2011
2012

FACULTY OF SCIENCES

Master of Statistics: Biostatistics

Masterproef

Exploratory analyses to assess the impact of a CMV infection on the immunogenicity of a flu vaccine

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Master Thesis nominated to obtain the degree of Master of Statistics , specialization Biostatistics

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Certification

I declare that this thesis was written by me under the guidance and counsel of my supervisors.

..... Date.....

Agbor Geraldine Manyi

We certify that this is the true thesis report written by **Agbor Geraldine Manyi** under our supervision and we thus permit its presentation for assessment.

..... Date.....

Prof. dr. Tomasz Burzykowski Internal Supervisor

..... Date.....

Ms. Carline Vanden Abeele External Supervisor

..... Date.....

Mr. Walthère Dewé External Supervisor

Acknowledgement

I am highly indebted to my supervisors Prof. dr. Tomasz Burzykowski, Mr. Walthère Dewé

and Ms. Carline Vanden Abeele for their immense contribution and their constant advice and guidance throughout this research work.

I thank the staff of Center for Statistics, Hasselt University , especially my Professors for the knowledge I have acquired through them.

I would like to express my profound gratitude to my parents Dr. and Mrs. Edmund Agbor for their invaluable support, constant advice and encouragement throughout.

I also thank my sibling Renzo, Edmund, Loredana and Audrey for constantly instilling in me the “Yes I can” spirit.

I appreciate my friends Adeline Ndifor, Jean Filbert, Wami Welcome, Sylvanus Fonguh, Victor Jong Lih, Doreen Abaine and the Cameroonian UHasselt community for the support and the memorable moments we shared.

Finally, I thank God for making all this possible.

Note of Confidentiality

Abstract

Introduction: Influenza virus infection, caused by influenza viruses, is a worldwide, highly contagious respiratory disease that is self-limiting in immunocompetent persons but life–threatening in immunocompromised subjects as well as in the older population. Of interest to the public health are H1N1 and H3N2 and the B strains of the virus. Research has shown that younger subjects respond better to influenza vaccines than the elderly. There are speculations that pathogens like the cytomegalovirus (CMV) may influence the response to influenza vaccine. The objective of this study is to investigate the relationship between the CMV status and response to the influenza vaccines; and to assess whether the response varies from one treatment group to another.

Data and Method: The data contains measurements for hemagglutinin inhibition serological tests to detect antibodies to influenza virus and serological tests based on ELISA technique to determine the CMV status. Measurements of the response to the vaccine were based on seroconversion, seroprotection and Geometric Mean Titers (GMT). Exploratory analyses were carried out using test statistics, graphical techniques, Linear mixed models and Logistic regression.

Result: Exploratory analyses with test statistics and graphical techniques revealed a potential difference between CMV seropositive and seronegative subjects in the response to influenza vaccine. Logistic regression models based on seroconversion provided evidence of an association between the CMV status and the response to influenza vaccine for strains H1N1 and B. Results from the linear mixed models provided evidence that the response to influenza vaccine varies between the treatment groups.

Conclusion: CMV seronegative subjects respond better to influenza vaccines than CMV seropositive subjects for strains H1N1 and B based on the seroconversion seroresponse. Response to the influenza vaccine varies between treatment groups based on the GMT seroresponse.

Table of Contents

1. Introduction

Influenza virus infection is a highly contagious respiratory disease that is self-limiting in immunocompetent persons but life–threatening in immunocompromised subjects as well as in the older population (Cox, N., and Subbarao, K. 1999). Influenza viruses, which circulate worldwide, are the etiological agents of seasonal flu. There are three types of these viruses: A, B and C. Types A and B viruses cause seasonal epidemics while type C infections cause a mild respiratory illness, a reason why type C is not included in influenza vaccines. Common symptoms include cough, sore throat, runny or stuffy nose, muscles or body aches, fatigue, headaches, amongst others (WHO, 2009). These viruses are able to undergo point mutation and antigenic variations, thus giving rise to various subtypes based on the different kinds and combinations of virus surface proteins. Influenza A|H1N1 and A|H3N2 are the predominant subtypes of Influenza A viruses circulating among humans (Bush, R. *et al*., 1999; and Webster, R. *et al.*, 1992).

Repetitive vaccination is considered the most effective way to avoid influenza, but unsatisfactory results in the efficacy have been consistent in the elderly, especially the frail, as compared to the younger subjects. However, the vaccines have shown to reduce hospitalization and mortality related with the infection (Govaert, T. *et al*., 1999; Wijma, G., and Lighart, G. 1996; and Bernstein E. *et al*., 1998). Hemagglutination inhibition tests which measure the anti-hemagglutinin antibody titers are used to assess the effectiveness of the influenza vaccine.

There are indications that influenza vaccine efficacy is low in the elderly as a result of immunosenescence. This is characterized by a decline in the protective immune response whereby memory T cells replace naïve T cells and also a decrease in the diversity and function of the T cell population (Kovaiou, R. *et al.*, 2007). A number of studies suggest that infection with the cytomegalovirus (CMV) contribute to the age-related changes in immunity (Pawelec, G. *et al*., 2009; and Looney, R. *et al*.,1999).

CMV is a herpes virus which infects humans of all ages with seroprevalence increasing with age. Once infected, the virus establishes a lifelong latency within the host and can periodically reactivate. Several strains of this virus exist hence there is also re-infection possibility (Staras, S. *et al*, 1994). CMV infection is asymptomatic and rarely causes illness in immunocompetent persons but can cause severe diseases in immunocompromised individuals, fetus and neonates. It can be transmitted through saliva, sexual contact, placental transfer,

breastfeeding, blood transfusion, organ transplantation or hematopoietic stem cell transplantation (Sia, I., and Patel, R., 2000).

Trzonkowski *et al.* (2003) and Olsson *et al.* (2000) indicated that infection with CMV accelerates aging of the immune system, thus contributing to poor responsiveness to influenza vaccine in the elderly. However, a recent study by den Elzen *et al.* (2011) failed to confirm this. In the context of the current study, a clinical trial was carried out in young adults and older subjects, using a standard vaccine versus a new adjuvanted influenza vaccine to assess the impact of CMV infection on the response to flu vaccine. Response to the influenza vaccines was based on the anti-hemagglutinin (HI) antibody titers against the 3 vaccine strains by using the following derived endpoints: the seroconversion rates (the percentage of vaccinees who had either a pre-vaccination titer <1:10 and a post-vaccination titer ≥ 1:40 or a pre-vaccination titer ≥ 1:10 and at least a four-fold increase in post-vaccination titer), and seroprotection rates (the percentage of vaccinees with a serum HI titer ≥ 1:40 that usually is acceptable as indicating protection). For a number of subjects the presence of CMV infection was assessed using 3 different ELISA tests: anti-CMV tegument proteins IgG, anti-gB IgG and anti-CMV IgM.

The objective of this study is to further investigate the relationship between the CMV status and response to the influenza vaccines; and to assess whether the response varies from one treatment group to another. Evidence that CMV infection actually affects the response to influenza vaccines may encourage pharmaceutical industries to produce influenza vaccines whose efficacies are not affected by CMV infections.

This report is structured as follows: Section 2 will comprise the description of the materials and methods, section 3 the presentation of the results, section 4 the conclusion and discussion, and section 5 and 6 the references and appendix respectively.

