The Effect of Systemic Levels of TNF-alpha and Complement Pathway Activity on Outcomes of VEGF Inhibition in Neovascular AMD

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ABSTRACT

Background/Objectives: Systemic levels of pro-inflammatory cytokines and activated complement components affect the risk and/or progression of neovascular age-related macular degeneration (AMD). This study investigated the effect of serum pro-inflammatory cytokine levels and complement pathway activity on the clinical response to vascular endothelial growth factor (VEGF) inhibition in neovascular AMD.

Methods: Sixty-five patients with a new diagnosis of neovascular AMD were observed over a six-month period in a single-centre, longitudinal cohort study. At each visit, the visual acuity score (VAS), central macular thickness (CMT), serum levels of CRP, pro-inflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6 and IL-8), and complement pathway activity were measured. Participant DNA samples were sequenced for six complement pathway single nucleotide polymorphisms (SNPs) associated with AMD.

Results: A statistically significant difference in VAS was observed for serum levels of TNF-α only: there was a gain in VAS (from baseline) of 1.37 for participants below the 1st quartile of mean concentration compared to a reduction of 2.71 for those above the 3rd quartile. Statistical significance was maintained after Bonferroni correction (*P* value set at <0.006). No significant differences in CMT were observed. Additionally, statistically significant differences, maintained after Bonferroni correction, were observed in serum complement activity for participants with the following SNPs: *CFH* region (rs1061170), *SERPING1* (rs2511989) and *CFB* (rs641153). Serum complement pathway components did not significantly affect VAS.

Conclusions: Lower serum TNF-α levels were associated with an increase in visual acuity after anti-VEGF therapy. This suggests that targeting pro-inflammatory cytokines may augment treatment for neovascular AMD.

Keywords

Age-Related Macular Degeneration; Cytokines; Complement; Tumour Necrosis Factor-alpha; Vascular Endothelial Growth Factor

INTRODUCTION

Age-related macular degeneration (AMD), a progressive retinal disease that results in the loss of central vision, is predicted to affect 288 million people worldwide by 2040 (1). Neovascular AMD (nAMD) is a result of choroidal neovascularisation (CNV) and leads to rapid vision loss. The mainstay of current treatment is inhibition of vascular endothelial growth factor (VEGF) (2). The evidence base for a genetic component in AMD is significant, and numerous single nucleotide polymorphisms (SNPs) have been associated with a patient's risk of developing AMD (3). SNPs in genes of the complement pathway, including the complement factor B (*CFB*) gene region (4, 5), the *C2* (4, 5) and *C3* (6) genes have been reported to affect the risk of developing AMD.

Uncontrolled activation of the complement pathway is limited by a set of complement regulatory proteins: Factor H and Factor I (encoded by the *CFH* and *CFI* genes respectively) regulate the alternative complement pathway (7), whereas the C1 inhibitor is a regulator of the classical pathway (8). Genetic variants at the Regulators of Complement Activation (RCA) locus on *chromosome* 1, which contains the *CFH* gene, contributes to AMD risk (9-11), in addition to the *CFI* gene region on *chromosome* 4 (12-14), and the *SERPING1* gene that encodes the C1 inhibitor (15, 16).

Studies have shown elevated levels of complement activation fragments to be independently associated with AMD (17-19). Furthermore, complement activation has been demonstrated to be associated with stage of AMD (20). In addition, systemic activation of the alternative complement pathway and complement components is associated with AMD genotypes (21), including the *CFH* SNP rs1061170 (Y402H) (19) and the *CFI* region SNP rs10033900 (17, 21). A meta-analysis by Hong ***et al.*** reported that treatment-naïve patients carrying the *CFH*

SNP, rs1061170 (Y402H), were more likely to achieve an improved outcome to anti-VEGF treatment (22). Furthermore, visual outcome was improved after anti-VEGF treatment for patients carrying a low-risk *CFH* genotype and low *CFH* risk score (23).

Expression of acute phase proteins and pro-inflammatory cytokines can also affect the risk of AMD development and/or progression: CRP is an acute phase protein and marker of systemic inflammation that is an independent risk factor for AMD (24). IL-6 is a known cytokine stimulus of CRP release by the liver (25), and both have been associated with AMD progression (26). CRP has been demonstrated to induce IL-8 expression by human retinal pigment epithelium (RPE) cell lines (27), and both IL-6 and IL-8 are expressed by RPE cells on complement activation (28), by degenerating RPE cells (29), and are associated with drusen formation (30). Systemic levels of IL-6 have been found to be associated with the progression rate of geographic atrophy secondary to AMD (31). Additionally, patients with AMD have been shown to express higher levels of circulating IL-1β than age-matched controls (32). IL-2 has been implicated in the pathogenesis of AMD as activation of IL-2 signalling pathways has been observed (33) and IL-2 contributes to extracellular matrix formation and the development of fibrosis in AMD (34). TNF-α, a pro-inflammatory cytokine that is known to mediate CNV formation in experimental models by upregulating VEGF expression by RPE cells (35), has also been demonstrated to promote the angiogenic drive of active CNV lesions (36). Patients with elevated levels of serum TNF-α have been shown to respond favourably to VEGF inhibition (32).

Although the studies mentioned above have investigated the role of complement pathway SNPs, complement pathway activity and systemic concentrations of pro-inflammatory cytokines on AMD pathogenesis, relatively few studies have investigated their functional

effect on outcomes of VEGF inhibition. The primary aim of this study was to investigate the effect of serum levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-2, IL-6 or IL-8) and complement pathway activity on the clinical response to VEGF inhibition in neovascular AMD. A secondary aim was to investigate the effect of complement pathway SNPs, associated with AMD, on serum complement activity in the same cohort of patients.

