Complotype affects the extent of down-regulation by Factor I of the C3b feedback cycle in-vitro

Short title: In-vitro effects of complotype

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Summary

Sera from a large panel of normal subjects were typed for three common polymorphisms, one in C3 (R102G) and two in Factor H (V62I and Y402H) that influence predisposition to age related macular degeneration and to some forms of kidney disease. Three groups of sera were tested; those that were homozygous for the three risk alleles; those that were heterozygous for all three; and those homozygous for the low risk alleles. These groups vary in their response to the addition of exogenous Factor I when the alternative complement pathway is activated by zymosan. Both the reduction in the maximum amount of iC3b formed and the rate at which the iC3b is converted to C3dg are affected. For both reactions the at-risk complotype requires higher doses of Factor I to produce similar down-regulation. Since iC3b reacting with the complement receptor CR3 is a major mechanism by which complement activation gives rise to inflammation the breakdown of iC3b to C3dg can be seen to have major significance for reducing complement induced inflammation.

These findings demonstrate for the first time that sera from subjects with different complement alleles do behave as predicted in an in-vitro assay of the down-regulation of the alternative complement pathway by increasing the concentration of Factor I. These results support the hypothesis that exogenous Factor I may be a valuable therapeutic for down-regulating hyperactivity of the C3b feedback cycle and thereby providing a treatment for age-related macular degeneration and other inflammatory diseases of later life.

Introduction

Over recent years it has become apparent that there are multiple polymorphisms among complement components which affect their function. The first of these to be described [1] was the C3 F/S polymorphism (R102G) where the F allele (102G) was shown to have increased functional activity [2]. It was shown much later that this increase in function is due to the decreased affinity of C3 convertases including C3F for Factor H [3]. Neither allele is hypomorphic so there is no difference in C3 levels due to the polymorphism [4] and data below.

In the last decade there has been a veritable explosion in the discovery of complement allotypes and their association with disease. These are reviewed by Harris et al [5] who coined the term complotype "to represent the pattern of genetic variants in complement genes inherited by an individual which alters risk for both inflammatory disorders and infectious diseases involving complement". Certain complotypes predispose very significantly to a group of inflammatory diseases, of which age related macular degeneration is much the most common. Some of the risk alleles are also associated with atypical haemolytic uraemic syndrome and a group of other kidney diseases now known collectively as C3 glomerulopathies [6] and quite possibly also to other inflammatory diseases of later life, including atherosclerosis [7] and systemic vasculitis [8].

The significant variant alleles that predispose to disease all share the property of increasing the activity of the C3b feedback cycle, which amplifies all complement pathways irrespective of triggering stimulus [9]. Significant alleles that occur at reasonable frequency include C3F, loss of function alleles in Factor H (62I and 402H). They also include a gain of function allele in Factor B (R32 W/Q) but because of

ascertainment difficulties described below it was not possible to include this polymorphism in the present study.

These hyperactive complotypes are likely to be advantageous in conferring resistance against infection in early life but in later life, once IgG antibodies have been formed to the infecting bacteria, become disadvantageous in predisposing to inflammatory diseases. There is therefore considerable interest in finding methods for reversing this hyperactivity of the C3b feedback cycle.

The C3b feedback cycle is discussed in detail in ([9]). Its rate is essentially controlled by the balance of two reactions: the positive feedback reaction where C3b reacts with Factor B which forms the alternative pathway C3 convertase after the Factor B is cleaved by Factor D; and the C3b breakdown reaction where C3b reacts with Factor H (or some other proteins that have co-factor activity for Factor I (CD46 and CR1)) which allows cleavage of C3b to iC3b by Factor I. In the total absence of Factor I the breakdown reaction cannot take place and all C3 is converted to C3b by the positive feedback reaction. However, raising the level of Factor I above the physiological level then has the reverse effect of reducing complement activation by accelerating the cleavage of C3b to iC3b. In the presence of CR1 there is also further cleavage of iC3b to C3c and C3dg. This final Factor I cleavage is important since it destroys the pro-inflammatory effects of iC3b.

It has long been known ([10, 11]) that raising the serum concentration of Factor I and/or of Factor H can down-regulate the feedback cycle, but from a therapeutic perspective, elevating Factor I is more likely to be effective. There are a number of reasons for this:

Firstly, the human serum concentration of Factor I is much lower than that of Factor H (reported around 35 µg/ml and 250 µg/ml respectively). Less Factor I protein would therefore be required to produce an effect.

Secondly, in some diseases and tissue sites, Factor H function is blocked by binding of dimers of Factor H-related proteins to cell surfaces for which they have a much enhanced avidity ([12]). This evidence indicates that this is a dominant pathogenic effect and increasing Factor H concentration is unlikely to overcome the effect on complement amplification.

The third reason, probably the most important, is that the main inflammatory mediator produced by complement activation, iC3b, is produced by Factor I in the breakdown cycle. iC3b reacts with the complement receptor CR3 on neutrophils triggering cell activation and phagocytosis. Augmenting concentrations of Factor H will lead to the formation of more iC3b in the first instance, and will not accelerate the breakdown of iC3b that is already present. Raising Factor I levels, on the other hand will both remove C3b from the feedback cycle and enhance the conversion of iC3b to C3dg ([9] and data in this paper). We are therefore pursuing the possibility of developing Factor I as a therapeutic and this is occurring against the background of the continuing therapeutic development (by King's College London) of Mirococept - a truncated CR1 derivative ([13]) - as a co-factor for the conversion of C3b to iC3b and of C3b to C3c and C3dg ([9]).

CR1 is the only co-factor that allows Factor I cleavage of iC3b to C3dg under physiological conditions ([9]) because the affinity of Factor H for iC3b is too low for it to act as co-factor. At reduced ionic strength, however, Factor H can act efficiently as co-factor for the cleavage of iC3b. When the breakdown of iC3b in serum, carefully cleared of cell fragments, is looked at after many hours of incubation it is not possible

to distinguish between minute contamination with soluble CR1 and extremely inefficient co-factor activity of Factor H – or both. Where iC3b has been fixed on red blood cell CR1 in-vivo (for example in immune complex diseases such as SLE or in cold autoantibody haemolytic anaemia) the CR1 on the cells is removed by proteolysis in the reticulo-endothelial system ([14]) and it is feasible that traces of this could appear in the serum.

As a preliminary investigation, in-vitro complement activation studies have been carried out on serum samples from subjects of three different complotypes, using the canonical alternative pathway activator - yeast cell walls (Zymosan). The effect of Factor I supplementation on iC3b formation and its subsequent breakdown to C3dg was measured. Most experiments were done without adding a source of CR1. This is normally present on human erythrocytes but the combination of a particulate activator and erythrocytes gave problems in the assay used. In some later experiments a soluble CR1 derivative (Mirococept) was used to provide an efficient co-factor for the cleavage of iC3b to C3dg by Factor I.

