tropical areas of Asia. Amongst the emerging viral infections, dengue, a mosquito-borne disease endemic in more than 100 countries including Pakistan, is a frequent cause of morbidity and mortality in most developing countries.<sup>11</sup>

The current study was planned to understand the polymorphism in CRP gene [Accession NO: NC\_000001.11]<sup>12</sup> and correlation of CRP levels during dengue infection, and to assess SNP rs199953854 in dengue patients at 201 allele position while focussing on T>G variation.

### Patients and Methods

The observational case-control study was conducted at Pir Mehr Ali Shah (PMAS) Arid Agriculture University, Rawalpindi, Pakistan, from October 2017 to October 2018, and comprised blood samples from dengue patients. The association of CRP polymorphism with CRP levels in paediatric and adult dengue patients was studied in line with literature.<sup>13</sup>

After approval from the ethics research committee of Rawalpindi Medical University (RMU), the sample size was calculated using sample an online calculator<sup>14</sup> at 95% confidence level and 7% confidence interval (CI) in an approximate population of 10,00,000. Samples were collected from patients diagnosed with dengue infection documented in local hospitals along with 200 randomly selected normal subjects to act as the control group. Those included were individuals examined and cleared as having no disease other than dengue infection. Patients with a history of chronic infections and/or any other underlying ailments or if taking any prescription medication were excluded.

After written informed consent, blood samples were

collected in the clean venepuncture with a single needle stick procedure after disinfecting the skin. The samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes with a purple top, and serum separator in tubes with a yellow top (ATLAS-LABOVAC italiano, Lot no: 20170602). The tubes were labelled with the name, date and time of collection. The collected blood was stored at 4°C until it was further processed for genomic deoxyribonucleic acid (DNA) extraction. Patient profile was built with demographic information and clinical details, including platelets count and disease-specific test details. Quantitative CRP analysis was done using Specific Protein Analyser (MD PACIFIC Imagin200 Tianjin New Industrial District, China) for hypersensitivity-CRP (Hs-CRP) test, and platelet counts were analysed from blood complete picture (CP) for all the cases and the controls.<sup>15</sup>

Tetra amplification-refractory mutation system (ARMS) primers for SNP rs199953854 were exclusively designed using online programme Primer3.<sup>16</sup> Primers designed were:

Outer Forward = 5'CATGAAGGATGCTCCACTGTTCTGTTC A 3';

Outer Reverse = 5'AGCAGTAAGGGAGTTTGCGCCACTATGT 3';

Inner T Forward = 5'TCTGGCCAAAAGCATGAGAGAGGCTA GT 3'; and

Inner G Reverse = 5'GCGAGACCAAGAAACACAACAGCTTC T 3'.

Primers were purchased (Macrogen company®, South Korea).

For DNA isolation, the method used was, as described in literature.<sup>17</sup> About 300µl of blood was mixed with 300ul of Cell Lysis buffer in an Eppendorf (extra gene, Taiwan) and was vortexed (WiSMix® VM-10 witeg Germany) for 15-20 seconds. The samples were centrifuged (Centrifuge Satorius Germany) at 1500 rpm for 7 minutes, and the supernatant was discarded carefully, keeping the pellet at the bottom to get a white precipitate of leukocytes. The entire process was repeated to eliminate any red blood cells (RBCs). Next, 400ul of Nuclear Lysis buffer (NLB) was added with 100µl of sodium chloride (NaCl) and 500ul of chloroform to the pellet. The whole mixture was well homogenised by vortex for 15-30 seconds and then centrifuged for 7 minutes at 5000 rpm which resulted in a layer-formation, separating the supernatant and the pellet. The supernatant containing DNA was separated into a new Eppendorf carefully. The supernatant was treated with 1000ul 70% ethanol and was kept at -20°C for 30 minutes and the tubes were then centrifuged at 14000 rpm for 8 minutes and ethanol was discarded carefully. In the white pellet of DNA at the bottom of the tube, 1000ul of pre-chilled absolute ethanol was added and was centrifuged again for 8 minutes at 14000 rpm. Ethanol was discarded and DNA pellet in the tube was inverted to dry the tubes completely. Afterward, 100ul of tris-EDTA (TE) buffer was

added and incubated at 37°C for 24 hours and the DNA was then quantified through Nanodrop.