2. Materials and Methods

2.1.Study Design

The material used for this analysis comes from a randomized active control phase III clinical trial where the subjects were randomly assigned to one of three treatment groups: FluNewEld (elderly subjects ≥65years administered the new influenza vaccine), FluEld (elderly subjects ≥65years administered the licensed influenza vaccine) and FluYng (young subjects 18-40years administered the licensed influenza vaccine) and followed up over time. Blood samples were collected at various time points: before vaccination - noted as day 0; and after vaccination at day 21, day 42 and day 180 and tested for the presence of antibodies to hemagglutinin using 3 different hemagglutination-inhibition (HI) tests, each for one of the influenza strains. Three serological tests, based on the method of ELISA, to detect the presence of CMV infection were also carried out on blood samples collected at day 0, day 42 and day 180.

2.2.Data set

The data set consists of 96 subjects, each randomly assigned to 1 of the 3 treatment groups described above. The following variables were considered for the current analysis: PID; the method, name, unit of measurement, cut-off values, raw results and numeric results for the various serological tests; time of visit; treatment groups; CMV status; among others, a description of which is presented in Table 1.

Table 1: Description of Variables in the Dataset considered in the study

Variabes	Description
PID	Subject Identification Number
Time (Day)	0, 21, 42 and 180
Group	Treatment groups: FluNewEld, FluEld and FluYng
cmvpos	CMV status, where 0=CMV seronegative on Day 0 and 1=CMV seropositive at day 0. Here, seropositive means being seropositive for both anti-gBCMV and anti-CMV tegument tests. If the anti-CMV tegument test result has 'Greyzone' at Day 0, the result is considered seropositive.
Num_res	Numerical results for the serological tests
Log_val	Logarithm of the numerical results

Raw_res	Raw results for the serological tests
Cut_off	Cut-off values for the serological tests.
Method	Hemagglutinin Inhibition (HI) - technique used to detect the presence of antibodies to influenza; while Enzyme-Linked Immnosorbent Assay (ELISA) - technique used for the CMV tests to detect the presence of CMV.
Serotest	Serological test codes: 1320 (FLU A/BRI/07.HA1 AB): HI test for H1N1 strain 1321 (FLU A/URU/07.HA3 AB): HI test for H3N2 strain 1322 (FLU B/BRI/07.HA AB): HI test for B strain 2050: ELISA test for anti-gB CMV 1126: ELISA test for anti-CMV tegument 2140: ELISA test for anti-CMV IgM
Seron_un	Names and units of measurements for serological tests
Protect	Protection Level for HI tests
P	Seroprotection status: 0=No and 1=Yes (titre ≥ protection level (variable 'PROTECT'))
R	Num_res/Num_res at Actref_1: if R≥4 it means the subject has seroconverted; if R<4 it means the subject had not seroconverted.

The variable R was used to identify the seroconversion status. It was dichotomized based on the definition that if R≥4 it means the subject has seroconverted and if R<4, then the subject has not seroconverted. It was then renamed R_1, where:

$$R\_1 = \begin{cases} 1 & if\ seroconverted \\ 0 & if\ not\ seroconveted \end{cases}$$

In addition to the binary response variables; seroconversion (denoted by R_1) and seroprotection (denoted as P), the Geometric Mean Titer (GMT) was also used as a response (continuous) variable in the analysis. The GMT is a surrogate for measuring the response to influenza vaccine. It is the anti-log of the mean of the log titer transformations. It does represent the magnitude of the immune response to the vaccine. R_1 and P only tell whether the subject has responded to the vaccine or not, they are not very informative with respect to the magnitude of the response. Antibody titers below the

cut-off values for the HI tests were given an arbitrary value of half the cut-off for the purpose of GMT calculation.

It is worth noting that HI titers are the reciprocal of the highest serial dilution which causes complete inhibition of agglutination.

A logarithmic transformation is frequently applied to the titers before analysis as it tends to normalize the distribution. Indeed some subjects can have very high immune responses, giving rise to a long-tail on the right hand side of the distribution. Therefore to ensure normality, a logarithm transformation was carried out on the values of the immune response (num_res), thus giving rise to the variable log_val.

2.3.Methodology

Exploratory analyses were carried out in order to gain insight into the data.

Boxplots were used to assess the relationship between PRE anti-CMV titers and response to influenza vaccine. Evolutionary graphs were used to show how the HI titers changed with time. Pearson correlation was used to account for the association between anti CMV titers and HI titers. Two-sample t-tests were used to test for differences between subjects with and without CMV based on the GMT seroresponse while chi-square tests of independence were used to test for differences between seronegative and seropositive CMV subjects for the seroconversion and seroprotection seroresponses.

2.3.1. Two-sample t-test

In this study, the two-sample t-test is used to test whether the mean of the CMV seropositive group is different from the mean of the CMV seronegative group. It is given by

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where s_p is the pooled sample standard deviation, $\bar{x}_1$ and $\bar{x}_2$ are the sample means of the groups for CMV seropositive and seronegative and n_1 and n_2 are the sample sizes for the CMV seropositive and seronegative groups. This statistic has the assumptions that the subjects are randomly and independently selected and the groups from which they are sampled have equal variances. It also assumes that the populations from which the samples are selected both have approximately normal distribution. Thus this statistics follows a t distribution with $n_1 + n_2 - 2$ degrees of freedom. The value obtained from

this statistic is compared with the t-table value with the same number of degrees of freedom. If this value is greater than the table value, the null hypothesis of equal means is rejected, otherwise not.

2.3.2. Chi-Square Test of Independence

Statistical independence in a 2x2 contingency table for example may either imply that the joint probabilities for the variables are equal to the product of their marginal probabilities; or that the conditional distributions of the response variable are identical at each level of the covariate (Agresti, 2002). In this case, it means that the response to influenza vaccine does not depend on the CMV status of a subject. If this condition is untrue, then there is an association between CMV status and the response to influenza vaccine.

In this case, the test is carried out by comparing the sample (observed) cell counts of subjects with (or without) CMV to the estimated expected counts of subjects with (or without) under the null hypothesis of independence. The larger the difference between the observed and expected frequencies, the stronger the evidence against the null hypothesis [17]. The test statistic used to make such comparisons have large-sample Chi-squared distributions. The test statistics for the hypotheses can be written as

$$X^2 = \sum_{i=1}^{I}\sum_{j=1}^{J} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} \quad i = 1,2 \; and \; j = 1,2$$

where *i* represents the seroconversion status (R_1=0/1) and *j* represents cmvpos (0/1).

O_{ij} is the number of observed counts for R_1=0 (or R_1=1) and cmvpos=0 (or cmvpos=1). E_{ij} is the number of expected counts for R_1=0 (or R_1=1) and cmvpos=0 (or cmvpos=1). E_{ij} is obtained from

$$E_{ij} = \frac{[i^{th}\, row\, total][j^{th}\, column\, total]}{[total\, sample\, size]}$$

2.3.3. Area Under the Curve (AUC)

In this study, the interest is not how subjects responded to the influenza vaccine over time, but the effect of CMV infection on the response to influenza vaccine. As a result, the longitudinal aspect of the data will not be used, a reason why the area under the curve (AUC) is considered. In its application, AUC expresses the longitudinal development of the outcome

variable (GMT) as one quantity by summarizing the evolutions of each subject, thus reducing the longitudinal problem into a cross-sectional one (Twisk, 2003). The AUC is calculated as

$$AUC_i = \sum_{j=1}^{n_i} (t_{i,j+1} - t_{i,j})(y_{i,j} + y_{i,j+1})\,/2$$

where n_i is the number of observations for subject i, y_i is the i^{th} measurement of HI antibody titers taken at time point t_i

For each subject, three values of AUCs were obtained, pertaining to the three HI serotests. These AUCs where incorporated into a mixed model to determine the covariate effects on GMT.