Materials and Methods

Sera studied

Sera were sourced through the Cambridge BioResource (Rec reference 04/Q0108/44, http://www.cambridgebioresource.org.uk/). DNA samples from the database were genotyped for three SNPs, one in C3 and two in Factor H, where there are known associations with susceptibility to age related macular degeneration and/or atypical haemolytic uraemic syndrome and/or C3 glomerulopathies ([3-6]). Factor B polymorphism is characterised by HindIII RFLP. SNPs in linkage disequilibrium with the HindIII sites can be used to tag this site. We used rs12757487 within the LD region to tag the HindIII site. However, these SNPs could not be investigated by this method since they failed to genotype on a number of different genotyping platforms. Table 1 shows the rs numbers and the nucleotide and amino acid variation that made up the final selection criteria as well as the number of subjects tested and the numbers in each of the selected complotype groups. Volunteers from the Cambridge BioResource were subsequently assigned to three groups according to their genotype at these three SNPs.   These are shown in detail in Table 1.   Briefly, Group A were subjects who are heterozygotes at all three SNPs (and make up 5.6% of the population studied); Group B were subjects who are susceptible homozygotes at all three SNPs (and make up 0.6% of the population studied); and
Group C were subjects who are protective homozygotes at all three SNPs (and made up 3.3% of the population studied).

Equal numbers of volunteers from each group were invited to participate in the study and donate a fresh sample of blood for sera isolation. Written informed consent was received from all volunteers who participated in the study under approved ethics application rec ref: HBREC 2012.06. Consent was received in all cases by Cambridge

BioResource research nurses. The blood was taken and the sera were separated as described ([15]) and stored at -80°C in small aliquots until they were used.

The experimenters remained blind to genotype throughout the study until all the sera had been collected and results had been obtained.

Quantitation of Factor I in the serum samples

The concentration of Factor I was measured in sixty-one sera using commercially available immunodiffusion plates (Bindarid plates from The Binding Site, Edgbaston, Birmingham, UK). The assay was recalibrated using as standards the Factor I purified from plasma and supplied by Comp Tech and the recombinant Factor I prepared by GlaxoSmithKline. It should be noted that there are differences in the way that the concentrations of Factor I can be expressed. The extinction coefficient at 280 nm can be calculated from the amino acid composition to be 1.53 for a solution of 1 mg/ml. This gives the concentration of the "core protein" but ignores the 13.7 per cent carbohydrate in the molecule. For the intact protein the OD used for calibration is 1.35 for 1 mg/ml.

Quantitation of C3, Factor H and Factor B in the serum samples

Antigenic quantitation of C3, Factor H and Factor B in the sera was performed by Dr. Sanja Ugrinovic at the Clinical Immunology Laboratories at Cambridge University Hospitals NHS Trust using standard nephelometric techniques.

Assay for Activation of the Alternative Complement Pathway – the "Clone 9 capture ELISA"

Factor I Two sources of Factor I were tested: Factor I purified from plasma prepared by Comp Tech (Complement Technology, Inc., Tyler, Texas, USA); and recombinant

Factor I prepared by GlaxoSmithKline (Stevenage, UK). The two preparations gave identical activity to protein ratios as measured by conglutination assays (data not shown). All the Clone 9 capture assays reported in this paper were performed using the recombinant Factor I.

Typically a serum from each genotype (A, B and C, eighteen sera in each group) was tested alongside each other on any one experimental day. The Clone 9 antibody ([16, 17]) recognises C3g which appears as a neo-antigen in iC3b and in C3dg but is not exposed in native C3 or in C3b. Therefore, these plates will capture iC3b or C3dg from the reaction mixture. The Clone 4 detection antibody (a monoclonal anti-C3c) ([17]) reacts with a conformational epitope in C3c and reacts with C3, C3b, iC3b and C3c. Therefore, only bound iC3b is detected by this antibody on the Clone 9 coated plate. When iC3b is converted to C3dg the C3c is released and with it the ability to be detected by the biotinylated-Clone 4 antibody.

Using this assay we were able to investigate the effect of adding Factor I to sera of the three different genotypes and also additionally adding a co-factor for the third Factor I cleavage. For this purpose we used Mirococept (previously known as APT070), which contains the first three SCRs of CR1 bound to a membrane-interactive amphiphilic peptide "tail" ([13]). CR1 and this product derived from it are essential co-factors that allow Factor I to cleave iC3b to C3dg and C3c. They also share with other complement control proteins (CD46 and Factor H) co-factor activity for the Factor I conversion of C3b to iC3b. There is very little, if any, soluble CR1 present in sera as is known from the long half-life (more than eighteen hours) of EAC43bi cells incubated in human serum before they lose their (iC3b requiring) reaction with conglutinin ([16]). This is unlike the situation in whole blood where there is CR1 present on the red cells.

Experimental protocol

Stage 1: The sera were thawed rapidly at 37°C, vortexed briefly, and then placed on ice. The sera were then treated with zymosan with the addition of recombinant Factor I (to 25 or 50 µg/ml) and/or Mirococept (APT070) (to 10, 20, 40 or 80 µg/ml). Zymosan (Sigma) was diluted in "Alternative Pathway buffer" (veronal buffered saline pH 7.2 containing 10mM EGTA and 2 mM Mg). The soluble additives were added to 500µl aliquots of serum prior to addition of zymosan to a final concentration of 5% and rotated in a 37°C incubator. At selected time points 40µl of each sample was removed into 10µl of 50mM EDTA. The sampling times were: 0, 30, 60, 120, 180, 240, 480 minutes. At each time point the samples were microfuged to remove the zymosan and frozen at -80°C until tested by ELISA.

Stage 2: Coating. Nunc Maxisorb microtitre plates were coated with a Clone 9 anti-C3g antibody (50 µl/well Clone 9 at 25 µg/ml in 0.1M carbonate/bicarbonate coating buffer pH 9.5), incubated overnight at 4°C, followed by 5 washes in PBS/0.05%Tween 20. The plates were blocked with PBS/0.05%Tween 20/4% dried skimmed milk (2 hours room temperature) then washed again as above.

Stage 3: Capture. 50 µl supernatants of the incubation mixtures as described above were loaded onto the plates and incubated 1 hour at room temperature.

Stage 4: Detection. Captured antigen (iC3b) was measured using biotinylated Clone 4 anti-C3c monoclonal antibody ([17]). After washing the plates (5X PBS/0.05%Tween 20), 50 µl biotinylated-Clone 4 at 5 µg/ml diluted in PBS/gelatin was added to each well and the plate incubated for 1 hour at room temperature. The plates were washed (5X PBS/0.05%Tween 20) before incubation with the Extravidin-peroxidase (Sigma Cat. No. E2886) diluted 1/1000 in PBS/gelatin for 1 hour at room temperature. The plates

were washed (5X PBS/0.05%Tween 20) and then incubated with 100 µl per well TMB substrate (Life Technologies) for 15-30 minutes. The reaction was stopped by the addition of 1M H_2SO_4 (50 µl per well) and the plates read immediately at 450nm on a plate reader. The maximum OD achieved in the first two hours of incubation is taken as a measure of the extent of activation of the alternative pathway and the percentage reduction of this level to its lowest level (usually at eight hours) as a measure of the breakdown of iC3b to C3c and C3dg.