MATERIALS AND METHODS

Study Approval, Registration and Regulation

This study was conducted in accordance with the Research Governance Framework for Health and Social Care (2005) and Good Clinical Practice. Ethical approval was obtained from the National Research Ethics Committee (NRES) South Central- Southampton A. This study adhered to the tenets of the Declaration of Helsinki. The University Hospital Southampton NHS Foundation Trust was the sponsor of this study, and The University of Southampton undertook the research study. All patient samples and data were anonymised for the purpose of this study. Patient DNA and serum samples were stored for future studies. Procedures for handling, processing and storage of patient data were in compliance with the UK Data Protection Act (1998).

Patient Recruitment, Consent, and Investigation

Patients were recruited to the study after informed consent by the ophthalmology department of University Hospital Southampton NHS Foundation Trust. Patients were invited to take part if they met the principle inclusion criteria for the study: 1) over the age of 50; 2) a new diagnosis of neovascular AMD in one eye, treated with an initial loading dose of three, monthly Ranibizumab intravitreal injections; 3) White ethnicity (to limit any effects of ethnic variation on outcomes of VEGF inhibition in neovascular AMD). The exclusion criteria

were: 1) bilateral diagnosis of neovascular AMD (one of the exploratory endpoints of the study was the development of nAMD in the second eye); 2) a macular co-pathology; 3) poor venous access that prevents a peripheral blood samples being taken.

All patients recruited to this study had a diagnosis of neovascular AMD, confirmed on fundus fluorescein angiography, that was made by a consultant ophthalmologist specialising in medical retina diseases. Indocyanine green angiography was carried out for patients to rule out polypoidal choroidal vasculopathy (PCV)- patients with PCV were not invited to take part in the study. Patients were eligible to enrol for the study after their third intravitreal Ranibizumab injection and subsequently invited to a baseline visit (**Figure 1**). Informed consent was taken from participants at this visit, and their demographic details, medical history and baseline LogMAR visual acuity score (VAS) was recorded (number of letters on an ETDRS chart). A baseline central macular thickness (CMT) was also measured using optical coherence tomography (OCT) (Topcon, Berkshire, UK). A blood sample was taken at the baseline visit for serum cytokine and genetic analysis. Participants were reviewed by a study investigator and received treatment with an intravitreal ranibizumab injection if they had active neovascular AMD. Following the baseline visit, participants attended for six, monthly follow-up visits. At each visit, the VAS and CMT was recorded, a blood sample was taken, and the patient reviewed by a study investigator before any treatment for active disease.

Detection of Serum Cytokine levels and Activated End Components of Complement Pathways

Serum was isolated from participant blood samples using standard density-gradient ultracentrifugation at 1355 x g for 10 minutes at 21°C (Eppendorf, Stevenage, UK). Patient

serum cytokine levels were measured using semi-quantitative assays by Meso Scale Discovery (Rockville, Maryland, USA) as per the manufacturer's instructions. All cytokine measurements were undertaken in triplicate using the assay, and cytokine measurements were within the reading range of the kit. Functional assessment of classical and alternative pathway complement activity in patient serum samples was undertaken using Wieslab semi-quantitative ELISA Assays (SVAR Life Sciences, Malmo, Sweden) as per the manufacturer's instructions. Measurement of activated end components of classical and alternative complement pathways was expressed as a percentage relative to the fluorescence intensity of the positive control, derived from human serum components, supplied with the testing kit.

Genetic Analysis

DNA was extracted from peripheral blood mononuclear cells of patient blood samples using erythrocyte lysis buffer (Fisher Scientific, Loughborough, UK) as previously described (37). DNA concentrations were measured using the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Sequence analysis of participant DNA samples was undertaken by LGC Genomics (Hoddesdon, UK) on the following six SNPs associated with the complement pathway and AMD risk: *CFH* region: rs1061170; *CFI* region: rs10033900; *SERPING1* / *C1-INH*: rs2511989; *CFB*: rs641153; *C2*: rs9332739; *C3*: rs2230199.

Statistical Analyses

The GraphPad Prism software version 8.2 (GraphPad Software, Lo Jolla, Ca, USA) was used for statistical analyses and graphical representation of the data obtained in this study. Assessment of normality of continuous variables was determined by quantile-quantile plots of the residuals using GraphPad Prism. The unpaired *t* test with Welch's correction was used

to determine statistically significant differences in changes of visual acuity scores, central macular thickness and percentage activity of activated end components of complement pathways compared to positive controls. Statistical significance was set at the $P<0.05$ value. As this is a preliminary / pilot study, the patient sample size was determined using a rationale laid out by S.A. Julious where a sample size of at least 12 is recommended (38). Our patient cohort was stratified into quartiles of approximately 16 in line with this recommendation.

RESULTS

Serum classical or alternative complement pathway activity and functional response to anti-VEGF intravitreal injections

A total of 65 patients with a new diagnosis of neovascular AMD were recruited to participate in this study (**Figure 1**). Participant demographics are summarised in **Table 1**. Study participants were stratified into quartiles according to average serum concentration of an inflammatory protein over seven study visits, in order to amplify the functional effects of small changes in serum concentration (**Table 1**). The study first investigated any significant differences in the visual acuity score (VAS) or central macular thickness (CMT) change from baseline at each visit between participants who had a mean serum concentration of classical pathway (**Supplementary Figure 1A-B**) or alternative pathway (**Figure 2A-B**) complement components below the first quartile and above the third quartile. There was a statistically significant difference in the VAS change from baseline, -2.78 (SD=7.01) vs. -0.34 (SD=8.51) for mean serum alternative pathway components (P=0.048), using an unpaired t test with Welch's correction (**Figure 2A**), but significance was not maintained after a Bonferroni correction was applied (P value set at <0.006).

