MERIT RESEARCH JOURNALS
www.meritresearchjournals.org

Merit Research Journal of Medicine and Medical Sciences (ISSN: 2354-323X) Vol. 3(9) pp. 432-452, September, 2015
Available online http://www.meritresearchjournals.org/mms/index.htm

Original Research Article

Expression of Physiologic Biomolecules following Anti-Malarial Cytokine-CpG Motif Oligodeoxynucleotide Gene Therapy

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Abstract

According to latest estimates, there were 198 million cases of malaria worldwide in 2013, with 82% of these cases occurring in Africa. There were approximately 584,000 malaria deaths worldwide. The outcome of infection with *Plasmodia* parasites is determined by the activities of various biomolecules, cytokines and other host-specific factors. *Plasmodia* parasites evade immunity and modulate immune systems to their advantage thereby exacerbating infection and disease. When combined in therapy, immunostimulatory unmethylated CpG motif oligodeoxynucleotides (ODNs) synergise with cytokines in the promotion of anti-parasitic mechanisms providing effective protection in various cases. The CpG ODNs enhance immune activities through ligation to plasmacytoid dendritic cell (pDC) Toll-like receptors (TLRs) such as TLR-9 and they activate both pDCs and B-cells, while cytokines modulate cellular behaviour. By altering cellular functionality, the cytokine-CpG ODN immunotherapy combination can alter the expression of physiologic factors and this can influence disease outcomes including severity of infection with malaria parasites. It was previously unclear how physiologic biomolecules like matrix metalloproteinases (MMPs) and angiopoietins (ANGPTs) could be influenced by the coincidental introduction of recombinant cytokines and CpG ODNs during malaria. This project studied cytokine-CpG motif ODN co-inoculation in BALB/c mice infected with *P. berghei* ANKA strain. Two BALB/c mice groups infected with virulent blood stage *P. berghei* ANKA strain parasites were given immunotherapeutic cytokine and CpG ODN combinations for five consecutive days while six other control groups with different treatments were included for comparison. The mice were monitored daily for clinical symptoms and parasitaemia development from day 1 postinfection. At ten days postinfection, all mice were humanely sacrificed for the extraction of EDTA-treated blood and plasma for measuring various physiologic factors. Results unraveled cytokine-CpG-based gene therapy as an enhancer of anti-*Plasmodial* activities accompanied by elevations in adiponectin, ANGPT1, neuropilin-1(NRP-1) and cyclooxygenase-2 (Cox-2) and delevations in ANGPT2, MMP-8 and MMP-9. These physiologic outcomes, which are largely agreeable with data from other studies, favour further investigations on combinatorial cytokine-CpG ODN gene therapy for potential inclusion into preventative and therapeutic anti-malarial interventions.

Key Words: BALB/c Mice, Cytokines, CpG Motif ODN, Malaria, *P. berghei* ANKA, Physiologic Biomolecules.

INTRODUCTION

There were about 219 million cases of malaria in 2010 accompanied by 660 000 malaria-related deaths. Africa is the most affected continent: about 90% of all malaria deaths occur there and malaria remains inextricably linked with poverty (Keating, 2012). According to the latest approximations, released in December 2014, there

were 198 million cases of malaria worldwide in 2013 (with an uncertainty range of 124 million to 283 million), with 82% of these cases occurring in Africa (www.who.com). Malaria parasites, *Plasmodia,* challenge the immune system via active subversion, immune escape and sequestration responses, via antigenic epitope variation and foiling of vaccine-induced immunity and attempts to develop vaccines against malaria continue to be challenged by problems of low efficacy, reactogenicity and low immunogenicity (Wang *et al.,* 2009). Investigations on biomolecules, immunobiological and biochemical profiles with the potential of improving anti-malarial vaccination and therapy encompass an active part of current malaria research.

The CpG oligodeoxynucleotide (or CpG ODN) short single-stranded synthetic DNA molecules contain a cytosine triphosphate deoxynucleotide ("C") followed by a guanine triphosphate deoxynucleotide ("G"). The "p" in "CpG" refers to the phosphodiester link between consecutive nucleotides, although some ODN have a modified phosphorothioate (PS) backbone instead. Unmethylated CpG motifs are powerful immuno-stimulants (Weiner *et al.,* 1997; Bauer *et al.,* 2002) and CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundant presence in microbial genomes and their rarity in vertebrate genomes (Bauer *et al.,* 2002). Co-administration of CpG motif oligodeoxynucleotides (ODNs) with cytokines like IL-18 and IL-12 can potentially generate more upregulated immunopotentiation compared to independent administration of these components. Such combinatorial gene therapies have the potential of synergizing the advantages of both CpG ODN gene treatment and cytokine effects *in vivo*. Protective CpG-based mechanisms are initiated through TLR-9 (Toll Like Receptor-9) pathways *in vivo* and cytokines co-inoculated with immunostimulatory CpG motif ODNs are strong elicitors of protection against parasitic infections (Li *et al.,* 2004). The CpG ODN motifs trigger and upregulate immune functionality via ligation to dendritic cell Toll-like receptors (TLRs) such as TLR-9. Before this report, the effects of cytokine-CpG co-injections on physiologic biomolecules, in the context of malaria were yet to be evaluated. Various physiological biomolecules have been investigated for their association with both protection or increased risk in the context of various infection and disease conditions including infectious disease conditions like malaria. These include molecules like adiponectin, angiopoietins, angiogenin and MMPs. Angiogenin, for example, is a potent stimulator of new blood vessels through the process of angiogenesis. Ang hydrolyzes cellular RNA, resulting in modulated levels of protein synthesis and interacts with DNA causing a promoter-like increase in the expression of rRNA. Ang is associated with cancer and neurological disease through angiogenesis and through activating gene expression that suppresses apoptosis (Steidinger *et al.,* 2011). Human postmortem studies give evidence of enhanced protein levels of MMP-1 in brains of cerebral malaria (CM) patients, whereas MMP-8 become increased in plasma of severe malaria patients. The activation of the human MMP-9 gene by *P. falciparum* has been demonstrated in microarray studies on whole blood from children with malaria (Prato *et al.,* 2011).

The immunopotentiating effects of CpG ODNs include direct induction of B cell proliferation and immunoglobulin (Ig) secretion, as well as activation of monocytes, macrophages, and dendritic cells to upregulate their expression of costimulatory molecules, which drive immune responses, and secretion of a variety of cytokines, including high levels of IL-12 (Weeratna *et al.,* 1999). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA, and activate multiple cell types including macrophages, dendritic cells, NK cells, and B lymphocytes. Immunostimulatory capabilities of CpG-ODNs make them useful therapeutics for immune adjuvant, inflammatory and allergic disease, and for immunoprotective agent. Nanoparticle-bound CpG ODNs have been successfully used to deliver drugs to targeted cells leading to pinpoint delivery and increased immunopotentiating outcomes (Kerkmann *et al.,* 2006; Chinnathambi *et al.,* 2012; Alexandre de Titta *et al.,* 2013), thereby increasing potential applications of such nanoscale-complexes in dealing with infectious diseases.

This investigation determined the effects of cytokine-CpG ODN co-inoculation on the expression of a myriad of physiologic biomolecules in *P. berghei*-infected BALB/c mice. *Plasmodium berghei*-infected BALB/c mice that were therapeutically co-administered with cytokines and CpG motif-containing ODNs were monitored for clinical symptoms, parasitaemia and RBC levels, which were then related to physiologic factors quantified in plasma using various bioassays. Cytokine-CpG ODN co-injection mediated increased protection associated with increases in adiponectin, ANGPT1, NRP-1 and Cox-2 and decreases in ANGPT2, MMP-8 and MMP-9.

MATERIALS AND METHODS

Study Site

This project was conducted at the Kenya Medical Research Institute's (KEMRI) Center for Biotechnology Research and Development (CBRD) and the Institute of Primate Research (IPR), in Nairobi, Kenya. The study was approved by the KEMRI ethical and scientific steering committees.

Study Design

This study characterised cytokine-CpG motif co-inoculation in mice BALB/c infected with *P. berghei* ANKA strain. There were eight groups of mice; two main experimental cytokine-CpG co-inoculation groups and six control groups that were used. The groups were designated as CpG/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei*; IL-18/ *P. berghei*; IL-12/*P. berghei*; CpG / *P. berghei*; *P. berghei*; CpG and uninfected mice groups. The cytokines (IL-12 and IL-18) were chosen due to their previously illustrated protective roles in parasitised murine hosts (Angulo *et al.,* 2002; Li *et al.,* 2004). Each mice group had 18 mice. The mice groups given names containing '*P. berghei*' were infected simultaneously with *P. berghei* parasites as described above. Parasitaemia (using Giemsa-stained blood films) and clinical characteristics were monitored on a daily basis in all these mice. One day one post-infection mice groups were treated as follows: the CpG/IL-18/ *P. berghei* group was treated with both CpG ODNs and IL-18, the CpG/IL-12/ *P. berghei* group was treated with both CpG ODNs and IL-12, the IL-18/ *P. berghei* and IL-12/ *P. berghei* groups were treated with IL-18 and IL-12 respectively, the CpG/ *P. berghei* group was treated with CpG ODNs, the *P. berghei* group remained untreated, the CpG group (uninfected) received CpG ODNs only, while the uninfected group remained untreated. Treatments were repeated for 5 days. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups constituted the main groups under investigation, while the other six groups were the control groups for comparison purposes. After ten days, all mice were anaesthetized and humanely euthanised (using KEMRI SOPs) for the extraction of EDTA-treated blood via cardiac puncture for isolation of plasma to be used in physiologic factor analysis. A SysmeX SF-3000® hematology analyzer was used to measure RBC levels in all mice.

Experimental Mice, Parasites, and Infections

Twelve week-old female BALB/c mice purchased from KEMRI and ILRI (International Livestock Research Institute) were intraperitoneally injected, using a needle of size 26 G, with $1x10^4$ virulent wild type *P. berghei* ANKA-parasitized red blood cells obtained from donor infected BALB/c mice. Parasitaemia and reticulocyte levels were monitored in all mice in the experiments every day. Blood for parasitaemia determination was extracted from the tail veins of the mice (approximately 50 µl per mouse) and used to prepare triplicate Giemsa-stained thin blood smears and parasitaemia were expressed as a percentage of at least 2000 RBCs (Barasa *et al.,* 2010; Helegbe *et al.,* 2011).

For anaesthetisation, the Ketamine/Diazepam mixture of drugs 1:1 by volume were administered 0.1 ml/kg to all mice for restraint, anesthetic induction or for allowing non-painful cardiac puncture procedures using needle size 26 G. This gave excellent muscle relaxation, and minimal respiratory or cardiovascular depression. When combined and administered as described, the dose was 5 mg/kg ketamine and 0.25 mg/kg diazepam. All mice were killed "humanely" by placing them on top of the cage and breaking their neck by applying firm pressure at the base of the skull and sharply pinching and twisting between thumb and forefinger while at the same time pulling backward on the tail.