Statistical Analyses

All the comparisons made were of ratios of amounts of iC3b generated in a particular serum in a single experiment. Ratios (which have variance in both numerator and denominator) have to be analysed using non-parametric methods and the appropriate test, the Kruskal-Wallis test, was performed using the Graph Pad Prism programs. Because of the influence of acute phase reactions the level of the individual components was normally distributed and the same non-parametric statistics have been used.

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Results

Antigenic concentrations of Factor I, C3, Factor H and Factor B

The concentration of Factor I in 61 serum samples was measured using immunodiffusion plates. Figure 1a shows the distribution of serum Factor I levels across the different genotypes. Although there is a trend in the mean levels of Factor I suggesting that genotype C>A>B, these differences are not significant on statistical analysis across all groups; nor when comparing just Genotype C with Genotype B. The mean level for the whole population was 45 µg/ml (10th – 90th percentile 34 – 52 µg/ml). This compares well with the mean level of 40 µg/ml (10th – 90th percentile: 27– 84 µg/ml) found by Reynolds et al ([4]) using a similar assay and performed in a complement laboratory of high repute; and is not greatly different from the long-standing estimate of 35 µg/ml ([18]).   These results show that the effect of complotype does not depend on systematic differences in the pre-existing levels of Factor I.

C3, Factor H and Factor B levels are shown in Figure 1b, c and d. There is no significant correlation between the levels of any of these components and genotypes. It is indeed the case that the median level in the C3S homozygotes (Genotype C) is slightly higher than in the C3F homozygotes (Genotype B).   There is one normal subject in the (protected) Genotype C group who clearly has an acute phase reaction with markedly elevated levels of C3, Factor H and Factor B and some elevation of Factor I.   It is however already known ([4]) that the genetic variants in C3 and Factor H that define the complotypes in this study do not show systematic differences in C3 or Factor H levels. Their differences are due to differences in activity not in levels of expression. Sera depleted of the relevant components and reconstituted with purified components of appropriate complotype (C3102G/fB32R/fh62V) have been reported to show a 6-fold enhanced haemolytic titre in an alternative pathway assay compared with sera reconstituted with complotype C3102R/fB32Q/fH62I([3]).

Down-regulation of Alternative Complement pathway by raising the concentration of Factor I

Recombinant human Factor I was added to serum from eighteen subjects of each of the three genotypes and complement was activated using zymosan as described. The amount of soluble iC3b (not bound to zymosan) was measured using the Clone 9 capture assay. The effect of adding various concentrations of Factor I to a single serum sample from each genotype is shown in Figures 2 (i) – 2 (iii) which also show a typical time course for the Clone 9 assay. The data in Figure 2 show that Genotype B serum is substantially more resistant to the action of added Factor I when compared to the genotype A and C serum. In order to assess the effect of 50 μg/ml supplementation of Factor I on complement activation, the OD at maximum (before 2 hours) and following conversion of iC3b to C3dg (8 hours) was compared. The measurements chosen to analyse the test data were:

i. the percentage reduction of optical density produced by the addition of 50 µg/ml Factor I to the maximum reading achieved in the first two hours;

ii. the percentage reduction at eight hours.

The first of these readings measures the reduction in the amount of iC3b produced by the zymosan activation; and the second measures the reduction in the amount remaining after conversion to C3dg is anticipated to be substantially complete.

The percentage reductions brought about by the addition of Factor I at both time points (Figures 3 and 4) differ highly significantly between the three groups, with Genotype C > Genotype A > Genotype B. The differences observed between the three genotype groups are even more striking considering that the sera taken were not controlled for age or body mass index or for the possible occurrence of any acute phase reaction (all of which can modify complement levels). Therefore, the data in this study indicate that

the genotype differences observed were considerably robust. A summary of all the results obtained with sera of the three different genotypes in their response to the addition of 50µg of Factor I is shown on Table 2.

All these tests were carried out using serum, which does not represent the physiological situation in-vivo where CR1 is present on red blood cells in the circulation, and additionally on leucocytes at sites of inflammation. Notably, CR1 is the only co-factor in humans which mediates the Factor I clip of iC3b to C3dg under physiological conditions ([19]). To assess the effect of added co-factor, we have used a "tailed" recombinant CR1 functional protein fragment, Mirococept. This compound was tested at several dilutions using only the amount of Factor I present in the serum and also with supplementation of Factor I at 25 and 50µg/ml. These results are shown in Table 3. It can be seen that Mirococept has major concentration-related effects on both the maximum iC3b deposition and on its breakdown, showing that CR1 is a powerful inhibitor at both stages. Nevertheless, even at high concentrations of Mirococept (80 µg/ml) (initial trials of Mirococept in perfusing kidneys before transplantation used 10µg/ml) ([20]), an effect of adding further Factor I is still apparent. It is clear, therefore, in the presence of co-factor, the down-regulatory action of Factor I supplementation will be greater.

Discussion

It is now well established that polymorphisms in complement components that can cause acceleration of the C3b feedback cycle predispose to a group of inflammatory diseases including age related macular degeneration and haemolytic uraemic syndrome and C3 glomerulopathies. In this study we have examined a number of sera from normal donors who have been genotyped for three of these polymorphisms which are fairly common:

the C3S/F polymorphism (R102G) where the rarer allele C3F (102G) with a frequency around 0.2 in Caucasoids and much lower in other racial groups confers susceptibility;

the Factor H polymorphism (Y402H) where the rarer allele (402H) with a frequency of 0.38 confers susceptibility;

the Factor H polymorphism (V62I) where the commoner allele (62V) with a frequency of 0.81 confers susceptibility.

Serum was taken from subjects who were homozygous for all three susceptibility alleles (Genotype B), or were homozygous for all three protective alleles (Genotype C), or were heterozygous at all three loci (Genotype A). Of the 9989 individuals tested who gave results at all three loci, 55 (0.6%) were Genotype B; 331 (3.3%) were Genotype C; and 558 (5.6%) were Genotype A. Together these make up just under 10 per cent of the population tested. These were normal volunteers not selected by family or clinical history for age related macular degeneration or renal disease. The susceptible Genotype B represents a very small percentage of the population.

The sera were tested in-vitro for the extent of inhibition mediated by supplementation of Factor I to the activation of the alternative pathway by zymosan. The production of iC3b

and its subsequent conversion to C3dg was tested in the absence or presence of a derivative of CR1 which provided co-factor activity for the split from iC3b to C3dg.

The study demonstrated that there were no significant differences in Factor I concentrations between the three genotypes as measured antigenically although individual sera varied fairly widely. There is also quite extensive within-group variation in the rate and degree of complement activation by zymosan. This is not unexpected since the donors will vary in age and sex and body mass index and their acute phase state was not assessed at the time they were bled.

On the other hand, there was a substantial and highly significant difference between the mean of the different genotypes on the magnitude of the lowering effect of Factor I supplementation on iC3b formation and on the conversion of iC3b to C3dg. The at-risk genotype (Group B) showed a smaller degree of inhibition by Factor I compared to the protected genotype (Group C) with the heterozygous genotype (Group A) showing intermediate values. This was the case both at the time of maximal activation within the first two hours, which is a measure of the formation of iC3b, and at eight hours which is a measure of the breakdown of iC3b to C3dg.