Tetra ARMS polymerase chain reaction (PCR) amplification of DNA the fragment was done in line with the protocol in literature,<sup>18</sup> using PCR Master Mix (WizPure TM PCR 2X Master Mix). Amplification of DNA was done on PCR machine (BIO-RAD TTM100 Thermal Cycler) using four primers over 30X cycle of initial denaturation at 95°C for 10 minutes followed by denaturation at 95°C and annealing at 65°C for 30 seconds. The final step included an extension at 72°C for 7 minutes. The amplified products were stored at 4°C, followed by electrophoretic analysis of PCR products at 90V. Data was analysed using SPSS 21.

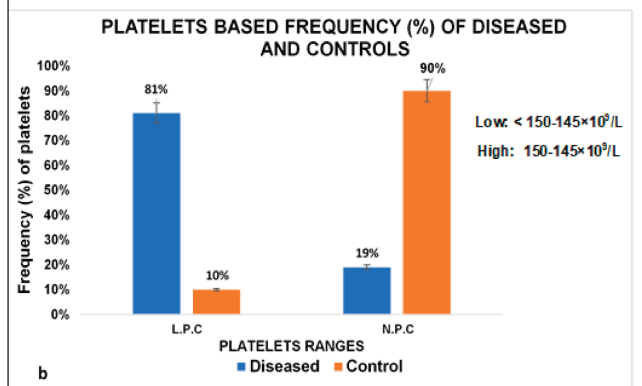
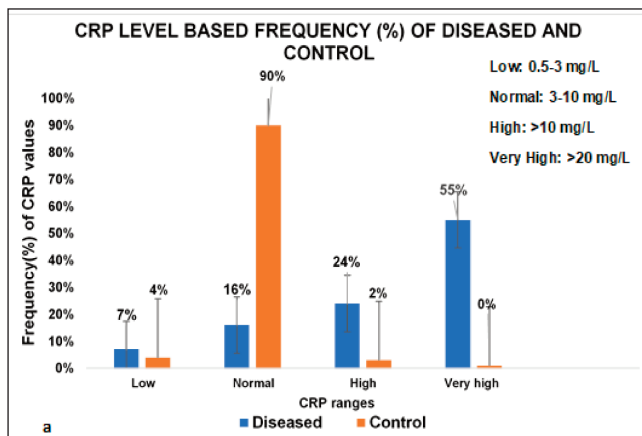
**Results**

Of the 400 subjects, there were 200(50%) cases and controls each. Among the cases, there were 24(12%) children aged 0.5-14 years with a mean age of 8.1±0.8 years; 37(18%) adolescents aged 15-25 years with a mean age of 18.8±0.3 years; 106(53%) adults aged 25-40 years with a mean age of 32.0±0.72 years; and 32(16%) old subjects aged 50-80 years had a mean age of

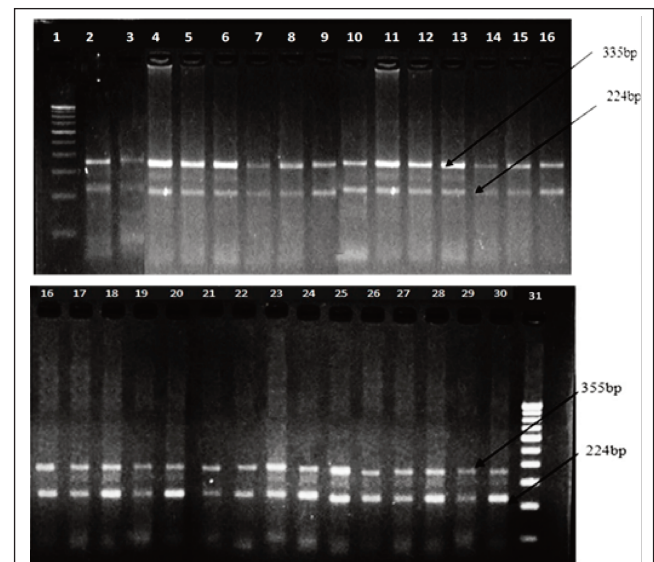
58.2±1.8 years. In the same age groups among the controls, the respective values were: 52(26%) with mean age 8.1±1.31 years, 64(32%) with mean age 19.8±0.8 years, 52(26%) with mean age 31.2±2.6 years and 32(16%) with mean age 50.5±0.5 (*p*>0.05). Of the 106(53%) adults among the cases, 42(40%) showed severe dengue infection. Overall mean CRP value was 44.6±30.2. Gender-based four groups of both the cases and the controls were made based on CRP levels. Among the cases, 14(7%) showed low levels of CRP <1mg/L, 20(15%) normal levels 1-3mg/L, 48(24%) high levels 3-10mg/L, and 106(54%) showed very high levels of CRP >20mg/L. Most of the subjects from control group showed the normal range of CRP with a mean of 6.4±0.9. Four (2%) showed low value, 90 (45%) showed normal CRP value, 2 (1%) showed high CRP value and 0 (0%) showed very high CRP values.