Recombinant Cytokines and CpG Motif Oligodeoxy-nucleotides (ODNs)

Recombinant murine cytokines (rIL-18, and rIL-12) were purchased and processed for intradermal inoculation according to manufacturer's specifications (Becton Dickinson, USA). The recombinant cytokines were reconstituted to final concentrations of 500 ng/mL in total volumes of 50 µl of PBS for delivery into each murine recipient. Synthetic CpG motif oligodeoxynucleotides (ODN) containing CpG motifs synthesized with a nuclease-resistant phosphorothioate backbone (Invivogen, USA) were purchased (Catalog number: CpG ODN M362) and used. The CpG ODN M362 sequence 5'-TCGTCGTCGTTC: GAACGACGTTGAT-3' (25 mer) contained the CpG motifs required for immunostimulation. Each BALB/c mouse was intramuscularly inoculated at the appropriate time with a 50µL preparation of the CpG ODN in phosphate buffered saline as previously described (De Rose *et al.,* 2002). After infection with *P. berghei* parasites on day 0, on the following day (day one), relevant mice groups were injected with 50 µg CpG ODN M362 by use of 27.5-gauge needles in volumes of 50 µl. The IL-12/CpG ODN and IL-18/CpG ODN immunotherapeutic combinations and the other treatments were repeated for 5 consecutive days from day 1.

Isolation of Plasma from EDTA-treated Whole blood

Murine whole blood obtained via cardiac puncture was collected into 1.5-ml EDTA coated Eppendorf® tubes containing 8 ul of 0.5 M EDTA that were chilled on ice and gently mixed. Gentle mixing of the blood and anticoagulant was done followed by storage on ice. Blood volume was estimated followed by the addition of an amount of 0.5 M EDTA so the final concentration of EDTA became 5 mM; for to 1 ml of blood, an additional 2 ul of EDTA was required. Centrifuging of the sample was then performed for 15 min at 3,000 rpm (1500 × g) at 4° C without braking to stop centrifuge. The supernatant (plasma) was carefully transferred into a 0.5 ml-Eppendorf® tube packed with glasswool and a hole at the bottom and spun at 3,000 rpm for 15 sec at 4° C. 12. The

plasma that was by then ready for analysis was stored in aliquots at –20◦ C. until used.

Physiologic Factor Bioassays

Sources of Antibodies and Kits for Physiologic Factor Bioassays

Bioassay kits for adiponectin and angiopoietin-2 detection were obtained from Abcam® while angiopoietin-1 kits were purchased from Biocompare®. The kits for angiogenin detection were purchased from Elabscience®), while those for NRP-1 were acquired from Cloud-clone Corp®). For quantifying COX-2, MMP-8 and MMP-9 bioassay kits from Cusabio® were used.

Adiponectin, Angiopoietin-1 and Angiopoietin-2 Bioassays

All materials and prepared reagents were equilibrated to room temperature (18 - 25°C) prior to use. Standards, controls and samples were assayed in triplicate and the assays were performed at room temperature (18- 25°C). Excess microplate strips were removed from the plate frame and returned immediately to the foil pouch with desiccant inside. The pouch was resealed securely to minimize exposure to water vapor and stored in a vacuum desiccators. Fifty microliters of adiponectin or angiopoietin-1 or angiopoietin-2 standards or samples were then pipetted per well and the wells of the 96-well pre-coated (with anti-mouse adiponectin or anti-mouse angiopoietin-1 or anti-mouse angiopoietin-2 antibodies) and pre-blocked microtiter plates were covered with sealing tapes and incubated for two hours (Abcam® and Biocompare®). The plates were then washed six times with 300 µL of 1x wash buffer automatically and the plates inverted each time and contents decanted; tapping it 4-5 times on absorbent paper towel to completely remove the liquid. Fifty microliters 1x biotinylated anti-adiponectin or anti-mouse angiopoietin-1 or anti-mouse angiopoietin-2 antibodies were added to each well and incubated for one hour. Washing was then done as described above and 50 µL of 1x Streptavidin HRP conjugate added to each well and incubated for 30 minutes.

The Dynatech MRX™ ELISA microplate reader programme was then set up in advance. Washing was then done as given above and 50 µL of TMB chromogen substrate added per well and incubated for about 10 minutes or till the optimal blue colour density became developed. Gentle tapping of the plate to ensure thorough mixing and breaking of the bubbles was done in the well with pipette tips and 50 µL of stop solution added to each well. Colour change occurred from blue to yellow and photo-absorbance on the microplate reader at a wavelength of 450 nm read immediately. The mean values of the triplicate readings for each standard and sample were calculated and used to generate a standard curve, using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line was determined by regression analysis using log-log or four-parameter logistic curve-fit. The unknown sample adiponectin, angiopoietin-1, angiopoietin-2 concentrations were determined from standard curves and the value multiplied by the dilution factor.

Angiogenin, NRP-1, COX-2, MMP-8 and MMP-9 Bioassays

One hundred microliters of standards, blank, or sample were dispensed per well. The blank wells received the reference standard and sample diluent. Solutions were added to the bottom of the pre-coated (with anti-mouse angiogenin or anti-mouse NRP-1 or anti- mouse COX-2 or anti- mouse MMP-8, or anti- mouse MMP-8 antibodies) and pre-blocked 96-well micro ELISA plate well (Elabscience®, Cloud-clone Corp.®, Cusabio®), touching of the inside walls and foaming was avoided as much as possible and mixing was gentle. The plates were then covered with the sealers provided and incubated for 90 minutes at 37℃. Liquids from each well were removed and, without washing, 100µL of biotinylated anti-mouse angiogenin, anti-mouse NRP-1, anti-mouse COX-2, anti-mouse MMP-8, or anti-mouse MMP-9 detection antibodies working solutions were delivered into each of the wells followed by coverage of the microtiter plates with the provided plate sealer. Gentle tapping of the plates was done to ensure thorough mixing and incubation done for 1 hour at 37°C. The plates were washed six times with 300 µL of 1x wash buffer automatically and then inverted each time and contents decanted; tapping was done 4-5 times on absorbent paper towel to completely remove the liquid. One hundred microliters of streptavidin HRP conjugate working solution were added into each well and the plates covered with plate sealers followed by incubation for 30 minutes at 37°C and a repeat of the washing process described above for five times. Ninety microliters of substrate solution were then added into each well and covered with new plate sealers. Incubation was done for about 15 minutes at 37°C whilst plates were being protected from direct light. The reaction times were shortened or extended according to the actual color change, but not more than 30 minutes; when apparent gradient appeared in standard wells, the reactions were terminated using 50µL of stop solution dispensed into each well causing the color to turn from blue to yellow immediately. The micro-plate reader was opened and activated with set parameters in advance. The optical

Table 1. Average parasitaemia levels in eight mice groups involved in the study. Clinical observations were quantified using an arbitrary scale and reported as either absent (–), mild (+), moderate (++), severe (+++) or extremely severe (++++).

	CpG/ IL-18/ *P. Berghei*	**CpG/ IL-12/ *P. berghei***	**IL-18/ *P. berghei***	**IL-12/ *P. berghei***	**CpG / *P. berghei***	***P. berghei***	**CpG ODN**	**Uninfected mice**
Parasitaemia (Means)	5.727	5.455	9.455	10.64	15.73	25.34	0.0	0.0
RBC (x 1000/ml)	10.3	10.1	9.32	9.6	8.34	6.08	10.2	10.53
Clincal Outcomes	**+**	**+**	**++**	**++**	**+++**	**+ + +++**	**-**	**-**
ANOVA	F (1.064, 10.64) = 10.23, P < 0.05							

density (OD value) of each well was determined at once, using a Dynatech MRX™ ELISA micro-plate reader set to 450 nm. Generated standard curves were then used to determine sample angiogenin, NRP-1, anti-COX-2, MMP-8 and MMP-9 concentrations.

Data Analysis and Management

Acquired data were analysed using the Graphpad Prism-6®, 2015 software. Group mean values of parasitaemia, and quantified physiologic factors were compared using one-way Analysis of Variance (ANOVA). Tabulated probability values of $P < 0.05$ were considered significant.

RESULTS

Parasitological and Clinical Outcomes

The two mice groups that were treated with cytokine-CpG co-inoculations experienced significantly ($P < 0.05$) lower total parasitaemia than the rest of the mice in the study. Their average parasitemia (5.591 %) was approximately three times lower than in the average parasitaemia (11.826 %) in the control experiment groups. The highest parasitaemia (15.73 %) was detected in the untreated *P. berghei* group followed by the CpG/*P. berghei* group (15.73 % (Table 1). Clinical parameters measured were scored arbitrarily on a scale of 1 to ten and each one to ten range score was represented on a clinical parameter score table as a single '+'. Analysed and scored symptoms included hair ruffling, appetite loss, intra-cage agility, diarrhea, skin turgor reduction, limb paralysis, convulsions. In the table 1 above, the higher the numbers of the '+' sign, the greater the symptomatic intensity. The most severe malarial symptoms were detected in the *P. berghei* group, coinciding with high parasitaemia levels and this group was followed closely by the CpG/*P. berghei* group in symptomatic severity. Amongst the malaria-infected groups, the least severe symptoms were experienced in the CpG/IL-18/*P. berghei* and CpG/IL-12/*P. berghei* groups followed by the IL-18/*P. berghei* and IL-12/*P. berghei* groups. RBC concentrations were lowest in the *P. berghei* mice group compared to the rest of the mice followed by the CpG/*P. berghei* group. RBC levels were significantly higher in the CpG/IL-18/*P. berghei* and CpG/IL-12/*P. berghei* groups (Table 1).

Physiologic Biomolecules' Concentrations

Adiponectin Levels

The concentrations of the adiponectin molecule ranged from 3.600 ng/ml (in the IL-18/ *P. berghei* mice group) to 12.24 ng/ml (in the CpG/IL-18/ *P. berghei* group). Significantly ($P < 0.0001$; $F (7, 336) = 3136$) high level concentrations were found in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-12/ *P. berghei,* CpG ODN groups; 12.24 ng/ml, 9.743 ng/ml, 10.49 ng/ml, and 10.36 ng/ml. These four groups together had a mean adiponectin level of 10.708 ng/ml. The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 3136$) adiponectin concentrations than the rest of the groups in these investigations. This group had a mean adiponectin concentration of 12.24 ng/ml. The IL-12/*P. berghei* group generated significantly higher concentrations of adiponectin than the rest of the groups (except the CpG/IL-18/ *P. berghei* group) and its mean levels were at 10.49 ng/ml. It was followed closely by the CpG ODN group which had 10.36 ng/ml mean adiponectin concentration. Only the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-12/ *P. berghei* and CpG ODN groups were found to have adiponectin levels of more than 9.500 ng/ml.