Our results demonstrate that complement genotype at just three loci has a striking influence on the regulation of alternative pathway complement activation by Factor I – the hyperinflammatory complotype being more resistant to down regulation by increased Factor I concentration . We have, furthermore, shown that the addition of a CR1-derived co-factor has a marked enhancing effect in the presence of physiological amounts of Factor I. This effect is further enhanced by the addition of further Factor I and the combination allows the total inhibition of complement activation.   We can

therefore conclude that Factor I supplementation can reverse, in-vitro at least, the effects that the risk complotype imposes on the alternative complement pathway. Further experiments on fewer sera using endotoxin as a soluble complement activator, (which allows the effect of erythrocytes as source of CR1 to be assessed) and using a range of Factor I concentrations are to be published separately.

The results obtained encourage further development of Factor I as a therapeutic to down-regulate the alternative pathway (or the C3b feedback cycle). Factor I supplementation may be particularly effective in those individuals who have a genetic predisposition to complement hyperactivity and who show any signs of the associated diseases.

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Figure Legends

Figure 1a. Factor I antigenic concentration in the different genotypes (61 samples). The concentrations were measured by single immunodiffusion on Binderid plates. The results for all sera are shown with the median and interquartile range. The slight trend towards lower levels from Genotypes C (low risk) to A (heterozygotes) to B (high risk) is not statistically significant whether analysed non-parametrically or parametrically. Non-parametric analysis using the Kruskal-Wallis test is shown in the Figure.

Figures 1b, c and d.   Antigenic concentrations of C3, Factor H and Factor B were measured nephelometrically in the Clinical Immunology Laboratory at the Cambridge University NHS Trust by Dr.   Sanja Ugrinovic.   Results are shown with median and interquartile range.   There were no significant differences between groups.

Figure 2 (i), (ii) and (iii). These figures show illustrative time course assays carried out with different concentrations of Factor I using three individual sera, one from each Genotype. The optical density at 450 nm is a measure of the amount of iC3b bound to the Clone 9 plate. A striking change between Genotypes is seen, particularly at the highest concentration of Factor I, where the Genotype B serum has maximum optical density twice that of either of the other Genotypes.

Figure 3. The percentage reduction produced by 50 µg/ml Factor I on the maximum OD 450 nm produced before two hours. The graph shows the individual results with the median and the inter-quartile range. Results were analysed non-parametrically and gave highly significant results by the Kruskall-Wallis test. This data shows the percentage reduction in the amount of zymosan-triggered iC3b produced after supplementation of sera with Factor I. Eighteen subjects in each group.

Figure 4. The percentage reduction in zymosan-triggered iC3b produced by the addition of 50 μg/ml Factor I on the OD 450 nm at eight hours. The graph shows the individual results with the median and the inter-quartile range. Results were analysed non-parametrically and gave highly significant results. This Figure therefore shows the efficiency with which added Factor I allows the final clip of iC3b to C3dg which again shows Genotype C>Genotype A>Genotype B. Eighteen subjects in each group.

Table 1 Sera Studied

rs number	Observed	Variant	Type	Description		Minor Allele Frequency	
				Minor allele	Number tested	Observed	Expected
rs800292	c/t SNP	Factor H	V62I	t (I) protective	10658	0.232	0.213
rs1061170	c/t SNP	Factor H	Y402H	c (H) susceptible	10551	0.383	0.42
rs2230199	c/g SNP	C3 SF	R102G	g (G) susceptible	10586	0.206	0.19

Number with 3 genotypes		9989	
Genotype C	Homozygous protective at all three loci	331	3.3%
Genotype A	Heterozygous at all three loci	558	5.6%
Genotype B	Homozygous susceptible at all three loci	55	0.6%

Table 2 Summary of results

		Genotype C (low risk homozygotes)	Genotype A (heterozygotes)	Genotype B (high risk homozygotes)		
FI concentration (μg/ml)* Shown in Figure 1a	**Median**	**46.00**	**43.00**	**43.00**	**Kruskal-Wallis statistic**	**2.706** **p=0.2584**
	25% Percentile	40.00	40.00	34.00		
	75% Percentile	52	52	52		
	Interquartile range	12.00	12.00	18.00		
C3 concentration (μg/ml) Shown in Figure 1b	**Median**	**1.20**	**0.99**	**0.98**	**Kruskal-Wallis statistic**	**3.707** **p=0.1567**
	25% Percentile	0.92	0.93	0.90		
	75% Percentile	1.43	1.25	1.14		
	Interquartile range	0.52	0.32	0.24		
Reduction by 50 μg/ml FI **of maximum reading (%)**	**Median**	**37.2%**	**29.1%**	**22.9%**	**Kruskal-Wallis statistic**	**19.66** **p=0.0001**
	25% Percentile	29.6%	20.3%	13.4%		
	75% Percentile	49.1%	39.7%	26.4%		
	Interquartile range	19.5%	19.4%	13.0%		
Reduction by 50 μg/ml FI **of 8 hr reading (%)**	**Median**	**53.5%**	**44.0%**	**28.2%**	**Kruskal-Wallis statistic**	**18.41** **p=<0.0001**
	25% Percentile	46.4%	32.6%	16.9%		
	75% Percentile	61.0%	50.4%	43.7%		
	Interquartile range	14.6%	17.8%	26.8%		

*Sera activated with Zymosan in Mg/EGTA buffer.
18 sera/group. Each serum is from a different individual.

Table 3 Effects of Factor I and Mirococept combinations on iC3b formation and destruction in a normal serum

Factor I concentration µg/ml		0	0		25	25		50	50
		MAX	MIN		MAX	MIN		MAX	MIN
Mirococept	**0**	100%	76%		99%	62%		83%	42%
concentration	**10**	65%	49%		74%	51%		63%	47%
µg/ml	**20**	63%	59%		42%	42%		33%	17%
	40	47%	37%		36%	25%		20%	16%
	80	31%	23%		16%	5%		3%	0%