Serum inflammatory protein concentration and functional response to anti-VEGF intravitreal injections

Study participants were also stratified into quartiles according to mean serum concentration of CRP or a pro-inflammatory cytokine (TNF-α, IL-1β, IL-2, IL-6 or IL-8) over the seven study visits. Statistically significant differences initially observed for VAS change (**Figure 2C**) for mean CRP concentration were not maintained after Bonferroni correction (*P* value was set at <0.006), and there were no significant differences in CMT change (**Figure 2D**) using the unpaired *t* test with Welch's correction.

Of the pro-inflammatory cytokines assessed, there was a statistically significant difference observed in the VAS change from baseline, 1.37 (SD=9.40) vs. -2.71 (SD=7.79), between participants for mean serum TNF-α concentration below the first quartile and above the third quartile respectively (*P*=0.0024), **Figure 2E**. Significance was maintained after a Bonferroni correction was applied (*P* value was set at <0.006). No significant difference was observed in CMT change in these participants (**Figure 2F**). Additionally, no significant differences were observed in the VAS or CMT change from baseline between participants for mean serum concentration of IL-1β (**Supplementary Figure 1C-D**), IL-2 (**Supplementary Figure 1E-F**), IL-6 (**Supplementary Figure 1G-H**), or IL-8 (**Supplementary Figure 1I-J**).

Complement Pathway SNPs and Activated Complement End Components

All 65 study participants underwent DNA sequencing for the detection of six complement pathway SNPs reported to affect AMD risk. Measurement of classical or alternative pathway complement activity in the serum was undertaken at each study visit. Complement activity was expressed as a percentage relative to the positive control (based on human serum components) of the assay and could thus exceed 100%. For the *CFH* SNP rs1061170, a

statistically significant reduction was observed: 1) in mean classical pathway complement activity in homozygous participants (16.7% reduction; n=24; *P*=0.0016) (**Figure 3A**); 2) in mean alternative pathway activity in both homozygous (20.1% reduction; n=24; *P*=0.0019) and heterozygous participants (19.4% reduction; n=35; *P*=0.0025), **Figure 3B**. For the *CFI* region SNP rs10033900, there was a statistically significant increase of 7.7%, after Bonferroni correction, in mean classical pathway activity, in both homozygous (n=16; *P*=0.0037) and heterozygous (n=32; *P*=0.0002) participants, **Supplementary Figure 2A-B.** For the *SERPING1* / *C1-INH* SNP rs2511989, there was a significant increase in only the mean alternative pathway complement activity of 11.8% in homozygous participants (n=11; *P*=0.005), maintained after Bonferroni correction **Figure 3C-D.**

A significant increase in mean classical pathway complement activity of 16.3% was observed, after Bonferroni correction (*P* value set at <0.008), in patients who were homozygous for the *CFB* SNP rs641153 (n=2; *P*<0.0001) and 9.7% who were heterozygous (n=10; *P*<0.0001), **Figure 3E**. Similarly, a significant increase in alternative complement pathway activity of 7.3% was observed, after Bonferroni correction, in heterozygous patients (n=10; *P*=0.0069), **Figure 3F**. No differences were observed for the *C2* SNP rs9332739 or *C3* SNP rs2230199 (**Supplementary Figure 2C-F**).

DISCUSSION

The primary aim of this study was to investigate the effect of serum pro-inflammatory cytokine levels and complement pathway activity on the clinical response to VEGF inhibition in neovascular AMD. After Bonferroni correction, a statistically significant difference was observed only in VAS (change from baseline) between participants stratified into quartiles by mean TNF-α serum concentration [a gain of 1.37 for participants below the 1st quartile

compared to a reduction of 2.71 above the 3rd quartile]. This was not associated with significant changes in CMT. This study supports both pre-clinical and clinic findings showing a small, but significant overall impact of systemic levels of TNF-α on CNV lesions and clinical responses to VEGF inhibition. In a previous study using a murine model of laser-induced CNV, inhibition of TNF-α with intraperitoneal injections of infliximab or etanercept led to significantly reduced CNV lesion size and pathological fluorescein leakage (39). Furthermore, in a non-controlled trial, infusions of the anti-TNF-α chimeric monoclonal antibody, infliximab, in neovascular AMD demonstrated non-progression of the disease in almost half of the treated patients and regression of exudative lesions without significant fibrous scarring (40). There was, however, no placebo group in this trial. Other small studies using intravitreal anti-TNF-α therapy combined with bevacizumab showed beneficial effects (41).

This study also investigated the effect of complement pathway SNPs, associated with AMD, on serum classical or alternative pathway complement activity in the same cohort of patients. A statistically significant, but modest, reduction (after Bonferroni correction) of classical pathway activity was observed in participants who were homozygous for the *CFH* region SNP rs1061170, in addition to a reduction in alternative pathway activity in participants who were either homozygous or heterozygous for this SNP. Furthermore, participants who were either homozygous or heterozygous for the *CFI* region SNP rs10033900 had a statistically significant increase in classical pathway activity, despite Factor I being better recognised as a regulator of the alternative pathway (7). Despite these differences, this study demonstrated no significant differences in VAS or CMT change from baseline between participants below the 1st quartile and above the 3rd quartile of mean serum classical or alternative pathway complement components. Therefore, although statistically-significant, modest, differences in

serum complement activity were observed in participants with *CFI*, *CFH* and other complement pathway SNPs tested, this did not translate to real-world, significant differences in visual response to anti-VEGF treatment.