Lower levels of adiponectin were detected in the IL-18/ *P. berghei,* CpG/ *P. berghei, P. berghei* and uninfected mice groups; 3.600 ng/ml, 4.814 ng/ml, 3.714 ng/ml and 4.271 ng/ml and these four groups all together had a mean adiponectin amount of 4.099 ng/ml. The 3.600 ng/ml level in the IL-18/ *P. berghei* group was the lowest detected concentration. The adiponectin levels in the CpG/IL-18/ *P. berghei* group were about 3½ times higher than levels in the IL-18/ *P. berghei* group (lowest detected) and 3 times higher than the lowest concentration groups as provided above. In the CpG/IL-12/ *P. berghei* group adiponectin levels were one-fold lower than in the IL-12/ *P. berghei* group and 2 times higher than the mean levels of the lower concentration

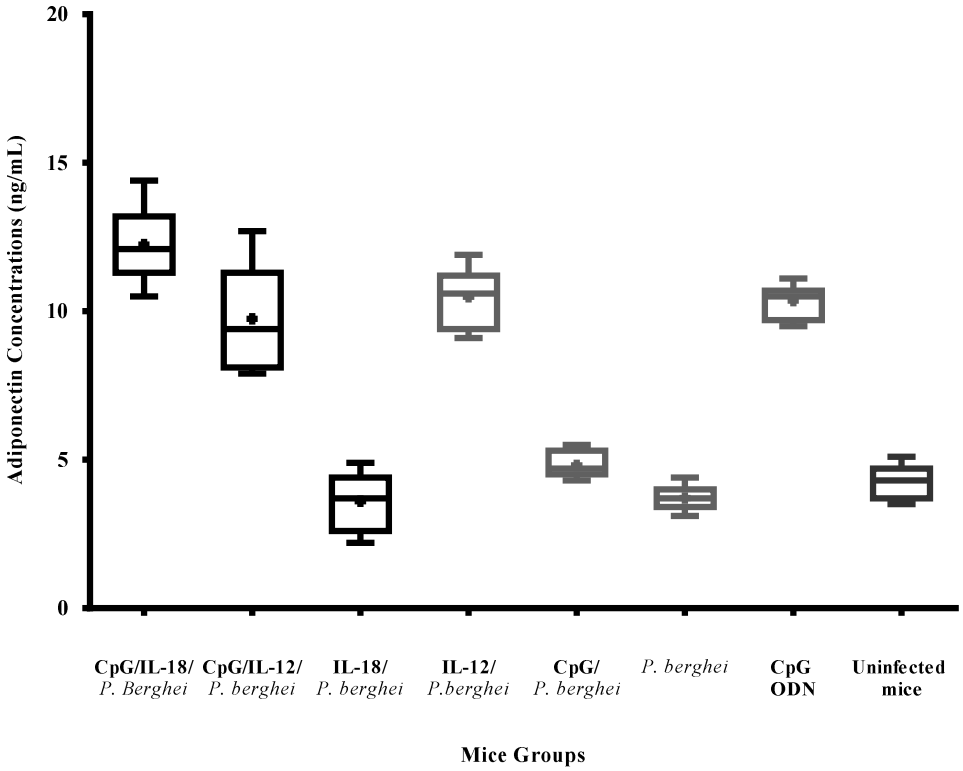

Figure 1. Adiponectin levels

groups (Figure 1).

The CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 3136$) adiponectin concentrations than the rest of the groups in these investigations. The adiponectin levels in the CpG/IL-18/ *P. berghei* group were about 3½ times higher than levels in the IL-18/ *P. berghei* group (lowest detected amount).

Angiogenin Levels

Quantified angiogenin concentrations ranged from 51.50 ng/ml (in the CpG/IL-12/ *P. berghei* mice group) to 715.00 ng/ml (in the *P. berghei* group). The *P. berghei* group produced significantly higher ($P < 0.0001$; $F(7, 336) = 9980$) angiogenin concentrations than all other groups in the study. This group had a mean angiogenin concentration of 715.00 ng/ml. This group had 2½ times higher angiogenin levels than the second highest group, the CpG/ *P. berghei* group, which had a concentration of 276.7 ng/ml.

Intermediate levels of angiogenin were detected in the CpG/ *P. berghei,* CpG ODN, and in the uninfected groups of mice; 276.7 ng/ml, 190.3 ng/ml and 166.9 ng/ml respectively, giving a mean value of 211.3 ng/ml. The lowest levels of angiogenin were measured in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei* and IL-12/ *P. berghei* mice groups; 64.20 ng/ml, 51.50 ng/ml, 94.03 ng/ml and 84.21 ng/ml respectively and these 4 groups had a mean angiogenin amount of 73.485 ng/ml. The angiogenin levels in the *P. berghei* group (the highest in the study) were about 11 times higher than levels in the CpG/IL-18/ *P. berghei* group, 13 times higher than in the CpG/IL-12/ *P. berghei* group,3-fold higher than in the intermediate concentration groups and approximately 10 times higher than the mean angiogenin level in the 4 lowest concentration groups above.

The mean angiogenin levels (276.7 ng/ml) in the CpG/ *P. berghei* group, second highest in the study, were about 4 times higher than levels in the CpG/IL-18/ *P. berghei* group, 5 times higher than in the CpG/IL-12/ *P. berghei* group, about 1-fold higher than the intermediate concentration groups and about 4 times higher than the mean angiogenin level in the 4 lowest concentration groups. The mean angiogenin level in the CpG/IL-18/ *P. berghei* was exceeded 1.5-fold by the mean level in the IL-18/ *P. berghei* group, whereas the mean level in the CpG/IL-12/ *P. berghei* group were 1.6-fold lower than in the IL-12/ *P. berghei* group (Figure 2).

The *P. berghei* group generated significantly higher

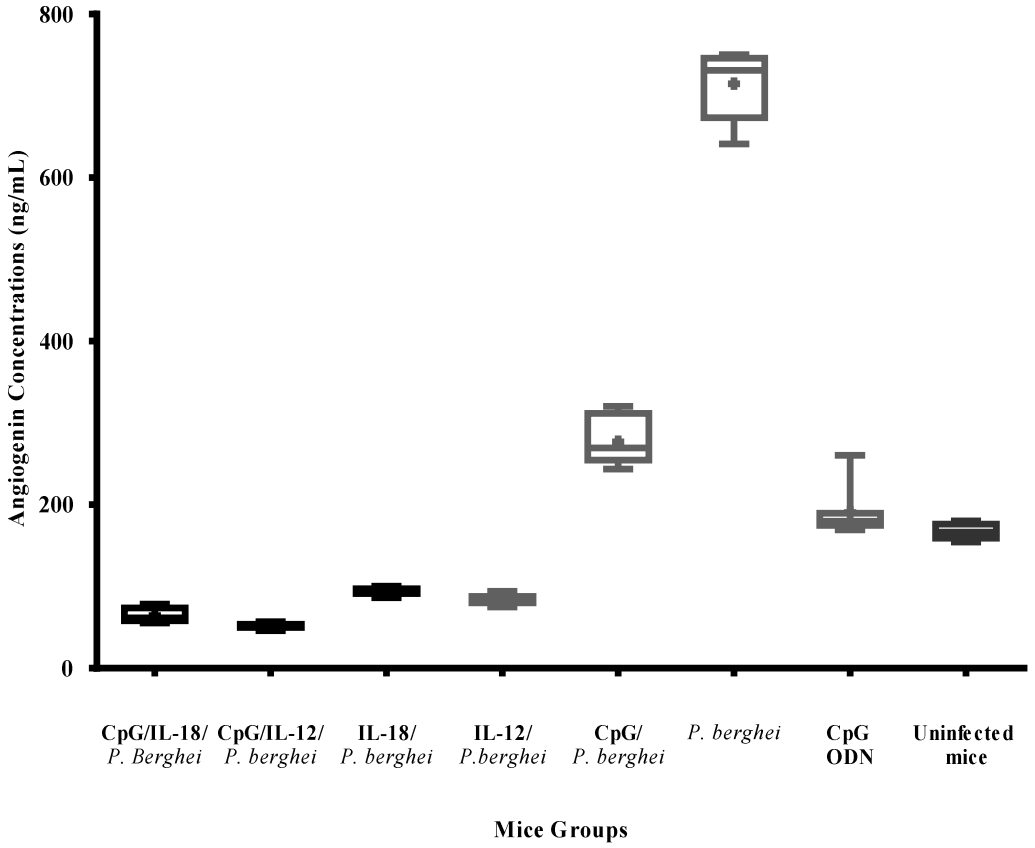

Figure 2. Angiogenin levels

(P < 0.0001; F (7, 336) = 9980) angiogenin concentrations than all other groups.

Angiopoietin 1 Levels

Angiopoietin 1 was detected in all investigated mice groups and overall its concentrations spanned from 1.769 ng/ml (in the *P. berghei* mice group) to 17.57 ng/ml (in the CpG/IL-12/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were the two significantly (P < 0.0001; F (7, 336) = 11862) high level concentration groups and they had a mean angiopoietin 1 level of 16.655 ng/ml. The CpG/IL-12/ *P. berghei* group had significantly higher (P < 0.0001; F (7, 336) = 11862) angiopoietin 1 concentrations than the rest of the groups in these investigations. This group had a mean angiopoietin 1 concentration of 17.57 ng/ml. The CpG/IL-18/*P. berghei* group produced significantly higher concentrations of angiopoietin 1 than the rest of the groups except the CpG/IL-18/ *P. berghei* group and its mean level was at 15.74 ng/ml. Only the CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were recorded with angiopoietin 1 levels of above15.5 ng/ml.

Intermediate levels of angiopoietin 1 were detected in the IL-18/ *P. berghei,* IL-12/ *P. berghei,* CpG/ *P. berghei*, CpG ODN and uninfected groups; 9.400 ng/ml, 8.343 ng/ml, 7.486 ng/ml, 6.143 ng/ml and 6.457 ng/ml respectively and these 5 groups had a mean angiopoietin 1 level of 7.566 ng/ml with values ranging from 6.143 ng/ml to 9.400 ng/ml. The lowest mean level of angiopoietin 1 was found in the *P. berghei* group; 1.769 ng/ml. The angiopoietin 1 levels in the CpG/IL-18/ *P. berghei* group were about one 1½ times higher than levels in the IL-18/ *P. berghei* group, 2 times higher than the intermediate concentration groups collectively (described above) and 9 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group angiopoietin 1 levels were 2 times higher than in the IL-12/ *P. berghei* group, more than 2 times higher than the mean levels of the intermediate concentration groups and 10 times higher than the two lowest concentration groups (Figure 3).

The CpG/IL-12/ *P. berghei* group had significantly higher (P < 0.0001; F (7, 336) = 11862) angiopoietin 1

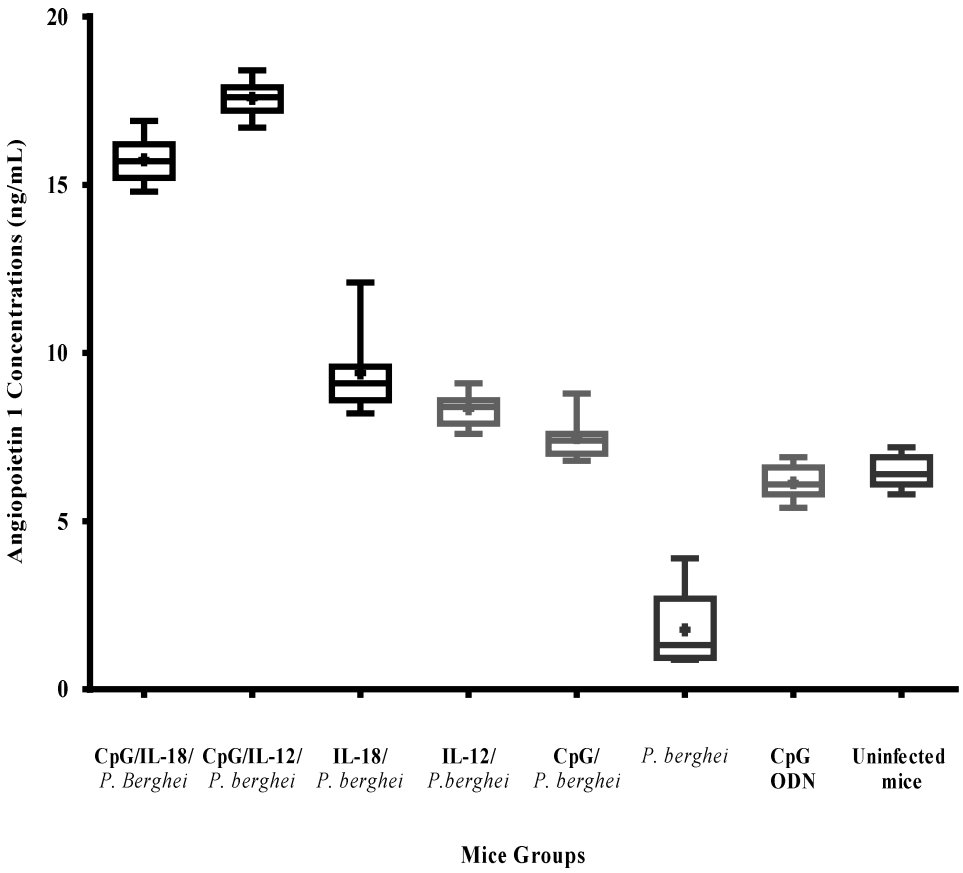

Figure 3. Angiopoietin-1 levels

concentrations than the rest of the groups in these investigations.