Values expressed as % of maximum value with no additions of Factor I or Mirococept
Max = maximum value within first two hours
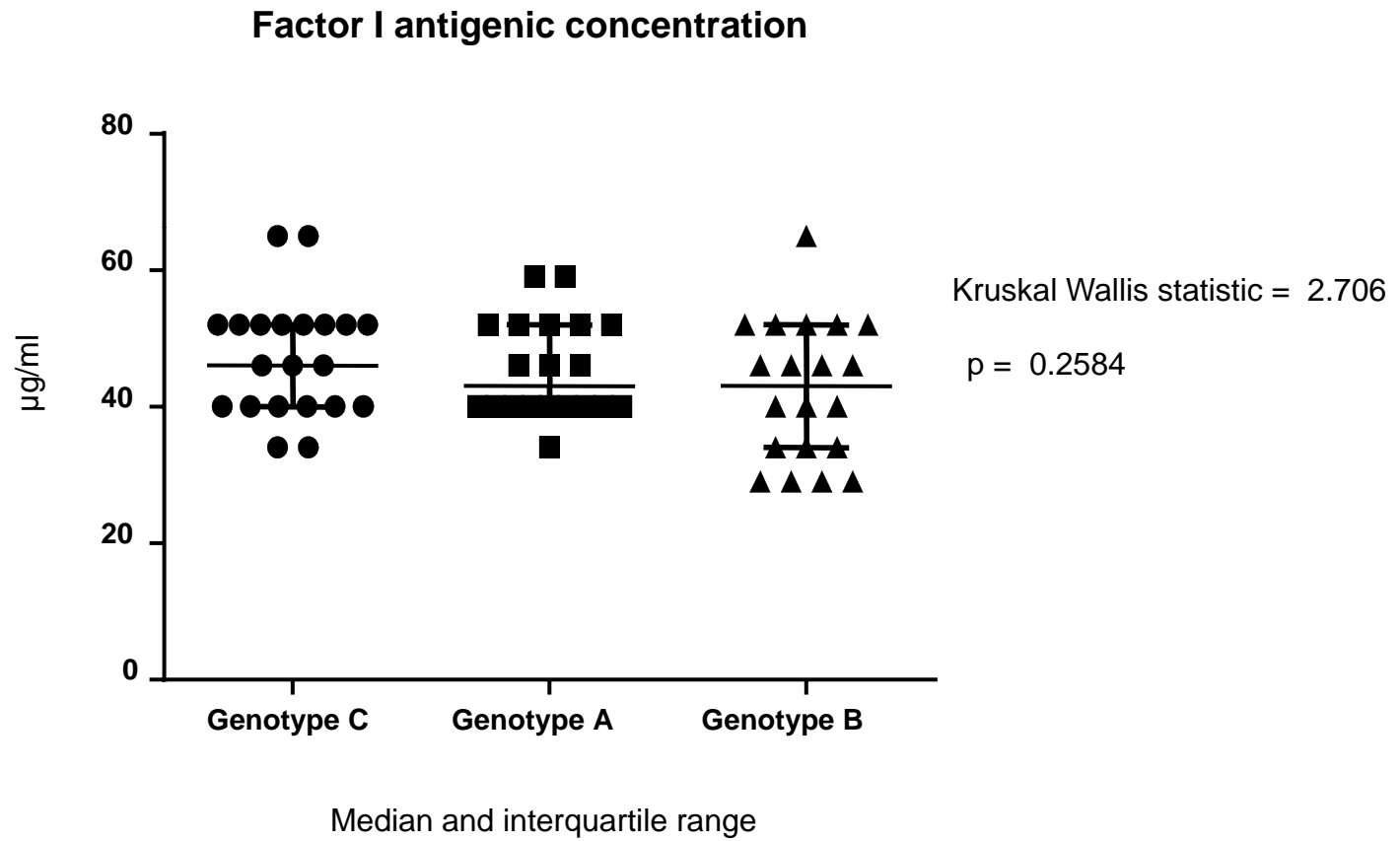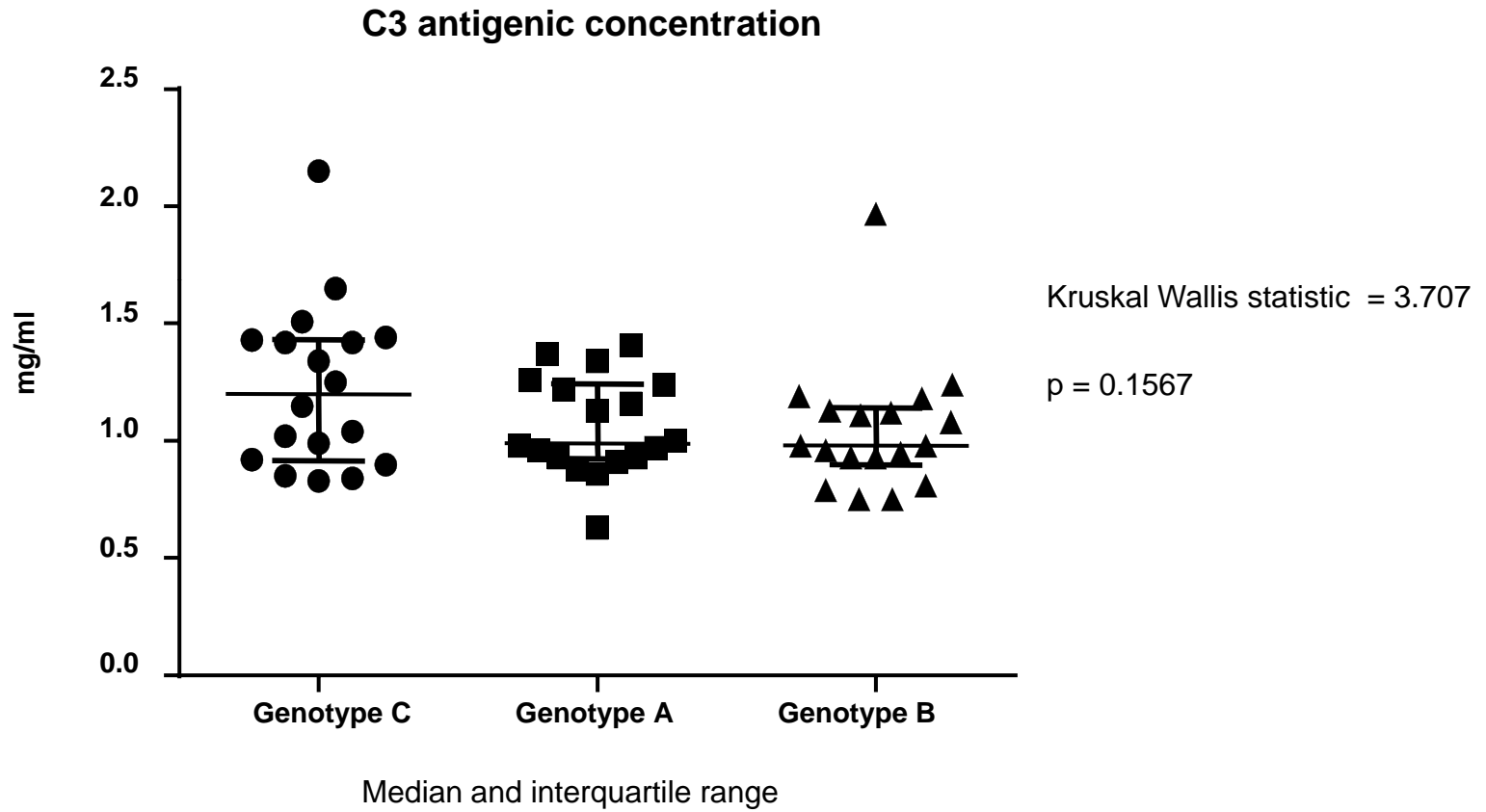Min = minimum value whenever it occurs