A recent study by Heesterbeek *et al*. demonstrated higher systemic levels of activated complement in patients with intermediate AMD (who demonstrated the highest serum complement activation), geographic atrophy and inactive neovascular AMD compared to patients with active nAMD (20). This raises the question of whether significant increases in complement activity were not observed in this study as all patients had active nAMD. Interestingly, it was demonstrated in a study by Keir *et al*., that anti-VEGF intravitreal injections in neovascular AMD patients resulted in increased levels of complement components (C3a, C4a and C5a) in the aqueous humour (42), and this was elevated in patients with earlier relapses of active nAMD compared to those with later relapses.

This study focused on measuring overall complement pathway activation (via the activated end products of complement activation) rather than a specific activated complement component, e.g. C3d. Commercially-available Wieslab assays (Svar Life Sciences) were used in this study, which are optimised to detect activation of the complement pathway using human serum. This assay was previously used to demonstrate a significant elevation in the activity of the alternative complement pathway in AMD patients with genetic variants in *CFB* and *C3* compared to controls (19). An alternative method to detect elevated systemic complement activation in our study could have been to calculate the C3d/C3 ratio from the plasma concentration of these complement components. This method was also used (in addition to the Wieslab assays) in recent studies to detect systemic complement activation in AMD patients with genetic variants (19, 20). It will be interesting to see whether there is any

difference between the Wieslab assay method and C3d/C3 ratio to measure systemic complement activation in our cohort of participants.

This study analysed individual genetic variants and their effect on complement pathway activation, which demonstrated some statistically-significant effects. Previous studies have demonstrated that the association of gene variants with complement activation in AMD patients may be stronger when undertaking haplotype analysis (43) compared to single variant analysis. An additive effect of complement pathway risk SNPs has been suggested to lead to an additive risk of disease (44). In the recent study by Heesterbeek *et al*, the association of AMD stage with complement activation was greatest in patients with haplotypes that were associated with the highest levels of complement activation (20). It will be interesting to undertake haplotype analysis to investigate the effect of overall complotype on the outcomes of VEGF inhibition in AMD in our patient cohort.

Studies have suggested that AMD pathogenesis is driven primarily by dysregulation of immune mediators locally within the eyes rather than circulating levels of these mediators. A study by Agawa *et al.* demonstrated that intravitreal anti-VEGF treatment (with bevacizumab) itself significantly raised intraocular levels of IL-6 and IL-8 (45), both implicated in AMD pathogenesis. A subsequent study, in contrast, demonstrated a reduced intraocular concentration of IL-6 after intravitreal aflibercept injection (46). Peripheral blood mononuclear cells (PBMCs), particularly monocytes, from AMD patients have been demonstrated to produce higher levels of IL-8 than age-matched controls (47), and it has been speculated that these cells could migrate to the macula to secrete additional IL-8.

375 The concept of AMD being a disease of systemic or local complement dysregulation was
376 previously discussed by our group nearly eight years ago (48). Studies have suggested that
377 SNPs associated with complement activation increase AMD risk by a combination of
378 systemic activation of complement and dysregulation of complement activation in local
379 tissues (49). It is unknown whether altered, systemic levels of complement in AMD are the
380 result of AMD-associated gene variants whose effects are expressed in all tissues, or the
381 result of circulating levels of complement that reach the choroid and retina to contribute to
382 AMD pathogenesis. It is also thought that AMD pathogenesis is driven by a combination of
383 locally-expressed complement factors (50, 51) in addition to systemic complement proteins
384 which lead to local effects in tissues, e.g. the FH-related proteins (FHR) such as FHR-4 (52).
385 This study did not demonstrate any statistically-significant functional effects, after
386 Bonferroni correction, of elevated or reduced systemic complement pathway activity on
387 outcomes of VEGF inhibition.
388
389 There were several limitations to this study, the most significant being the small cohort size
390 of 65 participants. The primary reason for this is the challenge in recruiting a large number of
391 patients for a study in which blood tests are taken every visit over seven months, in addition
392 to an intravitreal injection where required. Fortunately, no participants dropped out of this
393 study and all participant data from each visit was used in the analysis. Another limitation to
394 this study was taking blood tests (for serum cytokine and complement pathway activity) after
395 the loading dose of three, monthly intravitreal ranibizumab injections. The ethical regulations
396 of this study meant that the priority was for patients to receive their loading dose of
397 intravitreal anti-VEGF injections prior to enrolment in the study and for blood tests to be
398 undertaken subsequently. Although the biggest gains in visual activity usually take place
399 during the loading phase of intravitreal anti-VEGF injections, measurement of visual

outcomes took place in this study from the starting point of all participants having received their three, monthly intravitreal injections. Although there is emerging evidence of intravitreal ranibizumab injections affecting serum concentrations of pro-inflammatory cytokines (including a transient reduction of serum TNF-α levels) in patients with diabetic macular oedema (53), similar / significant evidence has not been demonstrated in the context of nAMD. Similarly, increased serum levels of TNF-α have been demonstrated in treatment-naïve patients with diabetic macular oedema (54), but not in nAMD patients (32).

In light of serum TNF-α levels being associated in this study with small, but significant effects on visual acuity after treatment with anti-VEGF intravitreal injections, it would be worth investigating this cytokine in larger cohorts to determine if this effect can be replicated. This would determine if systemic levels of TNF-α could be used to identify non-responders to anti-VEGF treatment.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest to disclose.

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AUTHOR CONTRIBUTION STATEMENT

AHK contributed to research design, data analysis and interpretation and manuscript preparation. COP, GDS, HG, MN, AJC and GM contributed to research design, data acquisition and research execution. AJL contributed to research design, data analysis and interpretation, and manuscript preparation.