Overall, 162(81%) cases showed low levels of platelets <150×10<sup>10</sup>/L and 38(19%) showed normal level 150-450×10<sup>10</sup>/L, whereas 20(10%) among the controls showed low platelets count due to reasons other than dengue and 180(90%) showed normal counts (Figure 1).

As for tetra ARMS PCR, the product size of outer primers was 355bp. For wild type (WT) T allele, products size was 224bp and for variant G allele, the product size was 186bp. On electrophoretic analysis, the product size for outer forward/outer reverse was 355bp, and for inner T forward/outer reverse was 224bp, whereas inner G reverse/outer forward was 186bp (Figure 2a). Tetra-ARMS with CRP forward outer primer and CRP reverse outer

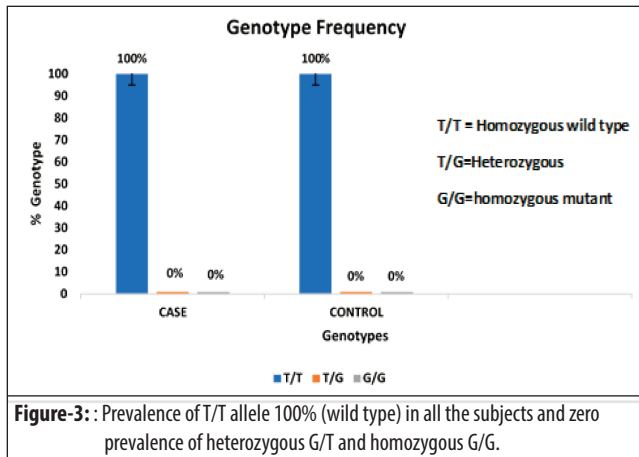


**Figure-1:** a) Different levels of C-reactive protein (CRP) among dengue patients and control subjects b) Platelets level of dengue patients and normal subjects.



(a) lane 2-16: Tetra-ARMS PCR products of dengue patients  
(b) Control subjects (16-30). 100bp ladder in lane (1&31).

**Figure-2:** 3% agarose gel electrophoretic analysis of Tetra- amplification-refractory mutation system (ARMS) polymerase chain reaction (PCR) for single nucleotide polymorphism (SNP) rs199953854.



primer gave PCR product of 355bp in lane 2-16. CRP forward inner and reverse outer primer gave PCR product size of 224bp. Comparison with ladder of 100bp (lane 1) showed that samples lane (2-16) were of 224bp for WT allele T. Similarly, ARMS PCR products for control samples in lane (16-30) were analysed by electrophoresis (Figure 2b). Comparison with ladder of 100bp in lane 31 showed that 16-30 samples were of 224bp of allele T.

Results of tetra ARMS PCR showed that the percentage for TT allele was 100%, while both T/G and G/G were 0% both in cases and controls (Figure 3).

## Discussion

The current study is novel in terms of trying to establish a correlation between CRP levels, platelets levels and CRP gene polymorphism against SNP rs199953854 in individuals with dengue infection. To the best of our knowledge, there is no known information in literature on the subject.