Figure 1a

Factor I antigenic concentration

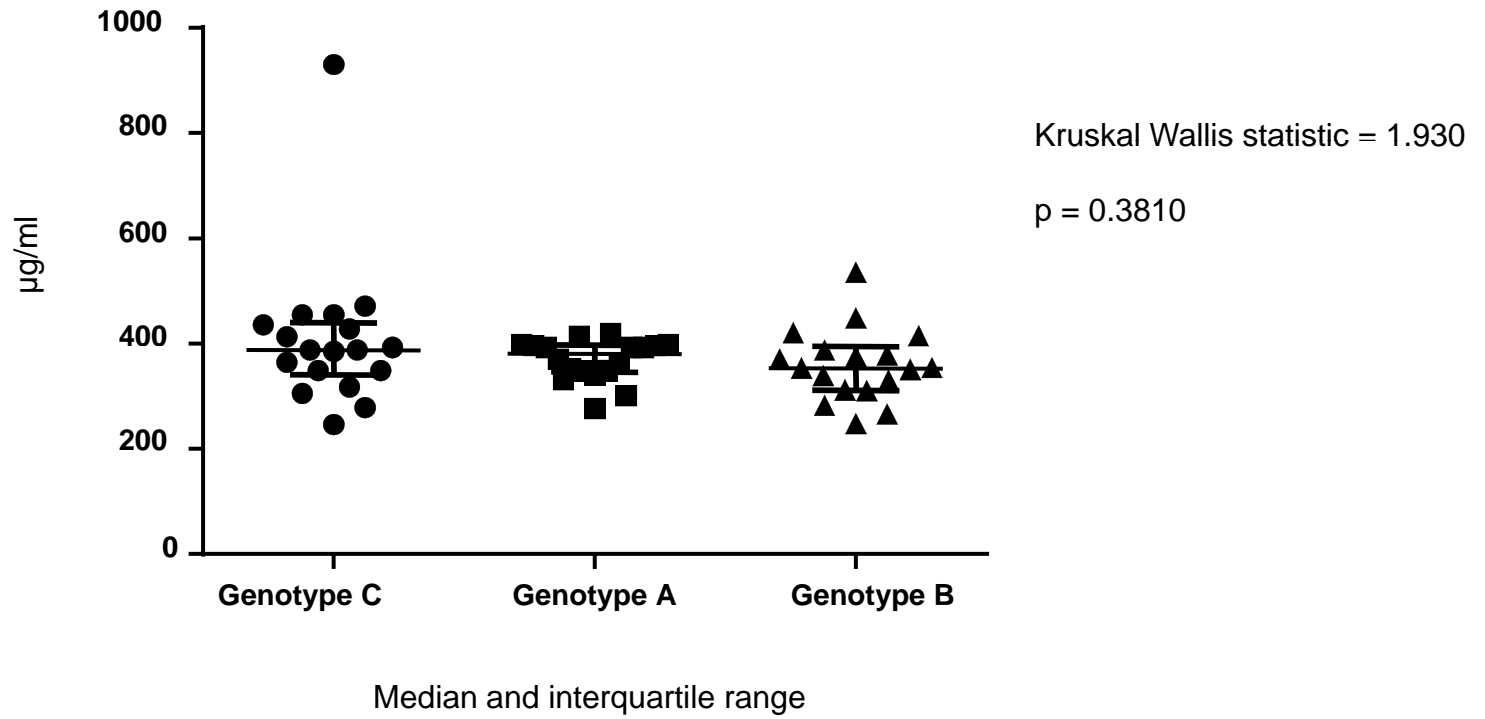Kruskal Wallis statistic = 2.706

p = 0.2584

Median and interquartile range

Figure 1b

C3 antigenic concentration

Kruskal Wallis statistic = 3.707

p = 0.1567

Median and interquartile range

Figure 1c

Factor H antigenic concentration

Kruskal Wallis statistic = 1.930

p = 0.3810

Median and interquartile range

Figure 1d

Factor B antigenic concentration

Kruskal Wallis statistic = 1.199
p = 0.5491

Median and interquartile range
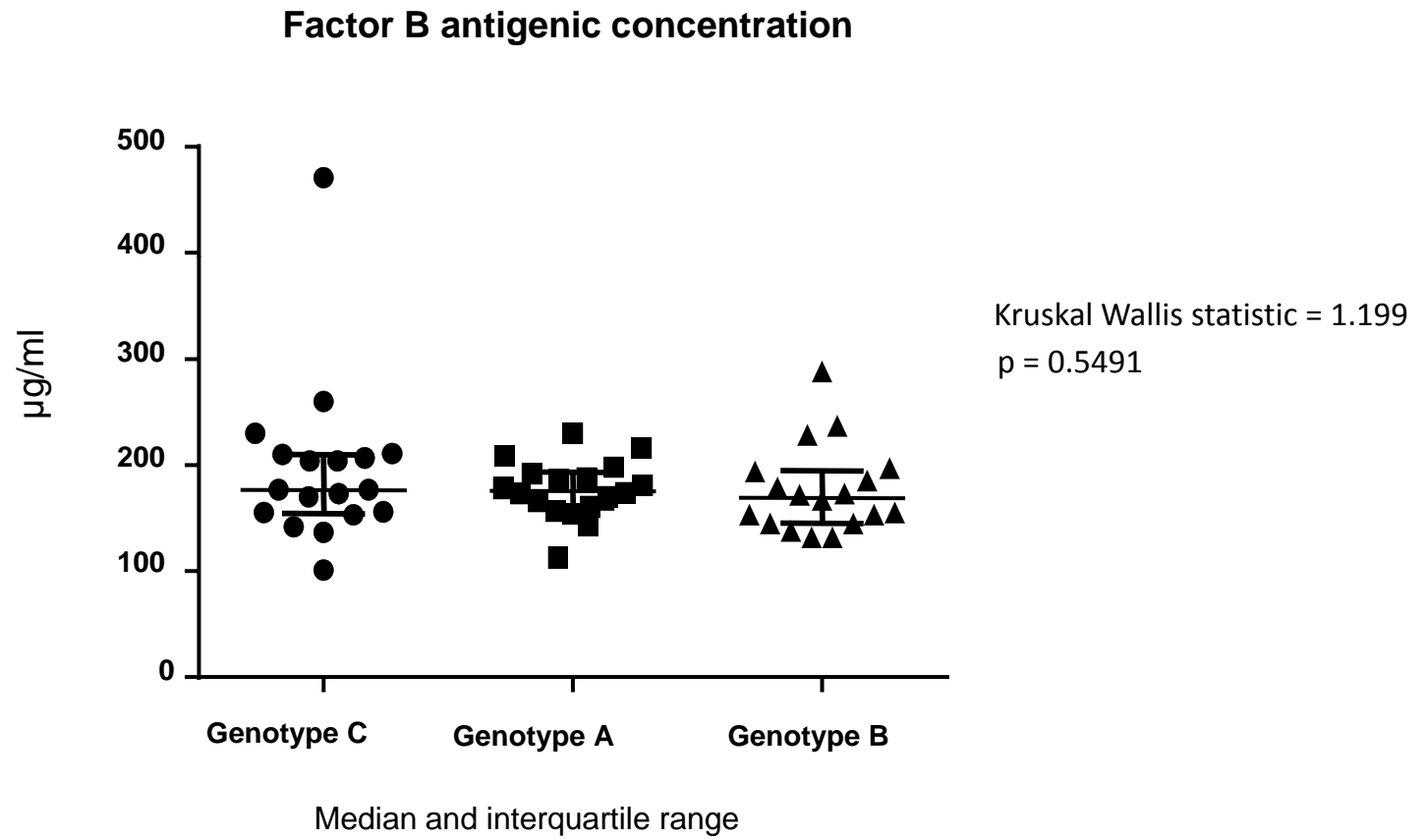
Figure 2 (i)