Supplementary information is available at *Eye*'s website.

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TITLES AND LEGENDS TO FIGURES

Figure 1. A flowchart diagram summarising the investigation pathway of 65 study participants with a new diagnosis of neovascular Age-Related Macular Degeneration (AMD). Peripheral blood samples were taken at seven visits (baseline visit and six follow-up visits), for serum and genotypic analysis.

Figure 2. Change in visual acuity score (VAS) and central macular thickness (CMT) associated with serum concentration of alternative complement pathway components and inflammatory proteins. Study participants were stratified into quartiles according to average serum concentration of an investigated inflammatory protein (including pro-inflammatory cytokine) or complement pathway-specific components over seven study visits. The change in VAS from baseline at each visit is plotted for all study patients who had a mean serum concentration of inflammatory protein or complement pathway component below the first quartile and above the third quartile. The percentage change in CMT from baseline at each visit is also plotted for the same study participants. Shown in parts **A-B** are the results for alternative complement pathway components and change in VAS or CMT from baseline at each study visit for patients below or above the indicated quartiles; **P*=0.048. Shown in parts **C-D** are the results for C-Reactive Protein (CRP) and change in VAS or CMT from baseline at each study visit for patients below or above the indicated quartiles; **P*=0.029. Shown in parts **E-F** are the results for Tumour Necrosis Factor-α (TNF-α) and change in VAS or CMT from baseline at each study visit. ***P*=0.0024. The unpaired *t* test, two-tailed, with Welch's correction, was used to determine whether there was a statistically significant difference in VA or CMT change from baseline between groups.

Figure 3. Classical or alternative complement pathway activity associated with single nucleotide polymorphisms (SNPs) in study participants. Study participants underwent DNA sequencing for the detection of six SNPs associated with the complement pathway and AMD risk. Assessment of serum levels of classical or alternative pathway complement components was undertaken on the same participants. The bar graphs **A**-**F** show the measurement of classical or alternative pathway complement activity on participants who express no SNP, are heterozygous, or homozygous for the following SNPs: *CFH* region: rs1061170 (**A**-**B**). *SERPING1* / *C1-INH*: rs2511989 (**C**-**D**). *CFB*: rs641153 (**E**-**F**). Measurement of activated end components specific for the classical or alternative complement pathway in serum samples is expressed as a percentage relative to the activity of the positive control. The unpaired *t* test, two-tailed, with Welch's correction, was used to determine whether there was a statistically significant difference in classical or alternative pathway components between groups who had no SNP, were heterozygous for the indicated SNP, or homozygous for the indicated SNP. **P*<0.05. ***P*<0.005. ****P*<0.0001.