2.3.4. Linear Mixed Models

In statistical methodology, there exist a wide range of models and modeling frameworks with different assumptions. In this report, attention will be given the mixed model framework and in particular the linear mixed model framework. This is mainly due to its advantages such as the assumptions (i.e. normality, linearity), the flexibility to allow more than one source of randomness, the ease of use, to mention but a few.

This model assumes that the vector of repeated measurements on each subject follows a linear regression model where some of the regression parameters are population-specific (fixed-effects) while other parameters are subject-specific (random-effects). The subject-specific regression coefficients reflect how each subject responds to the outcome variable (Verbeke and Molenberghs, 2000).

The general form can be written as:

$$Y_i = X_i\beta + Z_i b_i + \varepsilon_i$$

$$b_i \sim N(0, D_i)$$

$$\varepsilon_i \sim N(0, \Sigma_i)$$

$$b_1, b_2, ...b_N \; and \; \varepsilon_1, \varepsilon_2, ...\varepsilon_N \; are independent,$$

Where Y_i is the n-dimensional response vector for subjects $i_1 \leq i \leq N$, N is the number of subjects, X_i and Z_i are (n_i x p) and (n_i x q) dimensional matrices of known covariates respectively, β is a p-dimensional vector containing the fixed effects. D is a general (n x q) covariates matrix with (i, j) elements $d_{ij} = d_{ji}$ and Σ_i (n_i x n_i) covariance matrix which depends on i only through n_i

b_i is the q-dimensional vector containing the random effects and ε_i is an n_i-dimensional vector of residual components.

2.3.5. Logistic Regression

To investigate whether there is an association between CMV infection and the binary response variables seroconversion and seroprotection, ordinary logistic regression models were considered. In addition to being appropriate in modeling binary response in non-correlated data, they also have the elegant property of the parameter estimates being interpreted in terms of odds ratio. Ordinary logistic regression model is a part of the family of generalized linear models in which the response is assumed to have a binomial distribution and that the observations are independent. This method relates the explanatory variables, which are categorical in this study to the binary response variables seroconversion and seroprotection. The mean and response are related through a logit link function. The general logistic function is given as follows:

Let Y_i be the binary response for seroconversion or seroprotection for the i^{th} subject for a serotest and $\boldsymbol{X} = (x_{1,}x_2 and\ x_3)$ be the categorical explanatory variables cmvpos, group and cmvpos*group interaction respectively. Then the general starting model for $\pi(\boldsymbol{X}) = P(\boldsymbol{Y_i} = 1)$ at the different values of the predictor for a subject with index *i* is given by

$$logit\ P(\boldsymbol{Y_i} = 1) = \beta_0 + \beta_1 cmvpos_j + \beta_2 Group_k + \beta_3 cmvpos_j * Group_k$$

where *j*=0,1 representing the CMV status and *k*=FluNewEld, FluEld or FluYng. The **β**'s are the maximum likelihood estimates which are obtained as solutions to full likelihood equations. These **β**'s have an interpretation of log odds ratio. For more details, you can refer to Agresti, 2002 and Kutner *et al.* 2005.

3. RESULTS

In order to gain insight into the data, an exploratory data analysis was carried out. A total of 96 subjects were randomly assigned to the 3 treatment groups: 35 in the FluNewEld group, 36 in the FluEld group and 25 in the FluYng group. There were 24 CMV seronegative and 72 CMV seropositive subjects divided as follows: 29 (40.28%) CMV seropositive and 6 CMV seronegative subjects in the FluNewEld group, 32 (44.44%) CMV seropositive and 4 CMV seronegative subjects in the FluEld group, and 11 (15.28%) CMV seropositive and 14 CMV seronegative subjects in the FluYng group.

Table A1 in the appendix gives details of the sample size with respect to the HI and CMV serotests for the various treatment groups at the various time of visit. In the table, n stands for the number of subjects tested for HI antibodies. Cmvpos=0 stands for the number of subjects detected as CMV seronegative and cmvpos=1 stands for the number of subjects detected as CMV seropositive. Not all subjects have results for the CMV serotests at Day 180. This is not a problem because the CMV status was determined on Day 0.

Values of GMTs and coefficient of variation (CV) at the different time points and for the HI serotests are presented in table 2 below.

Table 2: GMT and Coefficient of Variation for the HI serotests at the different time of visit

		FluNewEld		FluEld		FluYng	
Serotest	Day	GMT	CV	GMT	CV	GMT	CV
1320							
	0	12.18	39.961	14.13	38.415	13.75	51.200
	21	97.43	25.463	60.48	32.934	232.63	20.684
	42	73.88	24.020	51.33	33.960	194.09	21.770
	180	29.68	30.380	30.52	37.161	78.90	32.349
1321							
	0	14.28	49.930	17.98	42.312	13.20	47.178
	21	329.63	22.849	164.57	33.373	162.08	31.382
	42	264.86	23.858	104.75	33.039	114.73	31.538
	180	104.46	30.748	59.91	37.787	81.08	32.631
1322							
	0	70.98	31.994	103.57	26.165	116.27	33.460
	21	615.15	14.385	456.78	19.685	1210.98	11.847
	42	426.50	15.795	380.44	18.060	943.52	12.760
	180	202.91	18.640	241.91	19.397	605.50	15.979

**CV=Coefficient of Variation*

From table 2, it can be seen that for serotests 1320 and 1322, generally the FluYng group seem to have higher GMTs than the FluNewEld and the FluEld groups. Meanwhile for the FluNewEld and the FluEld groups, there is no much difference in the GMTs. However, for serotest 1321, the values in GMTs are not consistently higher for one group as compared to the others.

The coefficient of variation (CV) describes the dispersion of the HI serotests in the different treatment group. The higher the CV, the greater the dispersion. From the above table, the dispersion fluctuates from group to group and it is generally higher in serotests 1320 and 1321 than in 1322.

In table 3, proportions and 95% confidence intervals are presented for seroconversion and seroprotection for the various HI serotest and treatment groups and at the various time points.

Table 3: Proportions and Confidence Intervals for Seroconversion and Seroprotection

		FluNewEld		FluEld		FluYng	
Seroresponse	Day	Proportion	CI	Proportion	CI	Proportion	CI
Seroconversion							
Serotest 1320							
	21	65.71	(49.17-82.26)	50.00	(32.84-67.16)	80.00	(63.15-96.85)
	42	62.86	(46.02-79.70)	41.67	(24.75-58.58)	80.00	(63.15-96.85)
	180	34.29	(17.74-50.83)	25.00	(10.14-39.86)	64.00	(43.78-84.22)
Serotest 1321							
	21	91.43	(81.67-100)	72.22	(56.85-87.59)	84.00	(68.56-99.44)
	42	91.43	(81.67-100)	63.89	(47.41-80.37)	80.00	(63.15-96.85)
	180	80.00	(66.06-93.94)	44.44	(27.39-61.50)	64.00	(43.78-84.22)
Serotest 1322							
	21	71.43	(55.68-87.17)	52.78	(35.65-69.91)	84.00	(68.56-99.44)
	42	68.57	(52.39-84.75)	50.00	(32.84-67.16)	64.00	(43.78-84.22)
	180	40.00	(22.93-57.07)	30.56	(14.75-46.36)	56.00	(35.09-76.91)
Seroprotection							
	0	25.71	(10.48-40.95)	30.56	(14.75-46.36)	24.00	(6.01-41.99)
Serotest 1320	21	88.57	(77.48-99.66)	77.78	(63.51-92.04)	96.00	(87.74-100)
	42	85.71	(73.52-97.91)	69.44	(53.64-85.25)	92.00	(80.57-100)
	180	51.43	(34.01-68.85)	52.78	(35.65-69.91)	76.00	(58.01-93.99)
Serotest 1321	0	28.57	(12.83-44.3)	33.33	(17.16-49.51)	28.00	(9.08-46.92)
	21	100.00	(100-100)	83.33	(70.54-96.12)	88.00	(74.31-100)
	42	97.14	(91.34-100)	80.56	(66.97-94.14)	84.00	(68.56-99.44)