FI Dose Response Assay

Legend:
- Serum+zymosan
- Serum+zymosan+FI(50ug/ml)
- Serum+zymosan+FI(100ug/ml)
- Serum+zymosan+FI(150ug/ml)
- Serum+zymosan+FI(200ug/ml)

Axis labels: OD 450nm (y-axis), time mins (x-axis)

Figure 2 (ii)

FI Dose Response Assay

OD 450nm vs Time mins
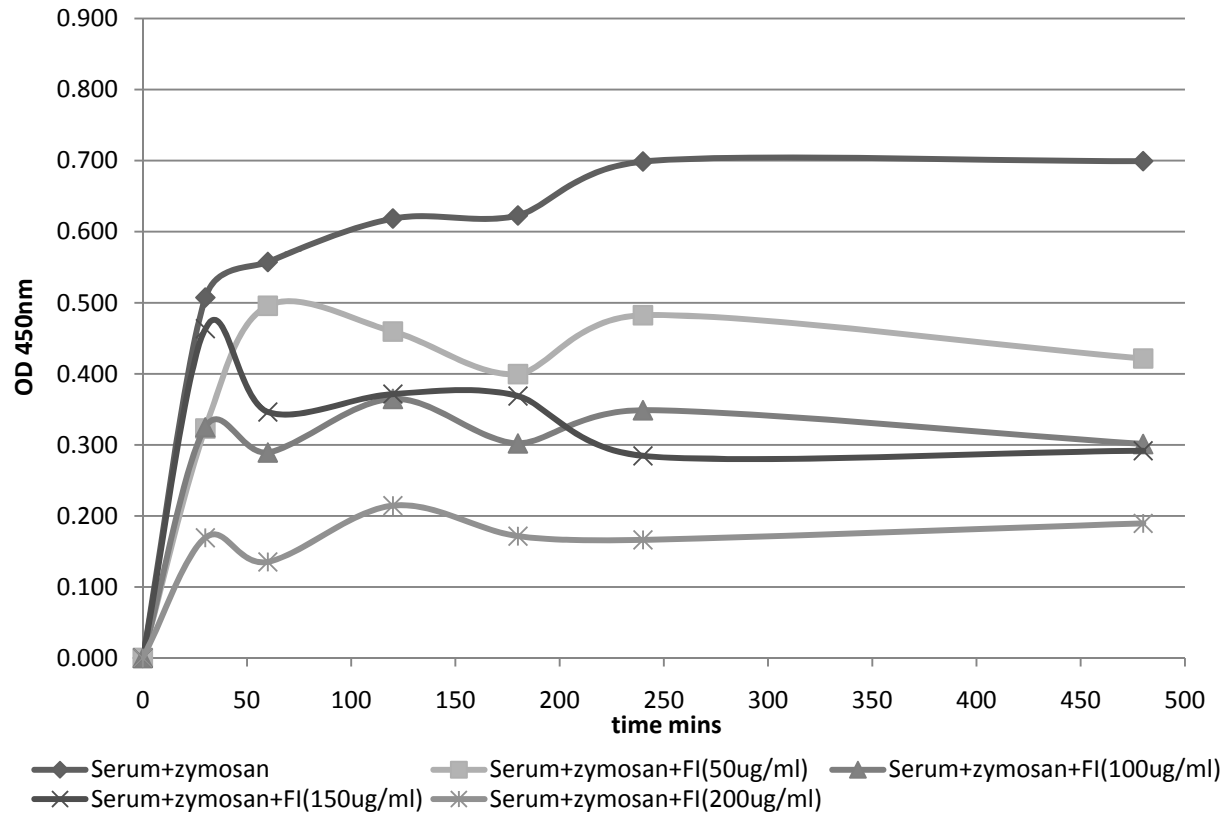
Legend:
- Serum+zymosan
- Serum+zymosan+50ug/ml FI
- Serum+zymosan+100ug/ml FI
- Serum+zymosan+150ug/ml FI
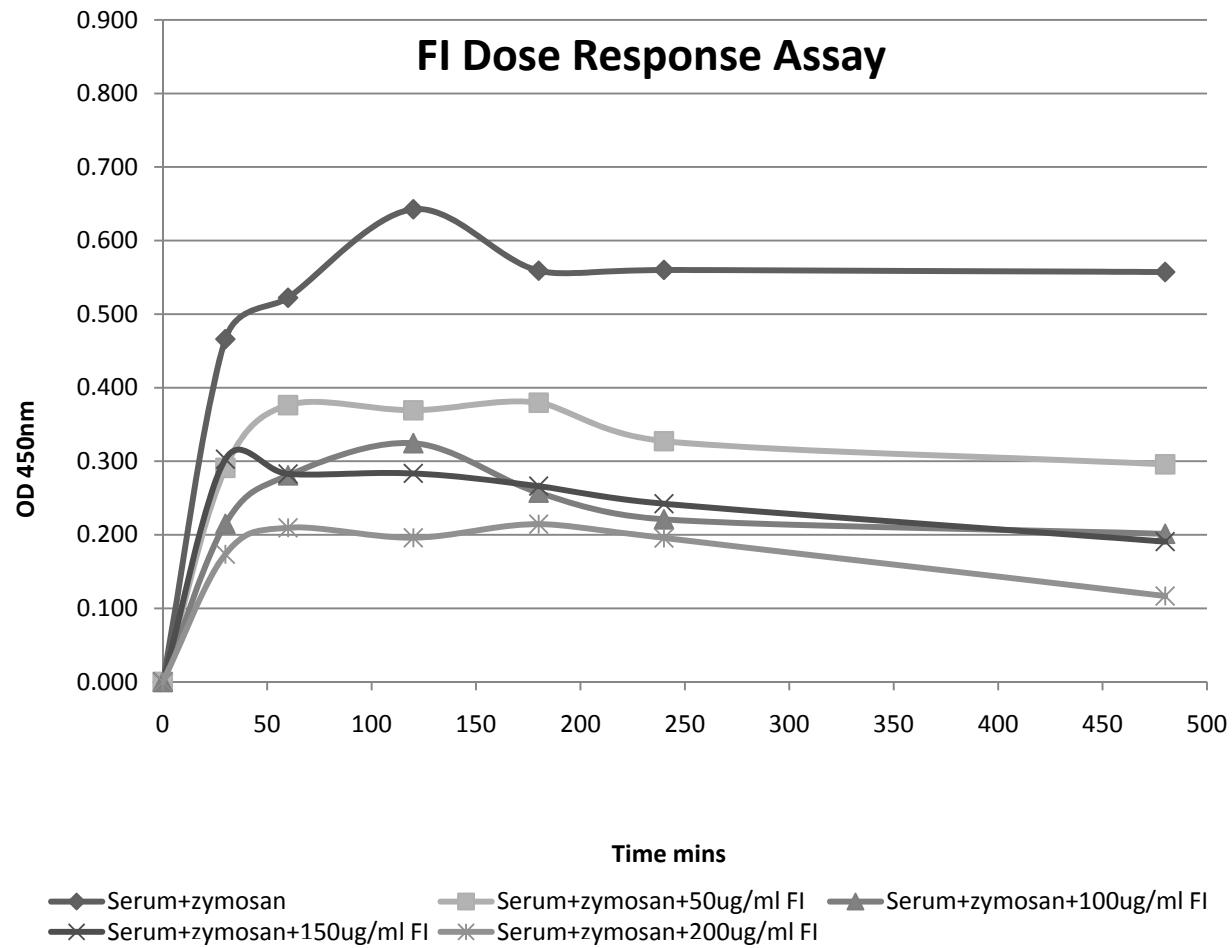- Serum+zymosan+200ug/ml FI
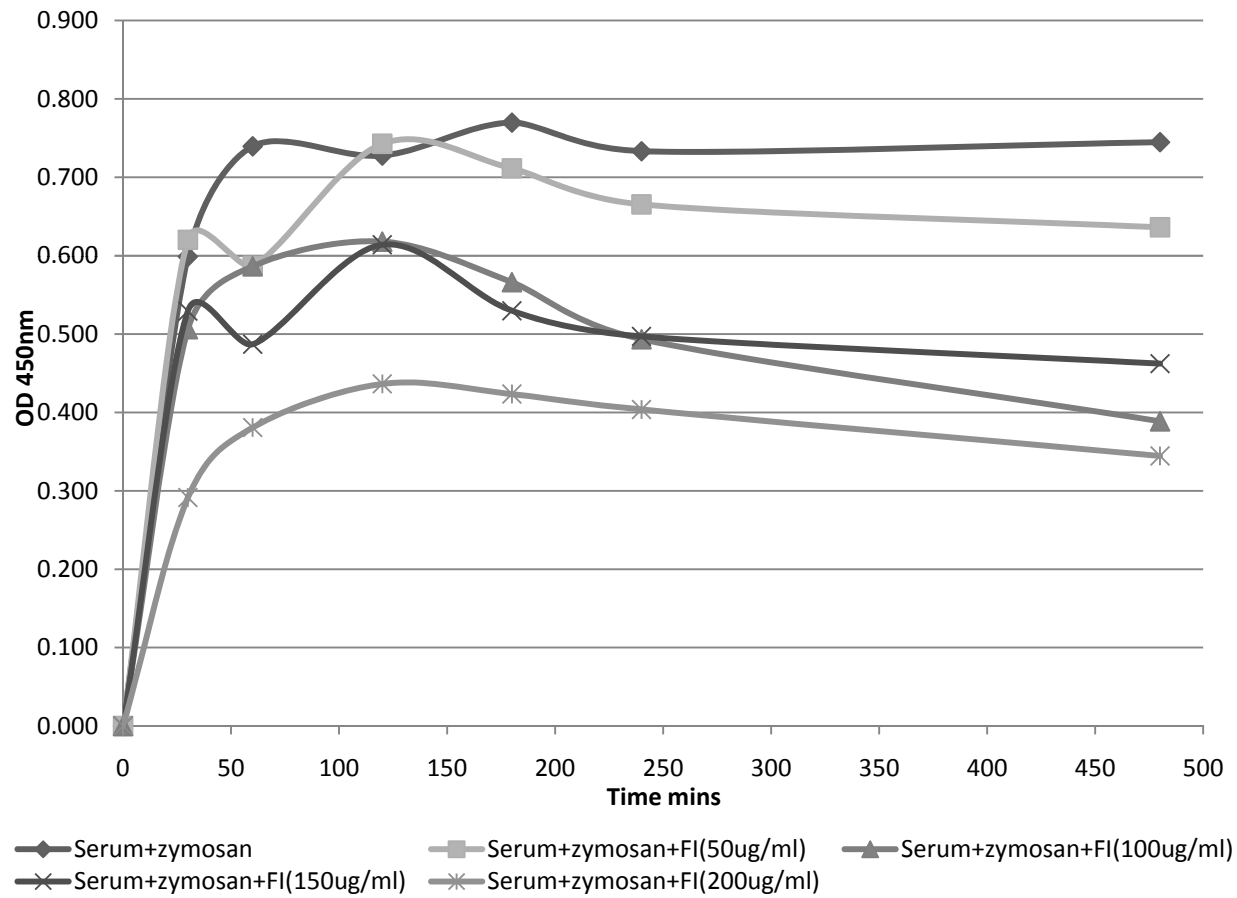
Figure 2 (iii)