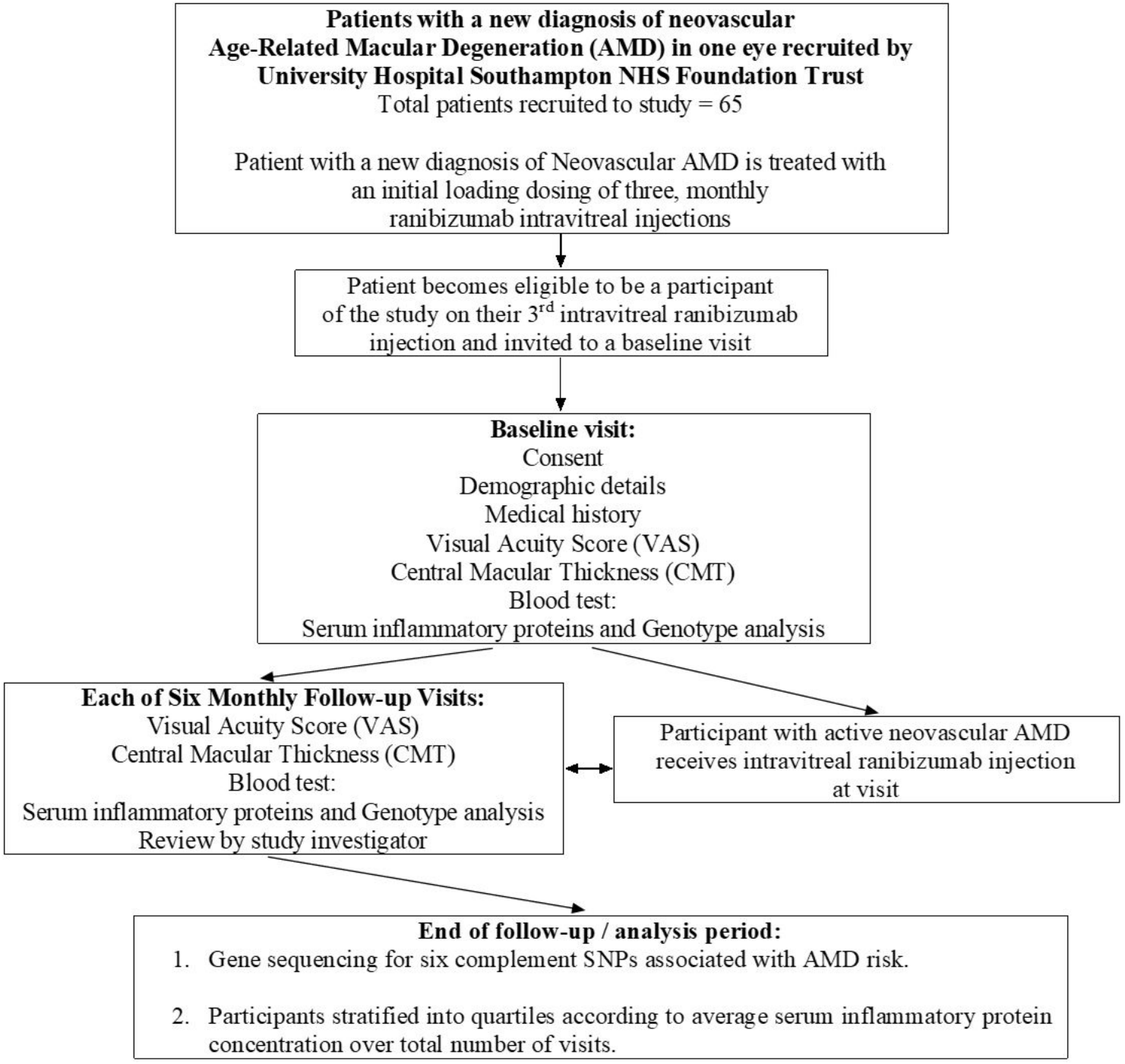
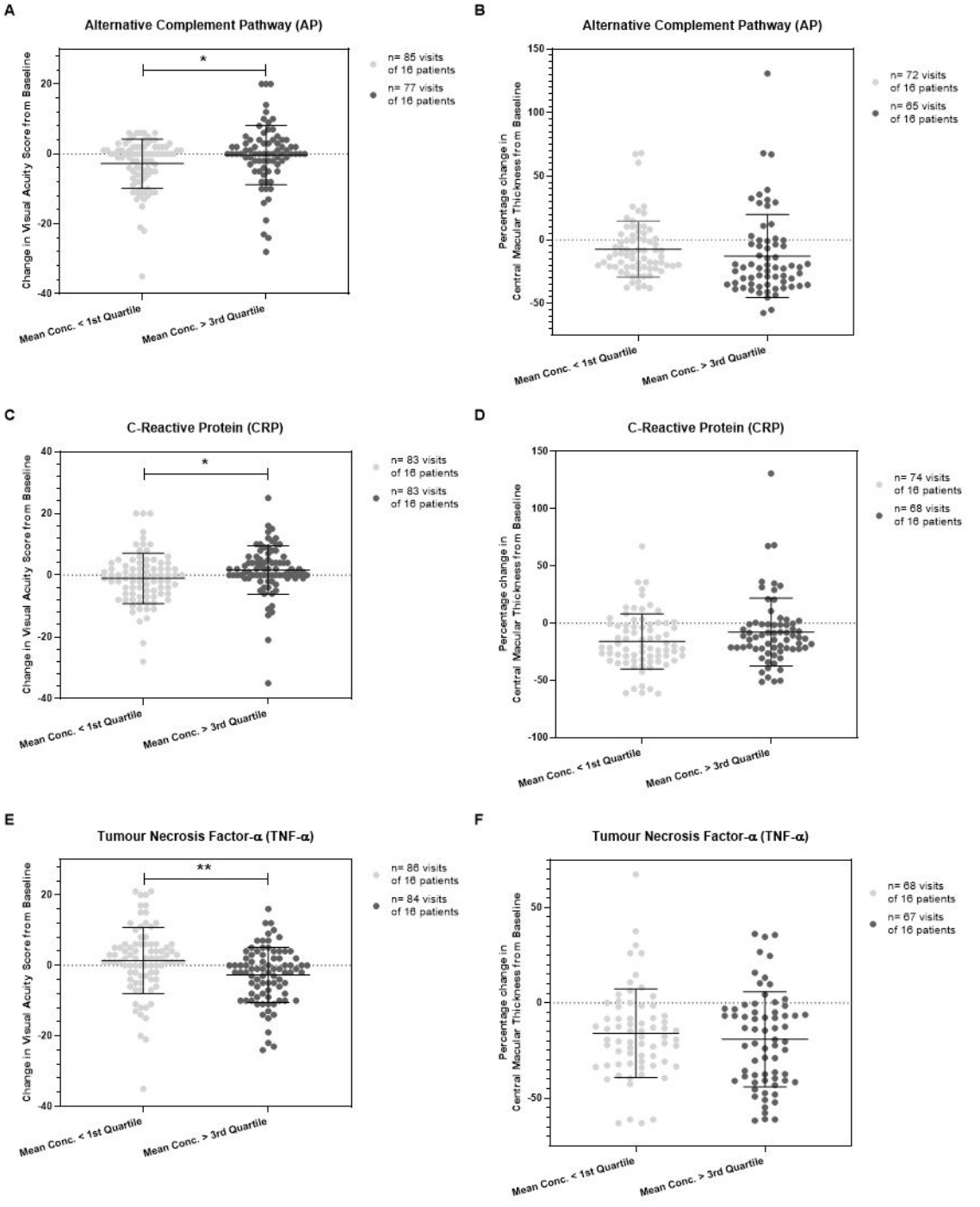