Angiopoietin 2 Levels

Angiopoietin 2 concentrations ranged from16.31 ng/ml (in the *P. berghei* mice group) to 1.326 ng/ml (in the IL-18/ *P. berghei* group). The outcomes in angiopoietin 2 levels were polar in nature with one pole having two highest concentration groups while the other pole had 6 low concentration groups. Significantly ($P < 0.0001$; $F (7, 336) = 23989$) high level angiopoietin 2 concentrations were found in the CpG/ *P. berghei* and *P. berghei* groups; 14.56 ng/ml and 16.31 ng/ml. These two groups together had a mean angiopoietin 2 level of 15.435 ng/ml. With a 16.31 ng/ml angiopoietin 2 mean level, the *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 3136$) angiopoietin 2 concentrations than the rest of the groups in these investigations. The CpG/*P. berghei* group generated significantly higher concentrations of angiopoietin 2 than the rest of the groups (except the *P. berghei* group) and its mean levels were at 14.56 ng/ml. Only the CpG/ *P. berghei* and *P. berghei* groups were found to have angiopoietin 2 levels of more than 14.500 ng/ml.

Lowest levels of angiopoietin 2 were detected in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* IL-12/ *P. berghei,* CpG ODN and uninfected mice groups; 1.644 ng/ml, 3.743 ng/ml, 1.326 ng/ml, 3.329 ng/ml, 1.386 ng/ml and 1.274 ng/ml respectively and all these six groups all together had a mean angiopoietin 2 amount of 1.905 ng/ml. The 1.274 ng/ml level in the uninfected group was the lowest detected concentration. The angiopoietin 2 levels in the *P. berghei* group were about 10 times higher than levels in the CpG/IL-18/ *P. berghei* group, 4 times higher than in the CpG/IL-12/ *P. berghei*and 9 times higher than the lowest concentration groups as mentioned above. The angiopoietin 2 levels in the CpG/*P. berghei* group were about 9 times higher than levels in the IL-18/ *P. berghei* group, about 4 times higher than in the CpG/IL-12/ *P. berghei* and 8 times higher than the lowest concentration groups as given above. In the CpG/IL-18/ *P. berghei* group angiopoietin 2 levels were one-fold lower than in the IL-18/ *P. berghei* group and 0.5-fold higher than the

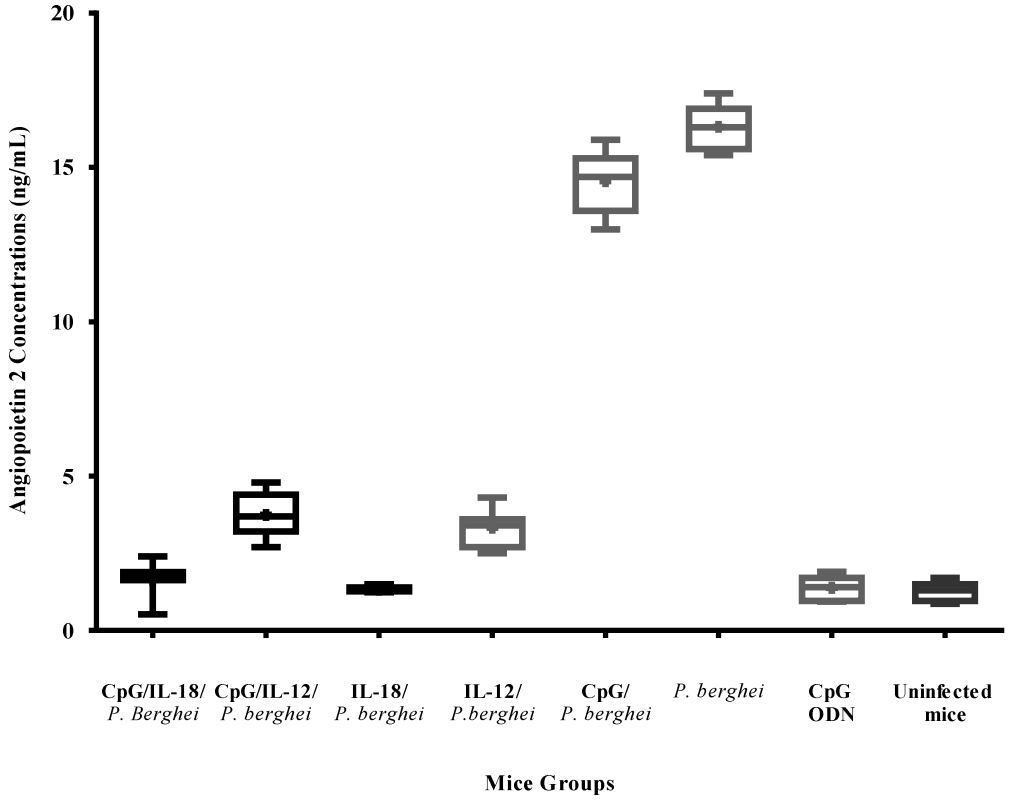

Figure 4. Angiopoietin 2 levels

mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group angiopoietin 2 levels were one-fold lower than in the IL-12/ *P. berghei* group and approximately 2 times higher than the mean levels of the lowest concentration groups (Figure 4).

The *P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 3136$) mean angiopoietin 2 concentrations, 16.31 ng/ml, than the rest of the groups in these investigations.

NRP-1 Levels

The neuropilin-1 (NRP-1) molecule concentrations ranged from 16.31 ng/ml (in the *P. berghei* mice group) to 1.326 ng/ml (in the IL-18/ *P. berghei* group). Polar outcomes in NRP-1levels were noted with one pole having three highest concentration groups while the other pole had 5 low concentration groups. Significantly ($P < 0.0001$; $F (7, 336) = 23989$) high level NRP-1 concentrations were found in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* and IL-12/ *P. berghei* groups; 12.66 ng/ml, 14.30 ng/ml, and 14.17 ng/ml respectively. These three groups together had a mean NRP-1 level of 13.71 ng/ml. With a 14.17 ng/ml NRP-1 mean level, the IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 99045$) NRP-1 concentrations than the rest of the groups in these investigations. The CpG/IL-12/*P. berghei* group generated significantly higher concentrations of NRP-1 than the rest of the groups (except theIL-12/*P. berghei* group) and its mean levels were at 14.30 ng/ml. Only the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* and IL-12/*P. berghei* groups were were found to have NRP-1 levels of more than 12.500 ng/ml.

Lowest levels of NRP-1 were detected in the IL-18/ *P. berghei,* CpG/ *P. berghei,P. berghei,* IL-12/ , CpG ODN and uninfected mice groups; 3.086 ng/ml, 2.214 ng/ml, 2.003 ng/ml, 3.086 ng/ml, 4.143 ng/ml respectively and all these five groups all together had a mean NRP-1 amount of 2.906 ng/ml. The 2.003 ng/ml level in the *P. berghei* group was the lowest detected concentration. The NRP-1 levels in the IL-12/*P. berghei* group were about 0.5 times higher than levels in the CpG/IL-18/ *P. berghei* group, 1-fold higher than in the CpG/IL-12/ *P. berghei* and about 5 times higher than the lowest concentration groups above. In the CpG/IL-18/ *P. berghei* group NRP-1 levels were 4-fold higher than in the IL-18/ *P. berghei* group and also 4-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group NRP-1 levels were one-fold higher than in the IL-12/ *P. berghei* group and

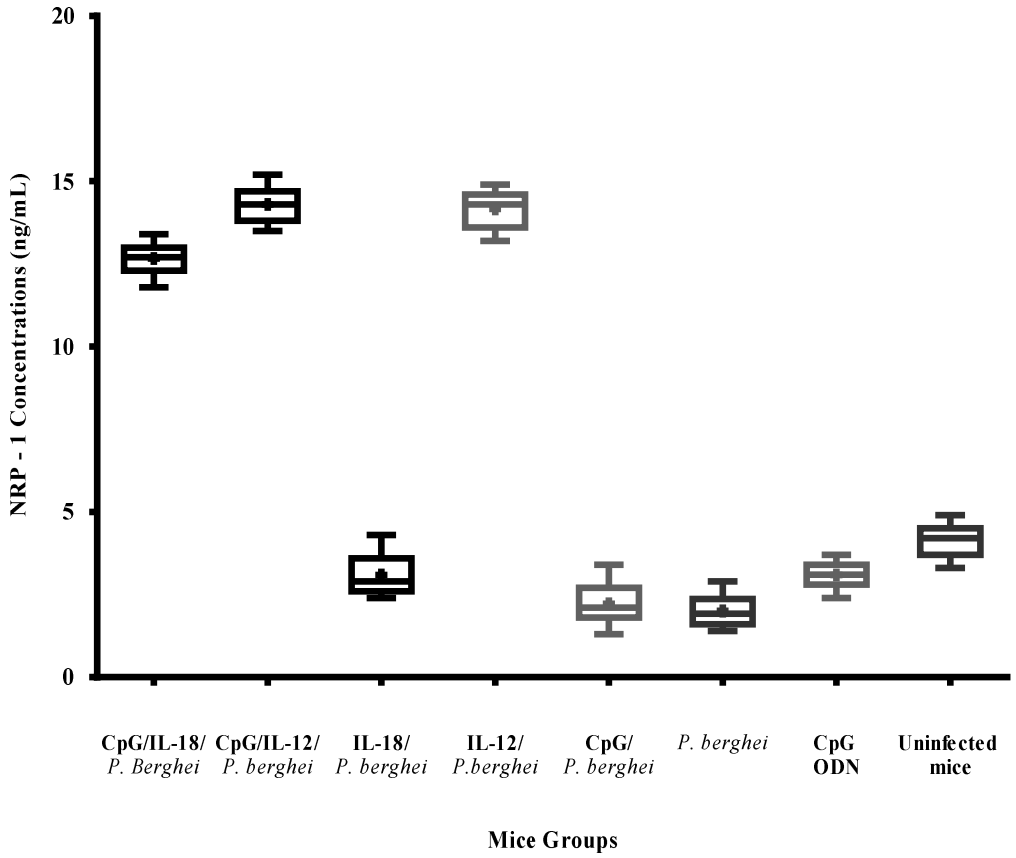

Figure 5. NRP-1 levels

approximately 5 times higher than the mean levels of the lowest concentration groups (Figure 5).

With a 14.17 ng/ml NRP-1 mean level, the IL-12/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 99045$) NRP-1concentrations than the rest of the groups in these investigations.

COX -2 Levels

Levels of COX -2 detected in these experiments ranged from 26.41 ng/ml (in the *P. berghei* mice group) to 139.6 ng/ml (in the CpG/IL-18/ *P. berghei* group). The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* were found to have significantly ($P < 0.0001$; $F(7, 336) = 16655$) higher concentrations of COX -2 and taken together, both groups had a mean COX -2level of 134.75 ng/ml. With a level of 139.6 ng/ml, the CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 16655$) COX -2 concentrations than the rest of the groups in these investigations. The CpG/IL-12/*P. berghei* group produced significantly higher concentrations of COX -2 than the rest of the groups except group 1 and its mean level was at 129.9 ng/ml. The CpG/IL-18/ *P. berghei* and CpG/IL-12/ *P. berghei* groups were the only groups recorded with COX -2 levels higher than 129.00 ng/ml.