FI Dose Response Assay

Figure 3
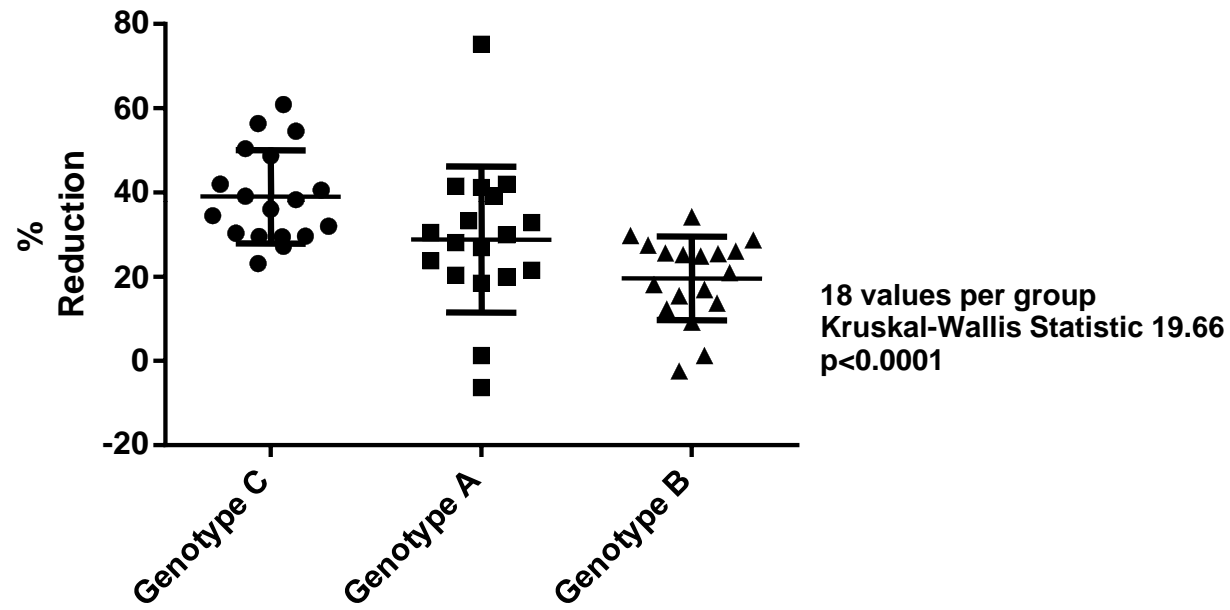Percentage reduction in zymosan-triggered iC3b by exogenous Factor I

Figure 4
Percentage reduction at 8 hours in zymosan-triggered iC3b by exogenous Factor I

18 values per group
Kruskal-Wallis statistic 18.41
p = .0001