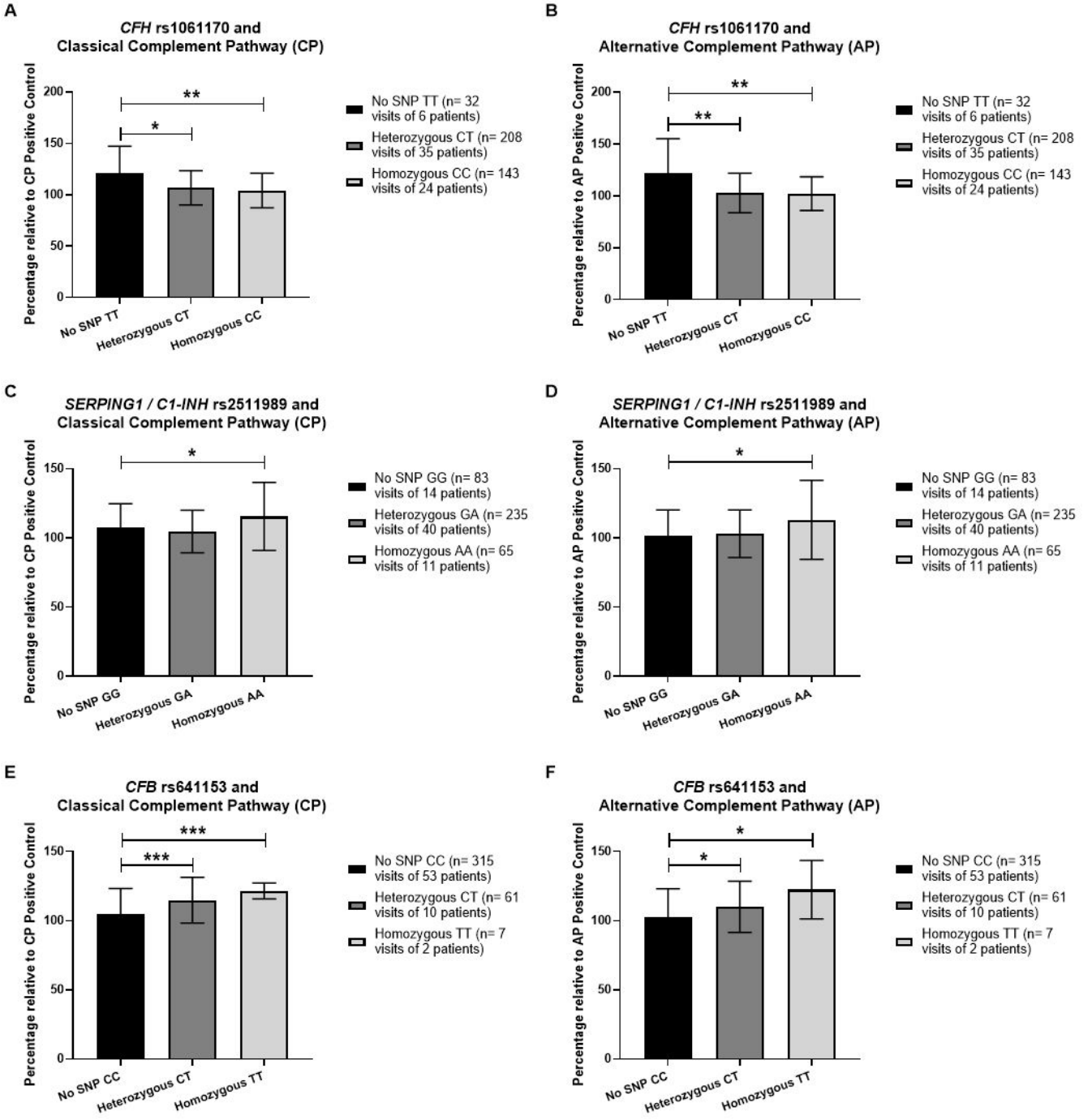