	180	77.14	(62.51-91.78)	69.44	(53.64-85.25)	76.00	(58.01-93.99)
Serotest 1322	0	74.29	(59.05-89.52)	86.11	(74.24-97.98)	84.00	(68.56-99.44)
	21	100.00	(100-100)	97.22	(91.58-100)	100.00	(100-100)
	42	100.00	(100-100)	97.22	(91.58-100)	100.00	(100-100)
	180	97.14	(91.34-100)	97.22	(91.58-100)	96.00	(87.74-100)

As can be seen from Table 3 above, and the proportion was generally higher for serotest 1321 than the other serotests for the FluNewEld and FluEld groups. For the FluYng group, the proportion of seroconversion was very similar for all the three serotests. With respect to the seroprotection seroresponse, higher proportions are generally seen in serotest 1322 as compared to the 1320 and 1321 serotests. Also, higher proportions seem to occur in the FluYng group than the FluNewEld and FluEld groups.

Boxplots were used to visually assess a possible relationship between PRE anti-CMV titers and the response to influenza vaccine.

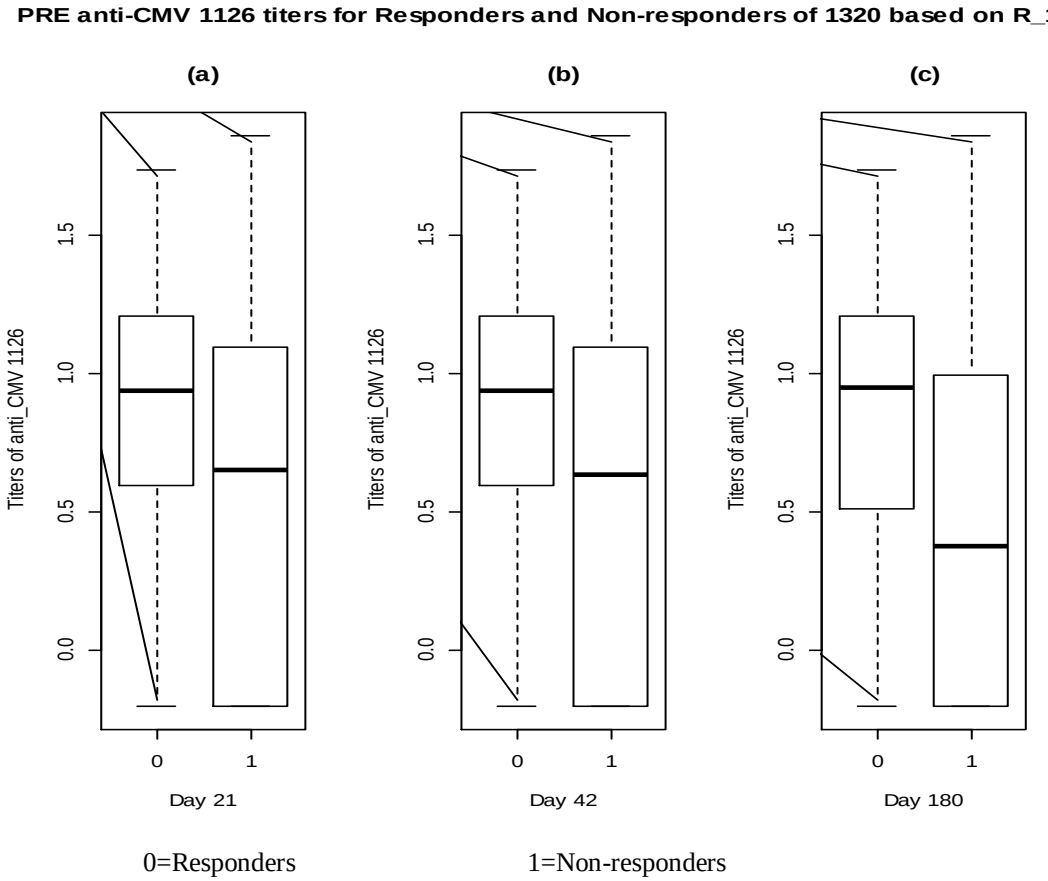
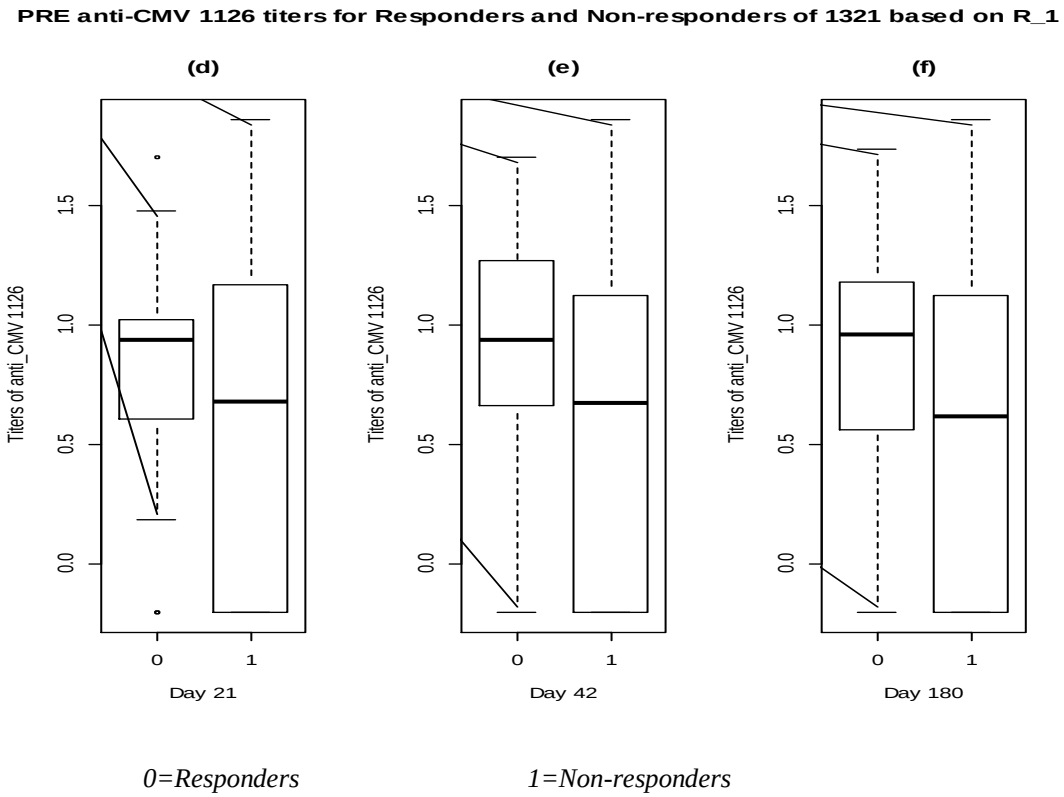


Figure 1: PRE Anti-CMV titers for Responders and Non-Responders for serotest 1320

From Figure 1a and 1c above, it can be seen that responders to the influenza strains H1N1 and B (1320 and 1322 respectively) seem to have a lower PRE anti-CMV titers than non-responders. This may be suggestive of an association between CMV infection and strains H1N1 and B. However, for the H3N2 strain, figure 1b (1321), responders have a higher PRE anti-CMV titer than non-responders.