Mid-levels of COX -2 were detected in the IL-12/ *P. berghei* group which averaged at 100.4 ng/ml. Low-levels of COX -2 was found in the IL-18/ *P. berghei,* CpG/*P. berghei, P. berghei,* CpG ODN and uninfected mice groups; 39.04 ng/ml, 39.56 ng/ml, 27.41 ng/ml, 47.67 ng/ml and 57.60 ng/ml, with the uninfected group having a slightly higher amount than the other low-level groups. Low-level groups together generated an average COX -2 concentration of 42.256 ng/ml. The COX -2 levels in the CpG/IL-18/ *P. berghei* group were about 4 times higher than levels in the IL-18/ *P. berghei* group, one-fold higher than the mid-level concentration group and 3 times higher than the two lowest concentration groups detailed above. In the CpG/IL-12/ *P. berghei* group COX -2 levels were 1.2-fold higher than in the IL-12/ *P. berghei* group (this was also the COX -2 mid-level group) and 3-fold higher than the two lowest concentration groups (Figure 6).

At a mean level of 139.6 ng/ml, the CpG/IL-18/ *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 16655$) COX -2concentration than the rest of the groups while the *P. berghei* group had the lowest mean concentration, 27.41 ng/ml.

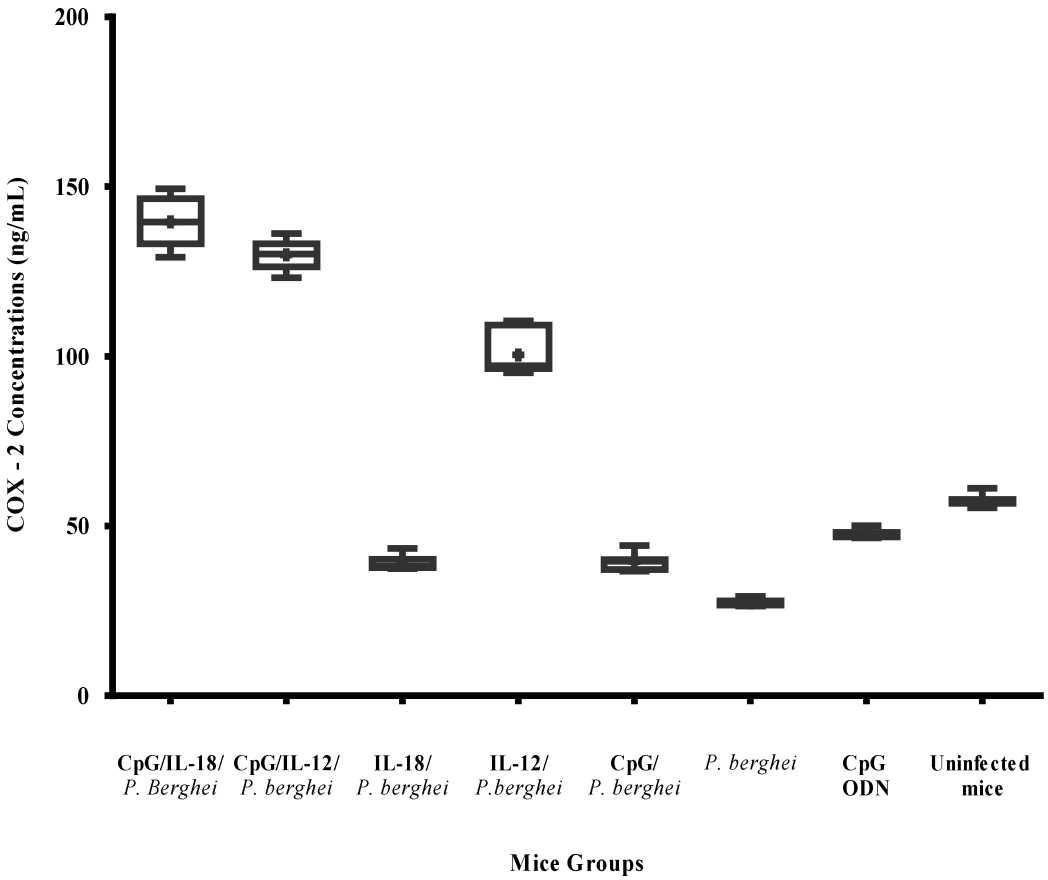

Figure 6. COX -2 levels

MMP - 8 Levels

Concentrations of MMP – 8 ranged from 26.31 ng/ml (in theIL-12/*P. berghei* mice group) to 298.0 ng/ml (in the CpG/*P. berghei* group). Findings indicated MMP – 8 levels also had polar distribution with one pole having two highest concentration groups while the other pole had 6 low concentration groups. Significantly ($P < 0.0001$; $F (7, 336) = 25568$) high level MMP – 8 concentrations were found in the CpG/*P. berghei* and *P. berghei* groups; 298.0 ng/ml and 296.9 ng/ml. These two groups together had a mean MMP – 8 level of 297.45 ng/ml. With a 298.0 ng/ml MMP – 8 mean level, the CpG/*P. berghei* group had significantly higher ($P < 0.0001$; $F (7, 336) = 25568$) MMP – 8 concentrations than the rest of the investigated groups. The *P. berghei* group generated significantly higher concentrations of MMP – 8 than the rest of the groups (except the *P. berghei* group) and its mean levels were at 296.90 ng/ml. Only the CpG/*P. berghei* and *P. berghei* groups yielded MMP – 8 levels of greater than 296.0 ng/ml.

Lowest levels of MMP – 8 were detected in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* IL-12/ *P. berghei,* CpG ODN and uninfected mice groups; 64.07 ng/ml, 46.29 ng/ml, 45.31 ng/ml, 26.31 ng/ml, 58.97 ng/ml and 56.89 ng/ml respectively and all these six groups all together had a mean MMP – 8 amount of 49.64 ng/ml. The 26.31 ng/ml level in the IL-18/ *P. berghei* group was the lowest detected concentration. The MMP – 8 levels in the CpG/*P. berghei* group were about 4.7 times higher than levels in the IL-18/ *P. berghei* group, 6.4 times higher than in the CpG/IL-12/ *P. berghei* and 6-fold higher than the lowest concentration groups as given above. The MMP – 8 levels in the *P. berghei* group were about 4.5 times higher than levels in the CpG/IL-18/ *P. berghei* group, 6.4 times higher than in the CpG/IL-12/ *P. berghei* and 9 times higher than the lowest concentration groups as mentioned above. In the CpG/IL-18/ *P. berghei* group MMP – 8 levels were 1.4-fold higher than in the IL-18/ *P. berghei* group and 1.2-fold higher than the mean levels of the lowest concentration groups. In the CpG/IL-12/ *P. berghei* group MMP – 8 levels were 1.7-fold higher than in the IL-12/ *P. berghei* group and approximately 0.9-fold higher than the mean levels of the lowest concentration groups (Figure 7).

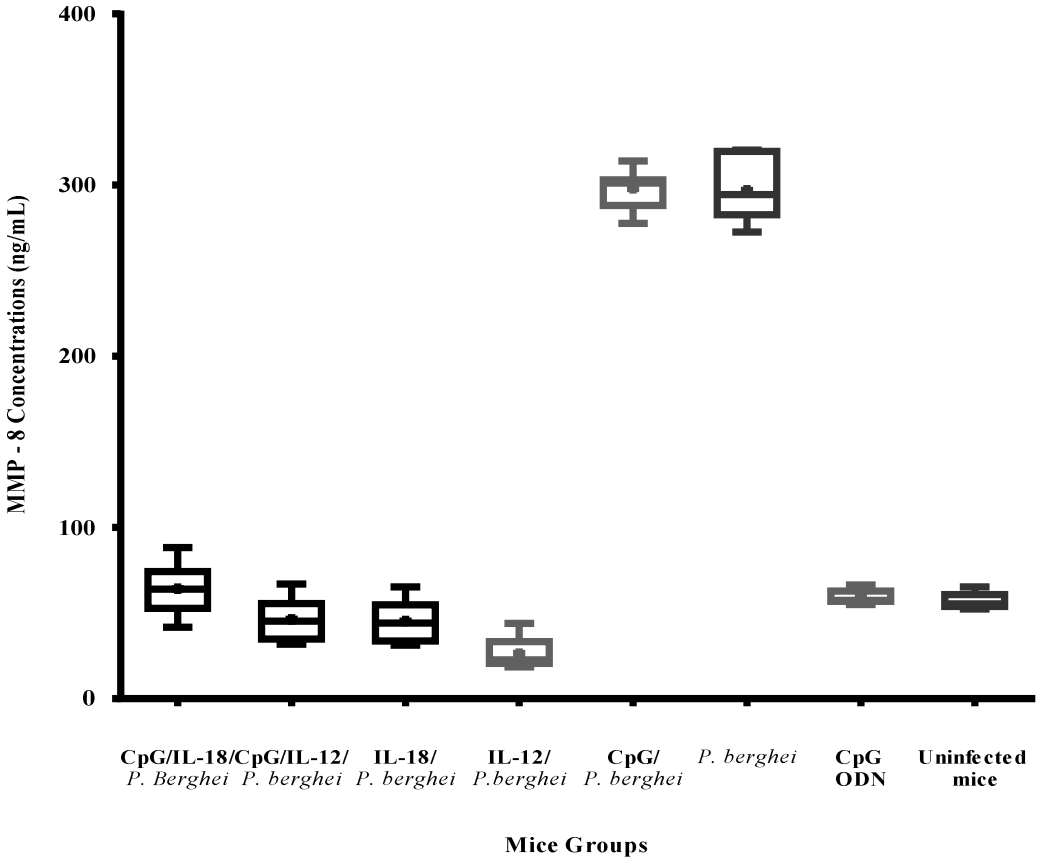

Figure 7. MMP - 8 levels

With a 298.0 ng/ml MMP – 8 mean level, the CpG/*P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 25568$) MMP - 8concentrations than the rest of the investigated groups. The 26.31 ng/ml level in the IL-18/ *P. berghei* group was the lowest detected concentration.

MMP - 9 Levels

Levels of MMP – 9 detected in these experiments ranged from 198.9 ng/ml (in the IL-12/*P. berghei* mice group) to 591.0 ng/ml (in the *P. berghei* group). The *P. berghei* and CpG/ *P. berghei* groups were found to have significantly ($P < 0.0001$; $F(7, 336) = 2726$) higher concentrations of MMP – 9 than the other mice groups and taken together, both groups had a mean MMP – 9 level of 496.95 ng/ml. With a level of 591.00 ng/ml, the *P. berghei* group had significantly higher ($P < 0.0001$; $F(7, 336) = 2726$) MMP – 9 concentrations than the rest of the groups in these investigations. The CpG/*P. berghei* group produced significantly higher concentrations of MMP – 9 than the rest of the groups except the *P. berghei* group and its mean level was at 402.9 ng/ml. The *P. berghei* and CpG/ *P. berghei* groups were the only groups recorded with MMP – 9 levels higher than 400.00 ng/ml (Figure 8).