CRP belongs to the family of APP proteins and is reported to be rapidly elevated in trauma, inflammation and infection.<sup>4</sup> We used SNP marker of the human CRP gene (rs199953854) to genotype 200 dengue patients and as many healthy individuals. Numerous studies have reported that CRP levels can vary due to the polymorphism in the CRP gene. To understand the correlation of CRP SNP in patients with dengue infection, it was confirmed that all the subjects in the experimental group were positive for dengue infection with the help of nonstructural protein 1 (NS1) antigen test, which is the optimal test used in Pakistan for the diagnosis.<sup>11</sup> We also performed quantitative analysis for CRP levels by high throughput CRP measurement. Patients were divided into multiple groups based on the age to parse out the correlation of CRP levels with age. In our study, 40% of adults had severe dengue infection. The effect of CRP gene variation on CRP levels in patients with dengue was also noted. The variation of CRP

levels among our groups showed that few patients were under treatment and their infection was somewhat improving and gave us the normal range of CRP. whereas 24% and 54% patients showed high and very high levels of CRP, respectively, who were not given any treatment yet, and their infection was at its peak. We also observed the low levels of platelets in our cohorts, and the decreased number of platelets in patients with dengue virus play an important role in the clinical outcome.<sup>19,20</sup> Thus, a correlation was made with the levels of platelets and the stage of dengue infection. Platelets count can also help in identifying the extent of improvement during the treatment. In our study, 91% of dengue patients showed low platelets count (LPC), showing they had severe dengue infection although it cannot be the only determinant factor in dengue infection as many sidelines, such as cancer, anaemia, toxic chemical, medicine, alcohol, viruses, genetic conditions, autoimmune diseases, infection, surgery and pregnancy, can result in LPC, or thrombocytopenia. Further, 9% dengue patients showed normal PC (NPC), showing their infection was at its end stage. Genetic differentiation among individuals can contribute to the variation in the production of platelets in the body.<sup>20,21</sup> CRP gene is polymorphic and has many SNPs. Multiple SNPs have been identified in various diseases like cancer, CVD, DM, cirrhosis and atherosclerosis. In the current study, novel SNP rs199953854 was selected at position 201T>G to check polymorphism and its correlation with the susceptibility of dengue infection and variation in CRP levels. While reviewing the population genetics of this SNP, it was found that there was 1% prevalence of rs199953854 polymorphism which is T>G in African Americans whereas, 0% in Caucasian and Asian populations retrieved from ENSEMBL Database 2018.<sup>22</sup> As this SNP was not studied previously in dengue patients, we selected it to confirm the prevalence in local population. Similar results were reported in another study with CC into CT SNP variants and CRP levels in vitamin D-deficient patients. Likewise, our data from tetra ARMS PCR is supported by the same study.<sup>23</sup> As no band for variant G allele was found during PCR, tetra ARMS PCR confirmed 0% prevalence of G allele in our patients who come under the umbrella of South Asian population as reported in ENSEMBL Database 2018. Conclusively, there is no polymorphism in SNP rs199953854, so no direct evidence can be made to correlate the variation in this SNP with CRP levels and risk of severity in dengue patients. Hence, our study showed no association between CRP levels and polymorphism in SNP rs199953854 which is similar to a study in patients of colorectal cancer.<sup>24,25</sup> To make an affirmative conclusion, a large-spectrum study can be designed keeping in consideration other reported CRP SNPs like rs766929411,

rs752054019, rs755051450 in dengue patients. In many studies, CRP is characterised as a potent marker in diseases like CVD, myocardial infarction (MI), atherosclerosis and cancer.

There are limitations to the current study, as platelets counts could vary due to gender and genetics of the patient and might require detailed genetic investigation to affirmatively attest our findings. The study was unable to define whether the 201 T>G polymorphism was a functional marker to establish linkage between variation of CRP levels in dengue patients. There might be functional variants elsewhere in the CRP gene as well. In future studies, the triallelic nature of SNP rs199953854 shall be explored as there might be a possibility that the triallelic SNP is affecting the levels of CRP in patients.

## Conclusion

CRP levels were found to be increased with suppressed platelets counts in dengue patients. SNP rs199953854 appeared to have no polymorphism.

**Disclaimer:** Part of the study was published in the Abstract Book of the First International Conference on Advancements in Biotechnology and Biocomputing, held at the International Islamic University, Islamabad, Pakistan, on February 7-9, 2019.