0=Responders *1=Non-responders*

Figure 2: PRE Anti-CMV titers for Responders and Non-Responders for serotest 1321

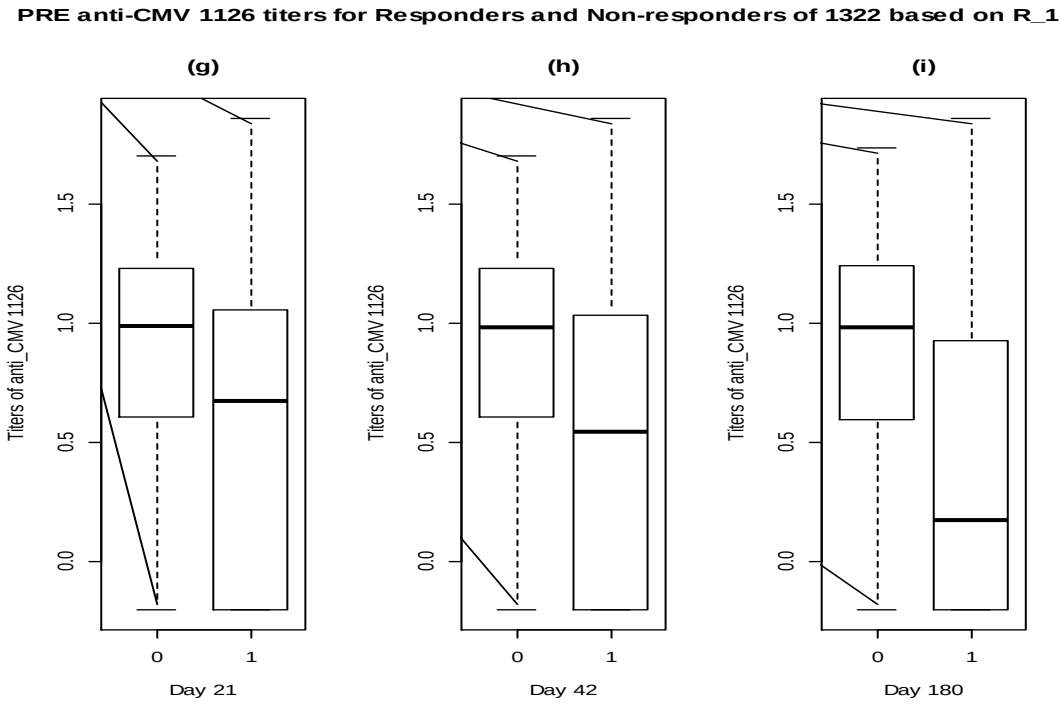


Figure 3: PRE Anti-CMV titers for Responders and Non-Responders for serotest 1322

It can be seen from figures 2 and 3 above that for all the influenza strains, responders have a lower PRE anti-CMV titer than non-responder. This is may be an indication that CMV infection contributes to poor responsiveness to influenza vaccine. Boxplots of PRE anti-CMV titers for responders and non-responders based on the seroprotection seroresponse are presented in figure A1 to A3 in the appendix.

Further exploratory analysis was carried out by comparing subjects with and without CMV based on seroresponses to influenza vaccination at day 21, 42 and 180. Differences between the CMV seropositive and seronegative subjects were tested using two-sample t-tests for the seroresponse GMT while Chi Square test of independence was used to test for an association between CMV infection and response to influenza vaccine based on the categorical seroresponses - Seroconversion and Seroprotection. The results of these analyses are presented in Table 4.

Table 4: Seroresponse to influenza vaccine for the different treatment groups.

	FluNewEld cmvpos		p-value	FluEld cmvpos		p-value	FluYng cmvpos		p-value
	0	1		0	1		0	1	
n	6	29		4	32		14	11	
GMT									
HI 1320									
Day 0	7.07(3.85-13.00)	13.63(9.19-20.23)	0.05	5.00**	16.09(11.2-23.13)	0.0001	15.22(7.47-31.01)	12.08(4.35-33.57)	0.69
21	106.77(23.05-494.50)	95.61(62.3-146.72)	0.87	226.27(5.23-9789.30)	51.28(34.182-76.93)	0.30	256.06(133.662-490.55)	205.88(93.734-452.2)	0.64
42	79.86(15.938-400.19)	72.7(50.94-103.75)	0.89	159.95(4.01-6376.39)	44.53(29.460-67.30)	0.35	194.87(104.06-364.9)	193.1(82.07-454.35)	0.99
180	31.75(8.882-113.48)	29.26(19.92-43.00)	0.88	51.74(2.48-1078.22)	28.57(18.568-43.97)	0.58	84.06(40.448-174.69)	72.78(24.170-219.18)	0.81
HI 1321									
Day 0	5.61(4.170-7.55)	17.33(10.257-29.3)	0.0003*	10.00(4.063- 24.61)	19.34(12.248-30.55)	0.11	11.04(5.485-22.22)	16.56(7.180-38.17)	0.42
21	226.31(45.977-1113.93)	356.3(217.5-583.6)	0.51	697.68(33.6-14492.5)	137.39(76.64-246.27)	0.19	220.58(85.287-570.51)	109.49(39.567-302.99)	0.28
42	285.08(43.581-1864.78)	260.8(161.8-420.6)	0.91	160(8.072-3171.57)	99.34(57.505-171.63)	0.65	152.29(59.993-386.60)	80.00(33.066-193.55)	0.28
180	80.00(12-533.2)	110.39(65.5-185.9)	0.69	67.27(5.831-776.17)	59.05(33.508-104.07)	0.88	121.87(55.991-265.25)	48.27(18.448-126.32)	0.11
HI 1322									
Day 0	56.57(11.69-273.81)	74.39(44.39-124.65)	0.69	51.48(25.584-103.60)	113.03(71.899-177.7)	0.03*	113.05(55.047-232.15)	120.51(31.148-466.22)	0.93
21	570.26(29.75-1122.33)	624.9(430.4-907.2)	0.78	1395.43(42-46185.1)	397.27(277.4-568.87)	0.34	1378.74(969.2-1961.38)	1026.65(498-2116.48)	0.43
42	380.74(110.9-1307.13)	436.64(306.8-621)	0.80	1174.1(98.2-14038.2)	330.46(235.6-463.46)	0.20	1024.35(688.6-1523.84)	849.8(407.515-1772.1)	0.63