Medium levels of MMP – 9 were detected in the CpG/IL-18/ *P. berghei,* CpG/IL-12/ *P. berghei,* IL-18/ *P. berghei,* CpG ODN and uninfectedgroup; 227.2 ng/ml, 215.6 ng/ml, 277.0 ng/ml, 261.9 ng/ml and 265.7 ng/ml respectively and these averaged at 207.9 ng/ml. Low-levels of MMP – 9 were found in the IL-12/ *P. berghei* group; 198.9 ng/ml. The MMP – 9 levels in the *P. berghei* group were about 2.6 times higher than levels in the CpG/IL-18/ *P. berghei* group, 2.7 times higher than in the CpG/IL-12/ *P. berghei,* 3-fold higher than the medium level average concentration and also 3-fold higher than the lowest concentration group (the IL-12/ *P. berghei* group). The MMP – 9 levels in the CpG/*P. berghei* group were about 1.7 times higher than levels in the CpG/IL-18/ *P. berghei* group, 1.8 times higher than in the CpG/IL-12/ *P. berghei*, 2-fold higher than the medium concentration groups and also 2-fold higher than the average concentration of the lowest concentration group (the IL-12/ *P. berghei* group).

In the CpG/IL-18/ *P. berghei* group MMP – 9 levels were 1.2-fold lower than in the IL-18/ *P. berghei* group, 1.09-fold higher than the mean of the medium level groups and 1.1-fold higher than the mean levels of the

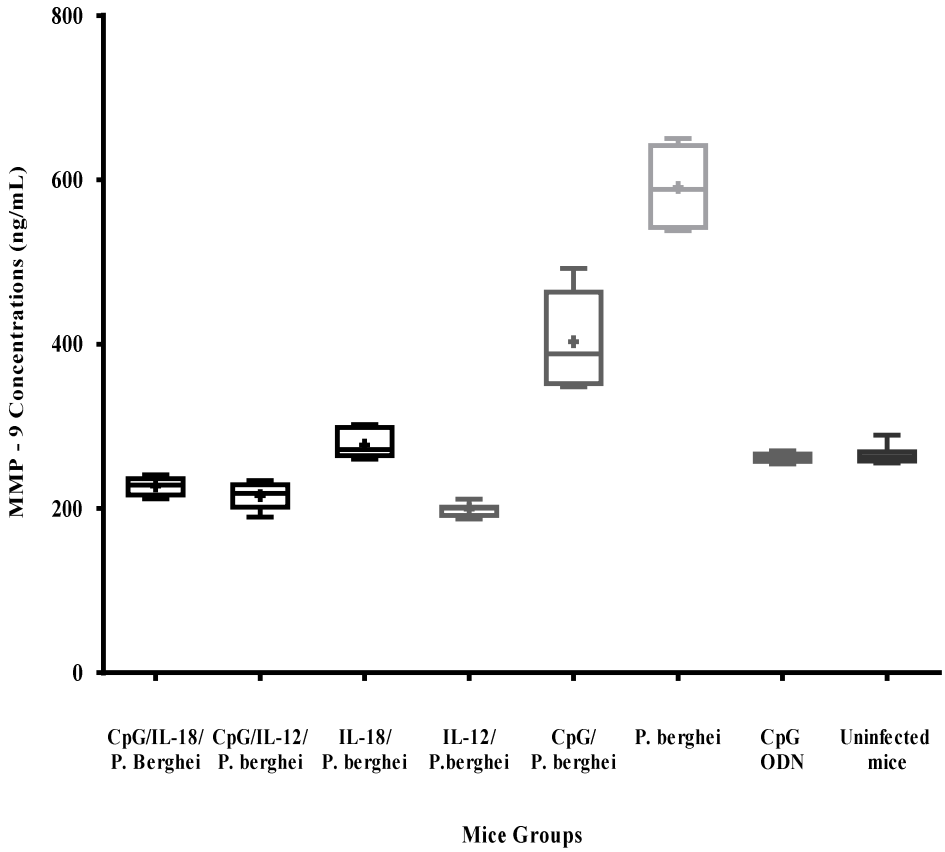

Figure 8. MMP - 9 levels

lowest concentration group. In the CpG/IL-12/ *P. berghei* group MMP – 9 levels were 1.08-fold higher than in the IL-12/ *P. berghei* group (this was also the lowest recorded concentration), 1.03-fold higher than the average level of the medium level groups.

The *P. berghei* and CpG/ *P. berghei* were found to have significantly ($P < 0.0001$; $F(7, 336) = 2726$) higher concentrations of MMP – 9 than the other mice group.

DISCUSSION

Generally, the physiologic biomolecules, adiponectin, angiopoietin 1, NRP-1 and COX-2 were demonstrably increased in the cytokine-CpG ODN combination groups, while angiogenin, angiopoietin 2, MMP-8 and MMP-9 were downregulated. Adiponectin, alternatively called APN, GBP-28, apM1, AdipoQ and Acrp30, is a protein that is involved in regulating glucose levels as well as fatty acid breakdown was noted to be more highly expressed in the cytokine-CpG co-inoculation groups and also in two control groups; the IL-12/ *P. berghei* and CpG ODN groups compared to the rest of the control groups. In studies carried out on *P. falciparum* malaria, it was found that although patients with cerebral malaria had greater amounts of adiponectin than those with uncomplicated malaria, patients with *P. falciparum* infection who had higher glucose production also had higher adiponectin levels and given adiponectin's ability to downregulate glucose generation, activation of adiponectin release during infection could be a mechanism of restraining the glucose production stimulating properties of hormones and cytokines acting during infection (Blumer *et al.,* 2005). The secretion of glucose counter-regulatory hormones and cytokines can complicate infections by triggering increases in glucose production. The insulin-sensitising, fat-derived adiponectin hormone such as the one detected in the cytokine-CpG coinoculation groups could play a regulatory role that counteracts the extent of parasite-activated glucose production (Blumer *et al.,* 2005).

Higher levels of adiponectin in the IL-12/ *P. berghei* and CpG ODN groups than in the rest of the controls implicate IL-12 and CpG ODN in a triggering role that leads to a higher presence of adiponectin in respective recipient murine hosts. However, adiponectin has also previously been noted to drive anti-inflammatory reactions, significantly reducing IFN-γ production in

human macrophages, and stimulating increases in the anti-inflammatory mediators IL-10 and IL-1RA in primary human monocytes, monocyte-derived macrophages, and dendritic cells (Wolf *et al.,* 2004). Experiments done in APN knockout mice revealed that APN deficiency protects mice from ischemia/reperfusion injury (IRI), suggesting a new role for APN in acute kidney injury and justifying further investigation for new mechanistic and therapeutic strategies in the fields of obesity and kidney diseases (Jieun *et al.,* 2013). APN is assembled into multimeric complexes within the endoplasmic reticulum and golgi compartments and after release it mostly circulates as a high-molecular weight complex, with serum levels ranging between 5–20 µg/ml in both humans and rodents. Besides adipocytes, it is also expressed by skeletal muscle cells, cardiac myocytes, endothelial cells, epithelial cells, and bone-forming cells (Jieun *et al.,* 2013). The lower levels of adiponectin recorded in *P. berghei*-infected mice treated with IL-18 could be expected, according to a previous study (Chendrasekar *et al.,* 2007) in which it was discovered that IL-18 suppresses adiponectin expression in $_3T_3$-L1 Adipocytes via a novel signal transduction pathway involving ERK1/2-dependent NFATc4 phosphorylation. Further, IL-18 was found to stimulate ERK1/2 phosphorylation and enzyme activity and pretreatment with the MEK inhibitor U0126, ERK1/2 inhibitor PD98059, or small interference RNA targeted to ERK1/2 attenuated ERK1/2 activation and NFATc4 phosphorylation and suppression of ERK1/2 or NFATc4 knockdown corrected IL-18-mediated adiponectin suppression (Chandrasekar *et al.,* 2007). However, in the current findings, despite IL-18 being accompanied by lower levels of adiponectin in the malaria-infected murine host, addition of CpG ODN into the setup triggered an increase in adiponectin concentrations, suggesting CpG ODN's ability to cause increased adiponectin concentrations. In another study it was reported that circulating IL-18 and adiponectin concentrations can be modulated by familiar foodstuffs in humans and that meal modulation of cytokines involved in atherogenesis could yield new ways for ameliorating atherogenetic inflammatory activity in diabetic patients (Esposito *et al.,* 2003). In another study leptin and adiponectin adipokines, but not the IL18 adipokine, were found to be related with insulin resistance in treated HIV-1-infected patients with lipodystrophy (Veloso *et al.,* 2012). With evidence from intervention studies with aipimox and insulin, it was uncovered that lipolysis reduces adiponectin and IL-18 adipokine levels (Lindegaard *et al.,* 2013).

Levels of the hormone are inversely correlated with body fat percentage in adults. However, a meta analysis was not able to confirm this association in healthy adults. The association in infants and young children is less clear. Circulating adiponectin concentrations increase during caloric restriction in animals and humans, such as in patients with anorexia nervosa. Adipose tissue within bone marrow, which increases during caloric restriction, contributes to elevated circulating adiponectin in this context. Transgenic mice with increased adiponectin show impaired adipocyte differentiation and increased energy expenditure associated with protein uncoupling. The hormone plays a role in the suppression of the metabolic derangements that may result in type 2 diabetes, obesity, atherosclerosis, non-alcoholic fatty liver disease (NAFLD) and an independent risk factor for metabolic syndrome. Adiponectin in combination with leptin has been shown to completely reverse insulin resistance in mice (Yamauchi *et al.,* 2001).

Quantities of the angiogenin (Ang) protein, also known as ribonuclease 5, a potent stimulator of new blood vessels through the process of angiogenesis, were significantly lower in the cytokine-CpG co-inoculated groups compared to the rest of the groups. Although at the time of this writing there was no other report on the role of angiogenin in malaria infection, Ang, a hydrolyzer of cellular RNA (causing protein synthesis modulation), and an actor on DNA causing a promoter-like increase in the expression of rRNA (Gao *et al.,* 2008), has been associated with cancer and neurological disease through angiogenesis and through activation of gene expression that suppresses apoptosis (Tello-Montoliu *et al.,* 2006).. Thus a higher presence of angiogenin in the excessively parasitized *P. berghei* control mice group may not come as a surprise, also because of the implicated roles of Ang in other disease scenariaos like multiple myeloma (Alexandrakis *et al.,* 2004) and neurodegenerative diseases (Gao *et al.,* 2008). Given the outcome with the *P. berghei*-infceted control group of mice, the lower Ang levels in the cytokine-CpG co-inoculated groups could be related to the control of parasitaemia in these two mice groups.

Angiogenin is a key protein implicated in angiogenesis in normal and tumor growth. Angiogenin interacts with endothelial and smooth muscle cells resulting in cell migration, invasion, proliferation and formation of tubular structures (Gao *et al.,* 2008). Ang binds to actin of both smooth muscle and endothelial cells to form complexes that activate proteolytic cascades which upregulate the production of proteases and plasmin that degrade the laminin and fibronectin layers of the basement membrane (Tello-Montoliu *et al.,* 2006). Degradation of the basement membrane and extracellular matrix allows the endothelial cells to penetrate and migrate into the perivascular tissue (Gao *et al.,* 2008). Signal transduction pathways activated by Ang interactions at the cellular membrane of endothelial cells produce extracellular signal-related kinase1/2 (ERK1/2) and protein kinase B/Akt (Gao *et al.,* 2008). Activation of these proteins leads to invasion of the basement membrane and cell proliferation associated with further angiogenesis. The most important step in the

angiogenesis process is the translocation of Ang to the cell nucleus. Once Ang has been translocated to the nucleus, it enhances rRNA transcription by binding to the CT-rich (CTCTCTCTCTCTCTCCCTC) angiogenin binding element (ABE) within the upstream intergenic region of rDNA, which subsequently activates other angiogenic factors that induce angiogenesis (Fu *et al.,* 2008).