**Conflict of interest:** None.

**Source of Funding:** None.

## References

1. Cerón JJ, Eckersall PD, Martínez Subiela S. Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Vet Clin Pathol* 2005; 34: 85-99.
2. Jain S, Gautam V, Naseem S. Acute-phase proteins: As diagnostic tool. *J Pharm Bioallied Sci* 2011; 3: 118-27.
3. Tillett WS, Francis T. Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J Exp Med* 1930; 52: 561-71.
4. Castelli GP, Pognani C, Meisner M, Stuani A, Bellomi D, Sgarbi L. Procalcitonin and C-reactive protein during systemic inflammatory response syndrome, sepsis and organ dysfunction. *Crit Care* 2004; 8: R234-42.
5. Lu J, Marjon KD, Mold C, Du Clos TW, Sun PD. Pentraxins and Fc receptors. *Immunol Rev* 2012; 250: 230-8.
6. Du Clos TW, Mold C. Pentraxins (CRP, SAP) in the process of complement activation and clearance of apoptotic bodies through Fcγ receptors. *Curr Opin Organ Transplant* 2011; 16: 15-20.
7. Agrawal A, Cha-Molstad H, Samols D, Kushner I. Overexpressed nuclear factor-kappaB can participate in endogenous C-reactive protein induction, and enhances the effects of C/EBPβ and signal transducer and activator of transcription-3. *Immunology* 2003; 108: 539-47.
8. Nimptsch K, Aleksandrova K, Boeing H, Janke J, Lee YA, Jenab M. Association of CRP genetic variants with blood concentrations of C-reactive protein and colorectal cancer risk. *Int J Cancer* 2015; 136: 1181-92.
9. Pai JK, Mukamal KJ, Rexrode KM, Rimm EB. C-reactive protein (CRP) gene polymorphisms, CRP levels, and risk of incident coronary heart disease in two nested case-control studies. *PLoS One* 2008; 3: e1395.
10. World Health Organization (WHO). Dengue. [Online] 2018 [Cited 2018 December 15]. Available from: URL: <http://www.emro.who.int/health-topics/dengue/index.html>.
11. Ranganath R, Peerapur BV. Evaluation of NS1 Antigen Detection for Early Diagnosis of Dengue Virus Infection in a Tertiary Care Hospital in Karnataka, India. *J Clin Diagn Res* 2016; 5: 710-7.
12. National Library of Medicine (US), National Centre for Biotechnology Information. [Online] 2004 [cited 2018 Dec 15]. Available from: URL: <https://www.ncbi.nlm.nih.gov/gene/1401>
13. Chen CC, Lee IK, Liu JW, Huang SY, Wang L. Utility of C-reactive protein levels for early prediction of dengue severity in adults. *Biomed Res Int* 2015; 2015: 936062.
14. Survey system com. [Online] [Cited 2017 October 2]. Available from: URL: <https://www.surveysystem.com/sscalc.htm>.
15. Bassuk SS, Rifai N, Ridker PM. High-sensitivity C-reactive protein: clinical importance. *Curr Probl Cardiol* 2004; 29: 439-93.
16. Köressaar T, Lepamets M, Kaplinski L, Raime K, Andreson R, Remm M. Primer3\_masker: integrating masking of template sequence with primer design software. *Bioinformatics* 2018; 34: 1937-8.
17. Chacon-Cortes D, Griffiths LR. Methods for extracting genomic DNA from whole blood samples: current perspectives. *Mol Biol Rep* 2014; 2014: 1-9.
18. Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res* 2001; 29: e88.
19. McConnell JP, Branum EL, Ballman KV, Lagerstedt SA, Katzmann JA, Jaffe AS. Gender differences in C-reactive protein concentrations-confirmation with two sensitive methods. *Clin Chem Lab Med* 2002; 40: 56-9.
20. Bain BJ. Platelet count and platelet size in males and females. *Scand J Haematol* 1985; 35: 77-9.
21. Daly ME. Determinants of platelet count in humans. *Haematologica* 2011; 96: 10-3.
22. Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, et al. Ensembl 2018. *Nucleic Acids Res* 2018; 46(D1): D754-61.
23. Myburgh PH. Interactions of CRP-SNPs with selected contributing factors in determining CRP concentrations in black South Africans. [Doctoral dissertation]. South Africa: North-West University; 2018.
24. Polterauer S, Grimm C, Zeillinger R, Heinze G, Tempfer C, Reinthaller A, et al. Association of C-reactive protein (CRP) gene polymorphisms, serum CRP levels and cervical cancer prognosis. *Anticancer Res* 2011; 31: 2259-64.
25. Liu ZQ, Chu L, Fang JM, Zhang X, Zhao HX, Chen YJ, et al. Prognostic role of C-reactive protein in prostate cancer: a systematic review and meta-analysis. *Asian J Androl* 2014; 16: 467-71.