180	239.77(76.93-747.28)	196(134.7-285.3)	0.69	538.17(46.6-6209.39)	218.90(153.8-311.55)	0.33	672.53(447.79-1010.07)	529.76(21.261-1315.98)	0.60
R_1>4									
HI 1320									
Day 21	6 (100%)	17 (58.62%)	0.08	4 (100%)	14 (43.75%)	0.05*	11 (78.57%)	9 (81.82%)	0.84
42	5 (83.33%)	17 (58.62%)	0.25	4 (100%)	11 (34.38%)	0.02*	11 (78.57%)	9 (81.82%)	0.84
180	4 (66.67%)	8 (27.59%)	0.07	2 (50%)	7 (21.88%)	0.22	9 (64.29%)	7 (63.64%)	0.97
HI 1321									
	6(100%)	26 (89.66%)	0.73	4 (100%)	22 (68.75%)	0.32	12 (85.71%)	9 (81.82%)	0.79
	6(100%)	26 (89.66%)	0.73	4 (100%)	19 (59.38%)	0.18	12 (85.71%)	8 (72.73%)	0.42
	5(83.33%)	23 (79.31%)	0.82	4 (100%)	12 (37.5%)	0.03*	10 (71.43%)	6 (54.55%)	0.38
HI 1322									
	5(83.33%)	20(68.97%)	0.48	3 (75%)	16 (50%)	0.35	13 (92.86%)	8 (72.73%)	0.17
	5(83.33%)	19(65.51%)	0.40	4 (100%)	14 (43.75%)	0.05*	11 (78.57%)	5 (45.45%)	0.09
	4 (66.67%)	10(34.48%)	0.14	3 (75%)	8 (25%)	0.04*	10 (71.43%)	4 (36.36%)	0.08
Titer≥40									
HI 1320									
	5 (83.33%)	26 (89.66%)	0.66	3 (75%)	25 (71.43%)	0.89	14 (100%)	10 (90.91%)	0.37
	4 (66.67%)	26 (89.66%)	0.14	3 (75%)	22 (68.75%)	0.80	13 (92.86%)	10 (90.91%)	0.86
	3 (50%)	15 (51.72%)	0.94	2 (50%)	17 (53.13%)	0.91	11 (78.57%)	8 (72.73%)	0.73
HI 1321									
	6(100%)	29 (100%)	0.42	4 (100%)	26 (81.25)	0.60	13 (92.86%)	9 (81.82%)	0.40

5 (83.33%)	29 (100%)	0.04*	4 (100%)	25 (71.43%)	0.52	12 (85.71%)	9 (81.82%)	0.79
3 (50%)	24 (82.76%)	0.08	3 (75%)	22 (68.75%)	0.80	11 (78.57%)	8 (72.73%)	0.73
HI 1322								
6(100%)	29 (100%)	0.42	4 (100%)	31 (96.88%)	0.61	14 (100%)	11 (100%)	0.91
6(100%)	29 (100%)	0.42	4 (100%)	31 (96.88%)	0.61	14 (100%)	11 (100%)	0.91
6(100%)	28 (96.55%)	0.82	4 (100%)	31 (96.88%)	0.61	14 (100%)	10 (90.91%)	0.37

**Significant at 5% level of significance. For GMT, differences are tested with two-sample t-test while for seroconversion and seroprotection, Chi square test was used.*

***No confidence interval was available for CMV seronegative subjects in the FluEld group on day 0 because they all had the same amount of HI titers for serotest 1320*

In Table 4, values of GMT are presented as means with 95% confidence interval while seroconversion and seroprotection rates are presented as numbers with their percentages in brackets.

A significant level of alpha=0.05 is used for all significance testing. Care has to be taken in interpreting the p-values because of the existence of multiple testing. As a result of multiple testing, there may be potentially high false significant results. Generally, subjects in the FluEld group generated antibody titers greater than 4-fold as compared to those of FluNewEld and FluYng groups. Moreover, there seem to be greater than 4-fold antibody titers generated for the H3N2 strain than for the H1N1 and B strains irrespective of the CMV status.

With respect to the seroprotection seroresponse, antibody titer greater than a titer of 40 was generated in strain B more than in strain H1N1 and H3N2.

Based on the GMT seroresponse, at baseline (day 0), there seem to be a statistically significant difference between subjects with or without CMV in the FluNewEld for strains H1N1 and H3N2. Also at baseline, for the FluEld group, there seem to be a difference between subjects with or without CMV for strains H1N1 and B. Average antibody titers are generally very large. This is possibly because some subjects have very large antibody titers. Confidence intervals are very large as well because of the large titers. Average titers are generally higher in the FluYng group than the FluNewEld and FluEld groups.

For the categorical seroresponses, the percentages in brackets are the proportion of subjects who have seroconverted or seroprotected for the various time points. For example; seroconversion rate for H1N1 strain on day 21 in the FluNewEld group, the percentage is obtained by dividing the number of subjects who have seroconverted by the total number of subjects in that group and multiplying this value by 100 (i.e 6/6 x100). The p-values are obtained from a 2X2 contingency table for seroconversion and cmvpos analysis using Chi-Square test of independence. Values for subjects who did not seroconvert and are not seroprotected are presented in table A2 of the appendix.

For some of the contingency tables, there were cells with zero counts as a result of sampling (sampling zero). Sampling zeros occur when there is no observation in the cell; i.e., $n_{ij} = 0$, but probabilistically you still have a chance of observing this value, P(observation in a cell) = $\pi_{ij} > 0$ (SAS help and manual, SAS9.2). As proposed by (Agresti, 2002), an adhoc procedure was adopted by adding 0.5 to each cell of such tables to enable a better computation of Chi Square p-values. There were also zeros in some row margins; the adhoc procedure was adopted here too.

With respect to seroconversion, there seemed to be an association between CMV infection and response to influenza vaccine for the H1N1 strains on days 21 and 42; for the H3N2 strain on day 180 and for the B strain on days 42 and 180 all in the FluEld group.

Based on seroprotection, there seemed to be an association between CMV infection and response to influenza vaccine in the FluNewEld groups for H3N2 strain on day 42.

The empirical distributions of the data for these comparisons were checked using histograms and for some, there seem to be deviations from normality. Also, the Levene's test for homogeneity of variance were carried out and for some, the variances were not equal. Furthermore, the sample sizes for some group comparison were unequal with large disparity. The sample sizes for some groups were very small. The t-test has been described as a robust test with respect to the assumptions of normality except when the differences in the sample size between the groups is greater than 1.5. (Laerd Statistics, 2012), which was the case for some group comparison, mostly in the FluNewEld and FluEld groups. Considering these aspects, definite conclusions should not be made from the results of the t-test. Likewise, in the Chi-Square tests, there were some 2x2 contingency tables which had more than 50% of the expected cell counts less than 5; hence valid inferences should not be made in such cases. These statistical tests (t-test and Chi-Square) were carried out for exploratory purposes, to give an insight into the data and an idea on the difference between subjects with and without CMV. Therefore, these limitations will be taken into account when selecting appropriate models.

Results of the above table can be illustrated graphically. A panel of average evolutionary profiles is presented in Figure 4.