Lower parasitaemia and both independent cytokine and cytokine-CpG combinations significantly influenced lower Ang concentrations while the absence of therapeutic cytokines (IL-12 and IL-18 in this case) or CpG provided conditions that permitted increased parasitaemia, as noted earlier and higher levels of the Ang protein. Cytokine influences on Ang presence have been reported elsewhere (Verselis *et al.,* 1999). In this previous study, the IL-6 inducer of acute-phase proteins, sinitiated the synthesis and secretion of angiogenin protein in human HepG2 cells within 24 hr following treatment, and this outcome was promoted by dexamethasone. Without half-life altering effects, IL-65 also increased the amount of angiogenin mRNA. The increase could be reduced by cycloheximide, and peaked at 12 hr following activation and returned to basal levels by 48 hr. IL-1 alone slightly reduced the basal production of angiogenin protein and mRNA, but impeded the response to IL-6 in the absence or presence of dexamethasone (Verselis *et al.,* 1999).

Ang has a prominent role in the pathology of cancer due to its functions in angiogenesis and cell survival. Since Ang possesses angiogenic activity, it makes Ang a possible candidate in therapeutic treatments of cancer. Studies of Ang and tumor relationships provide evidence for a connection between the two. The translocation of Ang to the nucleus causes an upregulation of transcriptional rRNA, while knockdown strains of Ang cause downregulation (Gao *et al.,* 2008). The presence of Ang inhibitors that block translocation resulted in a decrease of tumor growth and overall angiogenesis (Li *et al*., 2012). HeLa cells translocate Ang to the nucleus independent of cell density. In human umbilical vein endothelial cells (HUVEC), translocation of Ang to the nucleus stops after cells reach a specific density, while in HeLa cells translocation continued past that point (Tsuji *et al.,* 2005). Inhibition of Ang affects the ability of HeLa cells to proliferate, which proposes an effective target for possible therapies. Due to the ability of Ang to protect motoneurons (MNs), causal links between Ang mutations and Amyotrophic lateral sclerosis (ALS) are likely. The angiogenic factors associated with Ang may protect the central nervous system and MNs directly (Gao *et al.,* 2008). Experiments with wild type Ang found that it slows MN degeneration in mice that had developed ALS, providing evidence for further development of Ang protein therapy in ALS treatment (Li *et al*., 2012). Angiogenin expression in Parkinson's disease is dramatically decreased in the presence of alpha-synuclein (α-syn) aggregations. Exogenous angiogenin applied to dopamine-producing cells leads to the phosphorylation of PKB/AKT and the activation of this complex inhibits cleavage of caspase 3 and apoptosis when cells are exposed to a Parkinson's-like inducing substance (Steidinger *et al.,* 2010).

Angiopoietin 1 (ANGPT 1), a member of the angiopoietin family of vascular growth factors that play a role in embryonic and postnatal angiogenesis was higher in the cytokine-CpG co-inoculation groups than in the rest of the groups, thereby supporting previous evidence that increased levels of ANGPT 1 are associated with uncomplicated malaria (UM [Conroy *et al.,* 2009]). Conversely patients exhibiting complicated malaria, including those with severe (non-cerebral) malaria and cerebral malaria had lower levels of ANGPT 1; the current report has shown that ANGPT 1 levels were lowest in the *P. berghei*-infected control mice group. There was a reduced in ANGPT-1 levels in patients with SM (non-cerebral) versus CM and in participants with severe disease, ANGPT-2, but not ANGPT-1, levels correlated with cumulative organ injury scores; however, ANG-1 correlated with the presence of renal dysfunction and coma (Conroy *et al.,* 2009). The study found that whole blood ANG-1/2 levels are clinically informative biomarkers of malarial disease severity (Conroy *et al.,* 2009). Angiopoietin-1 and -2 levels have also been indicated to discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children (Lovegrove *et al.,* 2009). Mice groups with relatively higher parasitaemia, unlike in the recipients of cytokine-CpG co-administrations, generally had lower ANGPT 1 levels, agreeing with the widely held opinion that ANGPT 1/ANGPT 2 ratio is a valuable biomarker ratio in determining the severity of malaria outcomes. In previous research, a receiver operating characteristic curve analysis showed that ANGPT 1 and the ratio of ANGPT 1/ANGPT 2accurately discriminated CM patients from UM (Lovegrove *et al.,* 2009). Diagnostically, ANGPT 1 had a sensitivity and specificity of 100 % for distinguishing CM from UM and low ANGPT 1 levels at presentation predicted mortality levels in CM children (Lovegrove *et al.,* 2009).

Angiopoietin signaling pathways correspond with angiogenesis, the process by which new arteries and veins form from preexisting blood vessels. Angiogenesis proceeds through sprouting, endothelial cell migration, proliferation, and vessel destabilization and stabilization. They are responsible for assembling and disassembling the endothelial lining of blood vessels (Barton *et al.,* 2005; Alvez *et al.,* 2010). Angiopoietin cytokines are involved with controlling microvascular permeability, vasodilation, and vasoconstriction by signaling smooth muscle cells surrounding vessels (Scott *et al.,* 2010). There are now four identified angiopoietins: ANGPT1, ANGPT2, ANGPT4. In addition, there are a number of proteins that are closely related to angiopoietins

(ANGPTL1, ANGPTL2, ANGPTL3, ANGPTL4, ANGPTL5, ANGPTL6, ANGPTL7).

Angiopoietin-1 is critical for vessel maturation, adhesion, migration, and survival. Angiopoietin-2, on the other hand, promotes cell death and disrupts vascularization. Yet, when it is in conjunction with vascular endothelial growth factors, or VEGF, it can promote neo-vascularization (Fagiani and Christofori, 2013). In experiments done in the mouse model exhibited that the dysregulation of angiopoietins is associated with placental malaria and low birth weight (Silver *et al.,* 2010). That experiment showed that PM causes reduction of ANG-1, and increase of ANG-2, and an elevated ratio of ANG-2/ANG-1 in the placenta and the serum. *Plasmodium falciparum* infection was linked to lower ANG-1 levels a higher in ANGPT-2: ANGPT-1 ratio. Angiopoietin dysregulation was also connected to PM and LBW. Angiopoietin-2 and Angiopoietin-2/Angiopoietin-1 ratio was also identified as an indicator of potential severity of *Plasmodium vivax* malaria in patients with thrombocytopenia (Gomes *et al.,* 2014).

Deregulation of angiopoietin is common in blood-related diseases such as pulmonary hypertension, diabetes, sepsis, and malaria. This is demonstrated by an increased ratio of angiopoietin-2 and angiopoietin-1 in blood serum. For example, angiopoietin-2 is elevated in patients with angiosarcoma (Amo *et al.,* 2004). To be specific, angiopoietin levels provide an indication for sepsis. Research on angiopoietin-2 has shown that it is involved in the onset of septic shock. The combination of fever and high levels of angiopoietin-2 are correlated with a greater prospect of the development of septic shock. It has also been shown that imbalances between angiopoietin-1 and angiopoietin-2 signaling can act independently of each other. One angiopoietin factor can signal at high levels while the other angiopoieting factor remains at baseline level signaling (Alvez *et al.,* 2010). Angiopoietin-2 is produced and stored in Weibel-Palade bodies in endothelial cells and acts as a TEK tyrosine kinase antagonist. As a result, the promotion of endothelial activation, destabilization, and inflammation are promoted. Its role during angiogenesis depends on the presence of Vegf-a (Jeansson *et al.,* 2011). Serum levels of angiopoietin-2 expression are associated with the growth of multiple myeloma (Pappa *et al.,* 2013), angiogenesis, and overall survival in oral squamous cell carcinoma. Circulating angiopoietin-2 is a marker for early cardiovascular disease in children on chronic dialysis Kaposi's sarcoma-associated herpes virus induces rapid release of angiopoietin-2 from endothelial cells (Ye *et al.,* 2013).Research has shown angiopoietin signaling to be relevant in treating cancer as well. During tumor growth, pro-angiogenic molecules and anti-angiogenic molecules are off balance. Equilibrium is disrupted such that the number of pro-angiogenic molecules is increased. Angiopoietins have been known to be recruited as well as VEGFs and platelet-derived growth factors (PDGFs). This is relevant for clinical use relative to cancer treatments because the inhibition of angiogenesis can aid in suppressing tumor proliferation (Falcon *et al.,* 2009).

Levels of angiopoietin-2 (ANGPT 2) were significantly higher in the CpG/*P. berghei* and the *P. berghei* control groups compared to the rest of the mice groups, including the main experimental cytokine-CpG co-inoculation groups indicating that the cytokine-CpG therapeutic combination not only limits disease severity, but is also accompanied by increased levels of ANGPT 2 which has been widely associated with severe complicated malaria (Conroy *et al.,* 2009; Lovegrove *et al.,* 2009). Several studies have indicated that higher levels of ANGPT 2 coinciding with lower levels of ANGPT 1 that give a higher ANGPT 1/ANGPT 2 ratio are associated with complicated and severe malaria including increased CM and PM severity (Conroy *et al.,* 2009; Lovegrove *et al.,* 2009; Gomez *et al.,* 2014; Silver *et al.,* 2010). In the context of PM, ANGPT-2 levels in the placenta, and activates a strong increase in the ANGPT-2/ANGPT-1 ratio in the placenta and the serum. Severe *Plasmodium falciparum* infection PM and LBW have all been connected to higher in ANGPT-2:ANGPT-1 ratio (Gomes *et al.,* 2014). The current report gives a similar link in terms of ANGPT 1/ANGPT 2 ratio which was induced in the *P. berghei*-infected mice that were given cytokine-CpG ODN therapeutic co-inoculations. These mice had a significantly higher ANGPT 1/ANGPT 2 ratio compared to the heavily parasitised and severely symptomatic *P. berghei* control mice group in the, a scenario which has been shown to significantly support disease control and limitation of *Plasmodial* severity. Conversely, the CpG/*P. berghei* and the *P. berghei* control groups had a lower ANGPT 1/ANGPT 2 ratio, as a result of the relatively higher concentrations of ANGPT 2 and higher ANGPT 1 concentrations than ANGPT 2 that were detected in these two groups, and indeed this status coincided with highly severely outcomes, both in terms of parasitaemia and symptomatic manifestations.

Neuropilin-1 (NRP1), a membrane-bound co-receptor to a tyrosine kinase receptor for both vascular endothelial growth factor (VEGF; MIM 192240) and semaphorin family members was significantly increased in murine recipients of cytokine-CpG co-inoculations than in control groups in the study. Although up to the time of this publication there has been no direct indication of the role of NRP-1 in *Plasmodium* infection, NRP-1 has been implicated in versatile roles in angiogenesis, axon guidance, cell survival, migration, and invasion (Soker *et al.,* 1998). Neuropilins bind to class 3 semaphorins and vascular endothelial growth factor and are multifactorial non-tyrosine kinase receptors. Through interactions with class 3 semaphorins, NRP-1 and NRP-2 mediate axonal guidance in the developing nervous system and there is

upregulation of neuropilins in multiple tumour types with strong correlation existing with tumour progression and prognosis. In mediating tumour progression, neuropilins may directly impact on tumours or they may induce progression through their effects on angiogenesis (Lee, 2006). In the current experiments, lower NRP-1 concentrations were accompanied by higher *P. berghei* parasitaemia and increased severity of malaria while lower higher NRP-1 values accompanied lower parasitaemia and milder malaria, suggesting a disease limiting role of NRP-1 in the context of malaria. In immunity, NRP-1 is important in the formation of successful synapse between dendritic cells (DCs) and T cells, and it was identified as a biomarker that provides distinction between naturally occuring Treg cells originating in the thymus and peripheral adaptive Treg cells (Campos-Mora *et al.,* 2013).