Patients with a new diagnosis of neovascular Age-Related Macular Degeneration (AMD) in one eye recruited by University Hospital Southampton NHS Foundation Trust
Total patients recruited to study = 65
Patient with a new diagnosis of Neovascular AMD is treated with an initial loading dosing of three, monthly ranibizumab intravitreal injections
Patient becomes eligible to be a participant of the study on their 3rd intravitreal ranibizumab injection and invited to a baseline visit
Baseline visit:
Consent
Demographic details
Medical history
Visual Acuity Score (VAS)
Central Macular Thickness (CMT)
Blood test:
Serum inflammatory proteins and Genotype analysis
Each of Six Monthly Follow-up Visits:
Visual Acuity Score (VAS)
Central Macular Thickness (CMT)
Blood test:
Serum inflammatory proteins and Genotype analysis
Review by study investigator
Participant with active neovascular AMD receives intravitreal ranibizumab injection at visit
End of follow-up / analysis period:
1. Gene sequencing for six complement SNPs associated with AMD risk.
2. Participants stratified into quartiles according to average serum inflammatory protein concentration over total number of visits.

Table 1. Study Participant Demographics and Summary Table						
Demographics						
Number of patients recruited to study	65					
Patient sex	48% (n=31) male : 52% (n=34) female					
Patient age (mean, SD)	79.7 (SD = 8.6)					
Smoking Status	Current Smoker: 11% (n=7) Ex-Smoker: 46% (n=30) Non-smoker: 43% (n=28)					
Body Mass Index (BMI) (mean, SD)	26.8 (SD = 3.94)					
Co-morbidities	Hypertension: 47.7% (n=31) Asthma or COPD: 13.8% (n=9) Hyperlipidaemia: 13.8% (n=9) Diabetes (Type I or II): 12.3% (n=8) Nil co-morbidities declared: 16.9% (n=11)					
Mean Ranibizumab intravitreal injections over six, monthly follow-ups (mean, SD)	1.94 (SD = 0.98)					
Serum Inflammatory Protein; Units	**Serum Concentration (Cohort): 1st Quartile; Median; 3rd Quartile; Variance**					
C-Reactive Protein (CRP)	1.23 mg/L; 2.81 mg/L; 7.82 mg/L; 571.21 mg/L					
Tumour Necrosis Factor-α (TNF-α)	0.098 pg/ml; 0.136 pg/ml; 0.179 pg/ml; 0.078 pg/ml					
Interleukin-1β (IL-1β)	0.004 pg/ml; 0.010 pg/ml; 0.023 pg/ml; 0.012 pg/ml					
Interleukin-2 (IL-2)	0.009 pg/ml; 0.017 pg/ml; 0.033 pg/ml; 0.010 pg/ml					
Interleukin-6 (IL-6)	0.180 pg/ml; 0.300 pg/ml; 0.492 pg/ml; 2.723 pg/ml					
Interleukin-8 (IL-8)	0.964 pg/ml; 1.462 pg/ml; 2.067 pg/ml; 61.87 pg/ml					
Complement Pathway Activity*	**Percentage activity relative to positive control (Cohort) 1st Quartile; Median; 3rd Quartile**					
Classical Complement Pathway (CP)	95.57%; 102.2%; 117.1%;					
Alternative Complement Pathway (AP)	89.29%; 97.27%; 115.7%;					
Gene sequencing analysis undertaken on patient cohort						
Gene / DNA region	**Reference SNP**	**Chromosome and Position (bp)**	**Major / Minor Allele**	**MAF**	**OR**	**Reference**
CFH region	rs1061170	Chr 1; 196,659,237	T / C	0.61	2.41	11
CFI region	rs10033900	Chr 4; 110,659,067	C / T	0.52	1.18	11
SERPING1 / C1-INH	rs2511989	Chr 11; 57,134,901	G / A	0.45	0.63	15
CFB	rs641153	Chr 6; 31,914,180	C / T	0.05	0.54	11
C2	rs9332739	Chr 6; 31,903,804	G / C	0.02	0.46	11
C3	rs2230199	Chr 19; 6,718,387	G / C	0.24	1.53	11

* Measurement of classical or alternative complement pathway activity is expressed as a percentage relative to the fluorescence intensity of a positive control.
SD= Standard Deviation. SNP = Single Nucleotide Polymorphism. MAF = Minor Allele Frequency. OR = Odds Ratio of AMD.


A
Alternative Complement Pathway (AP)
Change in Visual Acuity Score from Baseline
*
n= 85 visits of 16 patients
n= 77 visits of 16 patients
Mean Conc. < 1st Quartile
Mean Conc. > 3rd Quartile
B
Alternative Complement Pathway (AP)
Percentage change in Central Macular Thickness from Baseline
n= 72 visits of 16 patients
n= 65 visits of 16 patients
Mean Conc. < 1st Quartile
Mean Conc. > 3rd Quartile
C
C-Reactive Protein (CRP)
Change in Visual Acuity Score from Baseline
*
n= 83 visits of 16 patients
n= 83 visits of 16 patients
Mean Conc. < 1st Quartile
Mean Conc. > 3rd Quartile
D
C-Reactive Protein (CRP)
Percentage change in Central Macular Thickness from Baseline
n= 74 visits of 16 patients
n= 68 visits of 16 patients
Mean Conc. < 1st Quartile
Mean Conc. > 3rd Quartile
E
Tumour Necrosis Factor-α (TNF-α)
Change in Visual Acuity Score from Baseline
**
n= 86 visits of 16 patients
n= 84 visits of 16 patients
Mean Conc. < 1st Quartile
Mean Conc. > 3rd Quartile
F
Tumour Necrosis Factor-α (TNF-α)
Percentage change in Central Macular Thickness from Baseline
n= 68 visits of 16 patients
n= 67 visits of 16 patients
Mean Conc. < 1st Quartile
Mean Conc. > 3rd Quartile


A
CFH rs1061170 and
Classical Complement Pathway (CP)
Percentage relative to CP Positive Control
200
150
100
50
0
**
*
No SNP TT
Heterozygous CT
Homozygous CC
No SNP TT (n= 32 visits of 6 patients)
Heterozygous CT (n= 208 visits of 35 patients)
Homozygous CC (n= 143 visits of 24 patients)
B
CFH rs1061170 and
Alternative Complement Pathway (AP)
Percentage relative to AP Positive Control
200
150
100
50
0
**
**
No SNP TT
Heterozygous CT
Homozygous CC
No SNP TT (n= 32 visits of 6 patients)
Heterozygous CT (n= 208 visits of 35 patients)
Homozygous CC (n= 143 visits of 24 patients)
C
SERPING1 / C1-INH rs2511989 and
Classical Complement Pathway (CP)
Percentage relative to CP Positive Control
150
100
50
0
*
No SNP GG
Heterozygous GA
Homozygous AA
No SNP GG (n= 83 visits of 14 patients)
Heterozygous GA (n= 235 visits of 40 patients)
Homozygous AA (n= 65 visits of 11 patients)
D
SERPING1 / C1-INH rs2511989 and
Alternative Complement Pathway (AP)
Percentage relative to AP Positive Control
150
100
50
0
*
No SNP GG
Heterozygous GA
Homozygous AA
No SNP GG (n= 83 visits of 14 patients)
Heterozygous GA (n= 235 visits of 40 patients)
Homozygous AA (n= 65 visits of 11 patients)
E
CFB rs641153 and
Classical Complement Pathway (CP)
Percentage relative to CP Positive Control
150
100
50
0
***
***
No SNP CC
Heterozygous CT
Homozygous TT
No SNP CC (n= 315 visits of 53 patients)
Heterozygous CT (n= 61 visits of 10 patients)
Homozygous TT (n= 7 visits of 2 patients)
F
CFB rs641153 and
Alternative Complement Pathway (AP)
Percentage relative to AP Positive Control
150
100
50
0
*
*
No SNP CC
Heterozygous CT
Homozygous TT
No SNP CC (n= 315 visits of 53 patients)
Heterozygous CT (n= 61 visits of 10 patients)
Homozygous TT (n= 7 visits of 2 patients)