Figure 4: Evolutionary Plots for cmvpos by HI serotests and treatment groups

Figure 4A1: Evolution of HI 1320 titers by CMV status for FluNewEld

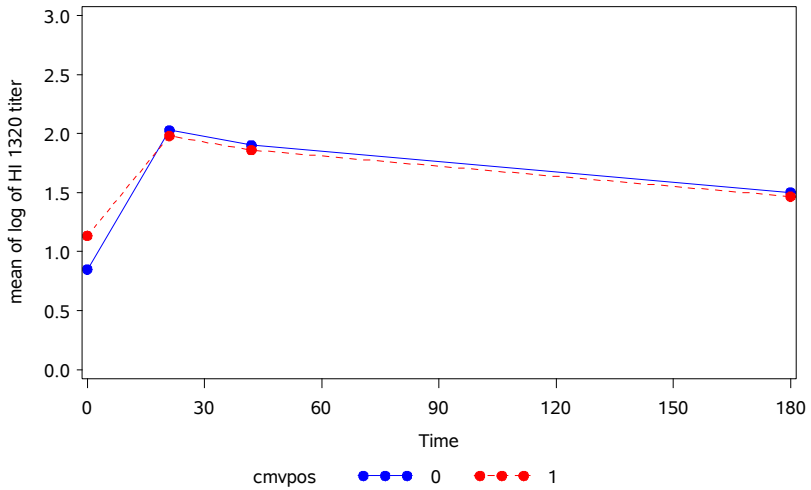


Figure 4A2: Evolution of HI 1320 titers by CMV status for FluEld

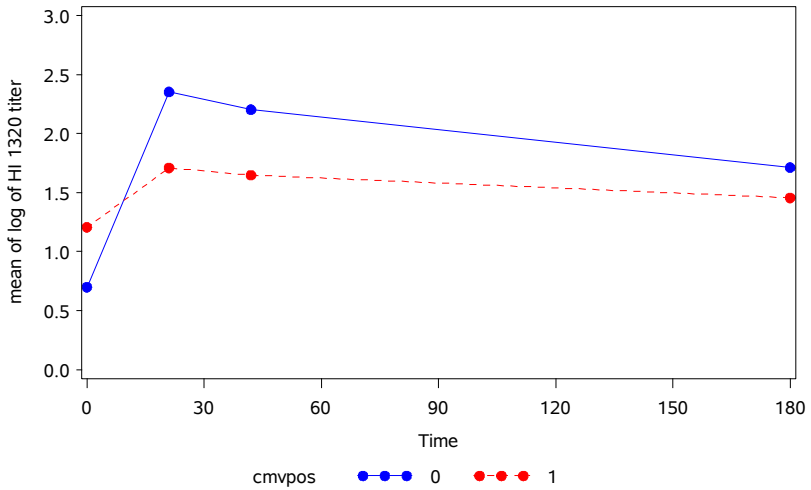


Figure 4A3: Evolution of HI 1320 titers by CMV status for FluYng

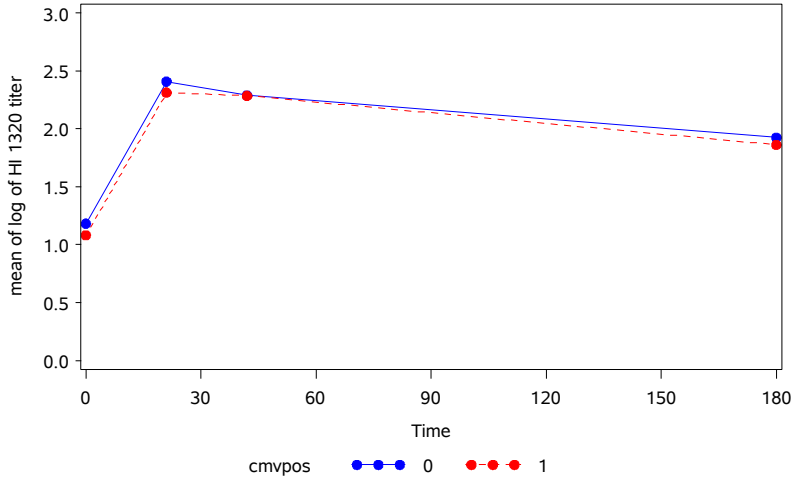


Figure 4B1: Evolution of HI 1321 titers by CMV status for FluNewEld

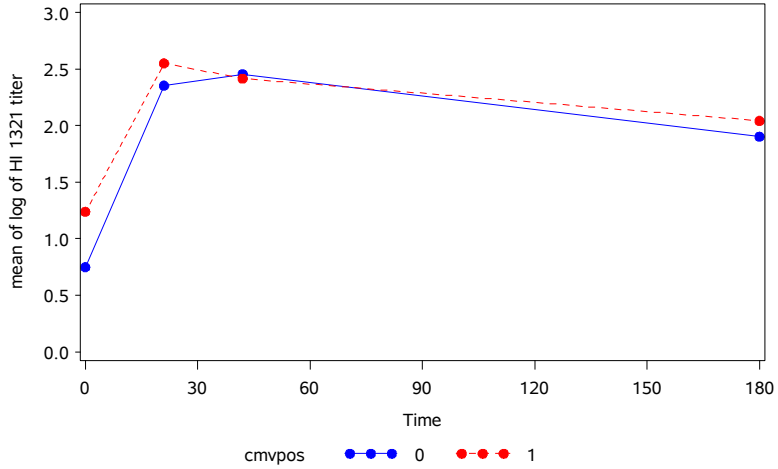


Figure 4B2: Evolution of HI 1321 titers by CMV status for FluEld

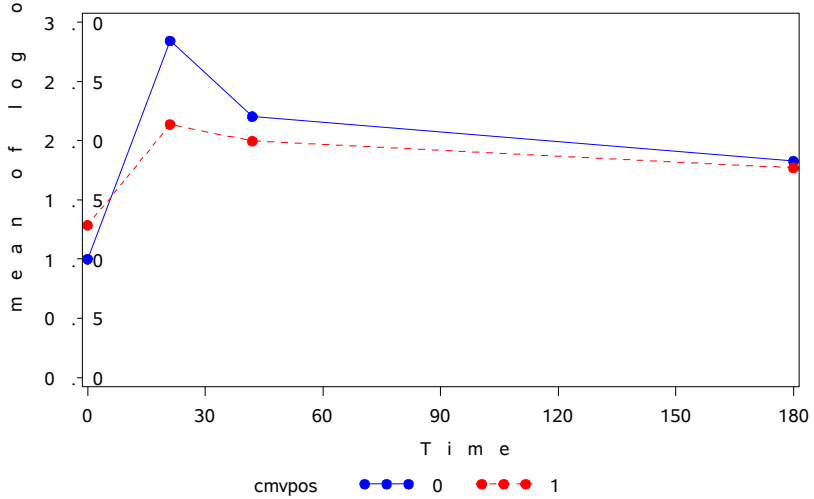


Figure 4B3: Evolution of HI 1321 titers by CMV status for FluYng

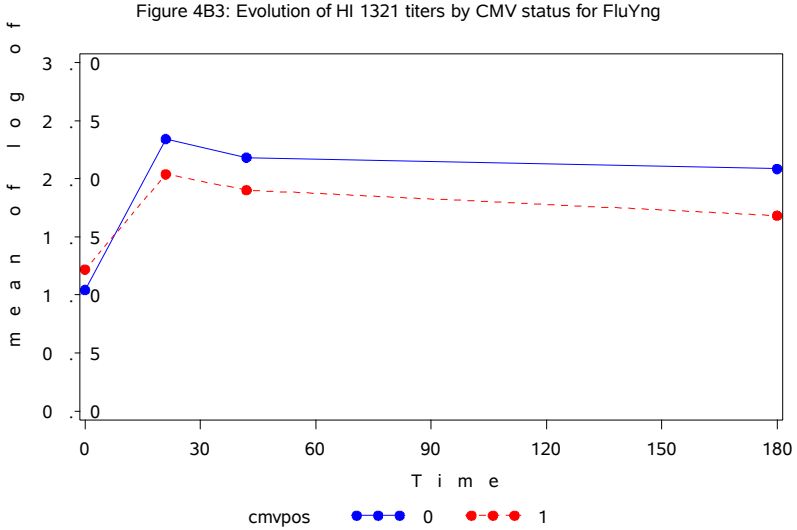


Figure 4C1: Evolution of HI 1322 titers by CMV status for FluNewEld

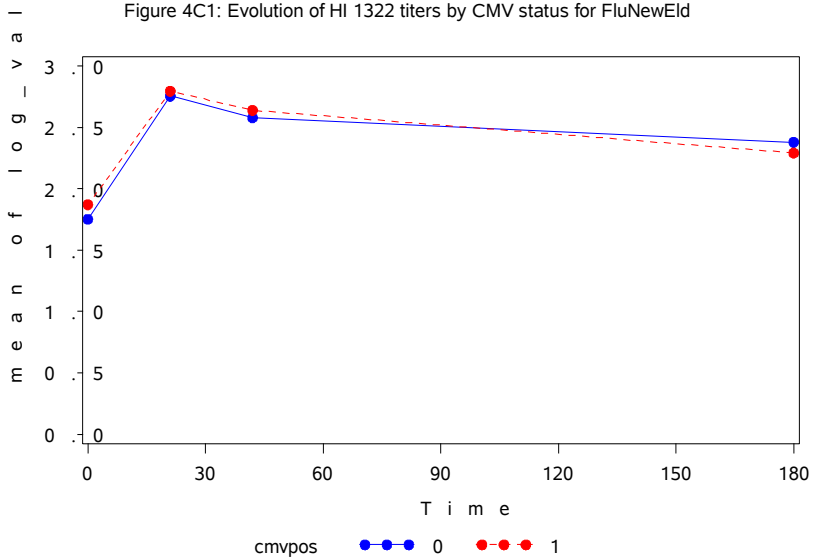


Figure 4C2: Evolution of HI 1322 titers by CMV status for FluEld

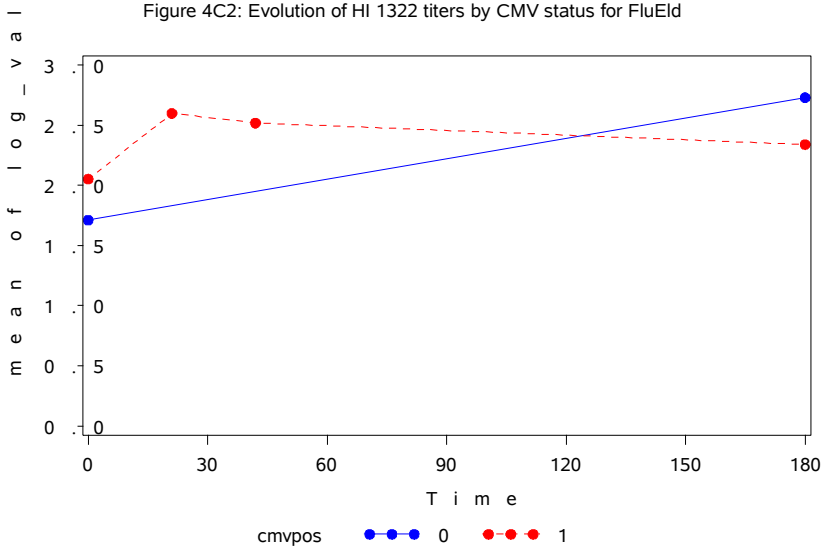


Figure 4C3: Evolution of HI 1322 titers by CMV status for FluYng

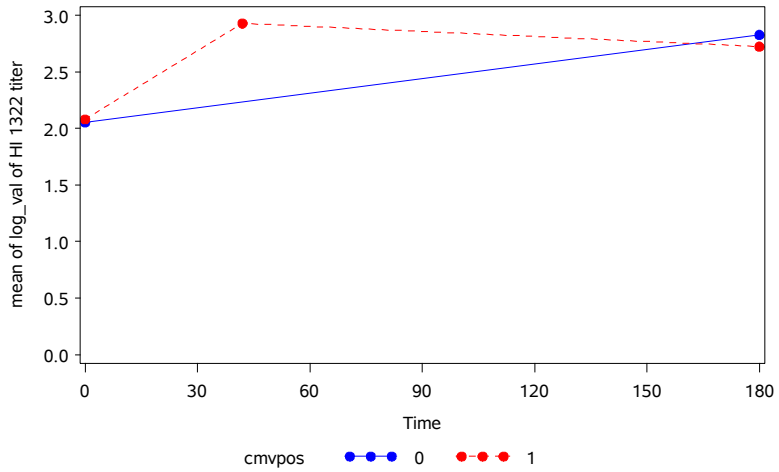


Figure 4 contains average evolutionary profiles for CMV seronegative and seropositive subjects by HI serotests and treatment groups. For serotest 1320, the average evolutionary profiles for the FluNewEld, FluEld and FluYng groups are presented in Figures 4A1, 4A2 and 4A3 respectively. Figures 4B1, 4B2 and 4B3 are average evolutionary plots for the FluNewEld, FluEld and FluYng groups respectively for serotest 1321, while Figures 4C1, 4C2 and 4C3 are average evolutionary graphs for the FluNewEld, FluEld and FluYng groups respectively for serotest 1322. For most the average evolutionary profiles, generally, there seems to be a steep increase in average antibody titers from day 0 to day 21 for both CMV seropositive and seronegative subjects. This is expected because when measurements were taken on day 0, no vaccine had been administered. When the vaccines were administered, antibodies were produced, causing an increase in the antibody titers.

For serotest 1320, there seem to be similar average evolutionary profiles for the FluNewEld and FluYng groups and a very small difference in the average antibody titer for CMV seropositive and seronegative subjects. Average antibody titers for CMV seropositive subjects seem to be higher than those of CMV seronegative subjects in the FluEld group with a much steeper increase for seropositive subjects than seronegative subjects.