The NRP-1 transmembrane glycoprotein has a multi-domain extracellular region, a single transmembrane helix, and a cytoplasmic domain and was first shown to be involved in the development of neurons. Through screening candidate molecules involved in retinotectal projection development, NRP-1, initially called A5, as an antigen preferentially expressed on superficial layers of the optic tectum of *Xenopus laevis* became identified (Campos-Mora *et al.,* 2013). These superficial layers are mainly made up of synapses, glial processes, dendrites and axonal ends of retinal neurons, or “neuropiles,” and hence the term “Neuropilin-1”. In mice, the olfactory, hippocampal, retinal, and sensory peripheral neurons express NRP-1; its expression levels depend on the development stage and establishment of neuronal circuits (Fujisawa *et al.,* 1995; Kawakami *et al.,* 1996). Signaling through Nrp1 expressed on neurons promotes neurite outgrowth *in vitro*, which can be inhibited using anti-Nrp1 antibodies (Campos-Mora *et al.,* 2013).

Cyclooxygenase (COX), also referred to as prostaglandin-endoperoxide synthase (PTGS), which is an enzyme (EC1.14.99.1) that is responsible for formation of prostanoids, including prostaglandins such as prostacyclin and thromboxane occurred at higher concentrations in the mice groups with cytokine-CpG ODN co-administration than in the controls agreeing with other data suggesting that the induction of COX-2 expression and prostaglandin synthesis has a protective effect against malarial conditions including CM (Ball *et al.,* 2006). In that experiment, COX-1 mRNA was induced in the brain in both CM and non-CM malaria models, but it was COX-2 mRNA that was specifically triggered in CM and blockage of COX-2 with celecoxib resulted in an earlier onset of CM. The "COX" abbreviation is more often encountered in medicine while the "PTGS" symbol is officially used for the prostaglandin-endoperoxide synthase (cyclooxygenase) family of genes and proteins, because the stem "COX" was already used for the cytochrome c oxidase family of genes and proteins.

Pharmacological inhibition of *COX* can provide relief from the symptoms of inflammation and pain. Non-steroidal anti-inflammatory drugs (NSAID), such as aspirin and ibuprofen, exert their effects through inhibition of COX. The names "prostaglandin synthase (PHS)" and "prostaglandin endoperoxide synthetase (PES)" are still used to refer to COX. Given the reported correlation relationship between low levels of COX-2 and increased *Plasmodial* parasitisation, the detection of lower levels of COX-2 in control mice groups that had relatively high parasitaemia including the untreated *P. berghei* group more symptomatic malaria could be expected. Moreover, in the murine- *P. berghei* ANKA model of CM, introduction of aspirin (a nonselective COX inhibitor) reduced the survival of *P. berghei* ANKA infected mice (Xiao *et al.,* 1999), providing further support to the current outcome that lower levels of COX-2 such as those witnessed in the CpG/*P. berghei* and *P. berghei* groups are linked to increased disease severity and parasitaemia.

Cyclooxygenases (COXs) catalyze the initial step of prostaglandin biosynthesis from arachidonic acid and COX-1 is widely, and usually constitutively, expressed. Cyclooxygenase 2 (COX-2 is constitutively expressed in the brain, kidneys, and gastrointestinal system and can also be induced by a variety of agents such as growth factors, bacterial endotoxin, and cytokines (Ball *et al.,* 2006). Therefore, COX-2 could be responsible for much of the synthesis of prostaglandin that occurs during inflammation. For COX-2, selective inhibitors that that are accompanied by less adverse GIT effects than nonselective COX inhibitors were developed (Buttgereit *et al.,* 2001). Bicyclo-prostaglandin E_2 (PGE_2) concentrations in plasma and secretion of COX-2 in peripheral blood mononuclear cells (PBMCs) of African children with *P. falciparum* malaria were upregulated in healthy children, and were reduced in children with SM (Perkins *et al.,* 2001). Chronic infection during PM has been associated with the upregulation of COX-2 and there is strong involvement of COX-2 in the recovery phases of PM (Demba Sarr *et al.,* 2010**).** In that study COX-2 and IL-10 were elicited during chronic placental infection, but were not linked to preterm delivery or low birth weight while increased haemozoin deposition was correlated with low birth weight (LBW) and decreased expression of COX-2 (Sarr *et al.,* 2010). In another experiment, accumulations of COX-2 expressing endothelial cells and COX-2 expressing astrocytes were linked to increased CM in brain parenchyma (Deininger *et al.,* 2001). The differential expression and accumulation of COX-1 and COX-2 in CM brains points to the a role of cyclooxygenases in the formation of fever, inflammation and granuloma of CM patients (Deininger *et al.,* 2001). Murine model experiments showed that specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis (Stolina *et al.,* 2000). Cyclooxygenase-2-issued prostaglandin E

(2) was found to enhance the production of endogenous IL-10, which downregulates dendritic cell functions (Harizi *et al.,* 2002).

The current study's results showing increased COX-2 levels in IL-18 conditions both with or without the co-administration of CpG, adds weight to the previously reported COX-2-inducing properties of IL-18 (Oku *et al.,* 2004). Cyclooxygenase enzymes which convert arachidonic acid to prostaglandins, include cyclooxygenase1 (COX1) and COX2 facilitate the rate-limiting step in arachidonic acid metabolism and expression of COX2 mRNA and protein is promoted in various cell types by inflammatory cytokines such like interleukin-1 (IL-1) and tumor necrosis factor; TNF (Takashi *et al.,* 2004), which are Th1 panel cytokines as are IL-18 and IL-12. It is therefore possible that the Th1 conditions availed by inoculation of IL-18 and IL-12 with CpG ODN caused the increased expression of COX-2, with is also associated with reduced parasitisation and uncomplicated malaria (Xiao *et al.,* 1999; Deininger *et al.,* 2001; Ball *et al.,* 2004; Sarr *et al.,* 2010**)**. In that previous research, the elevation of IL-18 in the peritoneal fluid of endometriosis patients and the stimulation of COX-2 in peritoneal monocytes by IL-18 indicated an IL-18 pathogenic role in endometriosis. Cyclooxygenase 2 is also a key enzyme for inflammatory cytokine-induced angiogenesis (Takashi *et al.,* 2004).

The Neutrophil collagenase, also known as matrix metalloproteinase-8 (MMP-8) or PMNL collagenase (MNL-CL), a collagen-cleaving enzyme which is present in the connective tissue of most mammals, was detected at higher concentrations in the CpG/*P. berg*hei and untreated *P. berghei* control groups than in the rest of the mice groups, signifying that elevated MMP-8 levels were correlated with increased parasitaemia and severe malaria (Dietmann *et al.,* 2008), as both these mice groups experienced significantly higher parasitaemia and symptomatic malaria than their cytokine-CpG-receiving counterparts. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases; other family members are adamalysins, serralysins, and astacins. The MMPs belong to a larger family of proteases known as the metzincin superfamily (Van Lint *et al.,* 2007). Collectively, these matrixin enzymes are capable of degrading all kinds of extracellular matrix proteins, but also can process a number of bioactive molecules. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine inactivation (Van Lint *et al.,* 2007). MMPs are also thought to play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiat-ion, angiogenesis, apoptosis, and host defense and MMP-8 has been linked to reduced malarial severity (Dietmann *et al.,* 2008; Van Den Steen *et al.,* 2006). Increased levels of both MMP-8 mRNAs and MMP-9+ cells were detected in severe *P. berghei* ANKA malaria in the murine model (Van Den Steen *et al.,* 2006), further agreeing with our findings of elevated MMP-8 in the CpG/*P. berg*hei and untreated *P. berghei* control groups and downregulated MMP-8 concentrations in the cytokine-CpG combination groups which also had lower parasitaemia and less symptomatic outcomes.

The CpG/IL-18/ *P. berghei*; CpG/IL-12/ *P. berghei*; IL-18/ *P. berghei*; IL-12/ *P. berghei* mice groups which all had less parasitaemia and milder malarial outcomes in comparison to the CpG/*P. berghei* and untreated *P. berghei* control groups, collectively had significantly downregulated MMP-8 levels than the latter groups, further linking cytokine-CpG-elicited reduction in MMP-8 with lower infection and parasitaemia characteristics. Previous reports have shown that increases in tissue inhibitors of metalloproteinase-1 (TIMP-1) and MMP-8 levels were associated with severe *P. falciparum* malaria outcomes TIMP-1 levels were increased in patients with severe malaria, compared with those in patients with uncomplicated malaria Raised TIMP-1 levels were strongly correlated with malarial severity, as determined by the simplified multiorgan dysfunction score (Dietmann *et al.,* 2008).

Like MMP-8, the matrixin factor matrix metallopeptidase 9 (MMP-9), also called the 92 kDa type IV collagenase, or 92 kDa gelatinase or gelatinase B (GELB), was expressed at higher concentrations in the CpG/*P. berghei* and untreated *P. berghei* control groups than in the rest of the mice groups, indicating that upregulated MMP-9 amounts were correlated with increased parasitaemia and severe malaria (Van Den Steen *et al.,* 2006). Similarly, microarray studies have revealed that there is increased activation of the human MMP-9 gene by *P. falciparum* in whole blood from children with severe malaria (Griffiths *et al.,* 2005), although MMP-9 increases in cerebrospinal fluid levels were not apparent, due to MMP-9's tight association with the extracellular matrix (Brown *et al.,* 2000). Current findings of reduced MMP-9 levels in mice groups that were given the IL-18/CpG/*P. berghei* and IL-12/CpG/*P. berghei* co-inoculations support the previous findings that that MMP-9 levels were strongly upregulated in C57BL/6 mice brain infected with *P. berghei* ANKA, which is a murine model of CMs with similar characteristics to human CM. Upregulated MMP-9 amounts were selective for the central nervous system, where they were linked to the vasculature and parenchyma while immunohistochemistry procedures revealed that higher amounts of MMP-9 were released by monocytic lineage cells (CD11b+ [Van Den Steen *et al.,* 2006]).

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, angiogenesis, bone develop-ment, wound healing, cell migration, learning and memory, as well as in pathological processes, such as arthritis, intracerebral hemorrhage, and metastasis

(Wang *et al.,* 2005; Van Vandooren *et al.,* 2013). Most MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens and other extracellular matrix proteins (Van den Steen *et al*., 2002). In rhesus monkeys the MMP enzymes are involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow. In mice MMPs have a role in tumor-associated tissue remodeling. Intervertebral disc proteins and thrombospondins regulate the effective levels of matrix metalloproteinases (MMPs) 2 and 9, which are key effectors of ECM remodeling (Hirose *et al.,* 2008).

CONCLUSION

Physiologic biomolecular analysis done in this project indicated that adiponectin, ANGPT1, NRP-1 and Cox-2 concentrations become upregulated in malaria-infected cytokine-CpG ODN recipients while angiogenin, ANGPT2, MMP-8 and MMP-9 levels are downregulated thereby being associated with reduced parasitaemia, mild malaria and less severe haematological outcomes. Likewise reductions in adiponectin, ANGPT1, NRP-1 and Cox-2 and increases in angiogenin, ANGPT2, MMP-8 and MMP-9 that prevailed in the absence of the cytokine-CpG ODN bitherapeutic interventions are associated with elevated parasitisation, severely symptomatic malaria.

ACKNOWLEDGEMENTS

We wish to acknowledge the KEMRI, IPR and MMUST teaching and technical staff for their support and technical assistance. We also thank the MMUST, KEMRI and IPR management for providing funding for this project.

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