For serotest 1321, average antibody titers seem to be lower for CMV seronegative subjects at day 0 than seropositive subjects for all the treatment groups. However, by day 21, average antibody titers seem to be consistently higher for CMV seronegative subjects than seropositive subjects for the FluEld and FluYng groups. For the FluNewEld group, average antibody titers are almost the same for the CMV seropositive and seronegative subjects from day 42 to around day 100, but after that, average titers are higher for CMV seropositive subjects than seronegative subjects.

For serotest 1322, average antibody titers are lower for seronegative subjects in groups FluNewEld and FluEld, while the average titers are almost for seropositive and seronegative subjects in the FluYng groups. However, in the FluNewEld group, there is no much difference in the average titers for seropositive and seronegative subjects. In the FluEld and FluYng groups, there is a consistent increase in average titers for seronegative subjects. By day 150 and 180, average titers are higher for seronegative subjects than seropositive subjects in the FluEld and FluYng groups respectively.

Further investigation was carried out to see if there was an association between CMV infection and seroresponse to influenza vaccine by using Pearson's correlation. The results are presented in Table 5.

Table 5: Correlation between PRE Anti CMV antibodies and Antibodies to Influenza Strains

Serotests	FluNewEld	p-value	FluEld	p-value	FluYng	p-value
CMV1126 vs H1N1	-0.14	0.43	0.26	0.12	-0.05	0.83
CMV 1126 vs H3N2	-0.10	0.59	-0.01	0.94	0.12	0.57
CMV 1126 vs B	0.03	0.88	0.44	0.01*	-0.08	0.71
CMV2050 vs H1N1	-0.07	0.69	0.13	0.46	-0.12	0.57
CMV2050 vs H3N2	0.09	0.59	0.09	0.60	-0.04	0.87
CMV2050 vs B	0.12	0.49	0.12	0.49	0.12	0.58

** Significant at 5% level of significance*

It can be seen from Table 5 that the correlation between CMV1126 and strain B in the FluEld group, has a coefficient of 0.44 which is significant. This suggests a positive correlation with moderate magnitude. Hence an increase in CMV1126 antibody titer may lead to an increase in antibodies to strain B. For other combinations of CMV antibodies and antibodies to influenza strains, there seem to be no evidence of correlation.

3.1 Area Under the Curve (AUC) and Mixed Model

Three AUCs values for the three HI serotests were computed for each subject and the logarithm to base 10 of these values were used in the mixed model to investigate whether CMV infection affects the response to influenza vaccine. Parameter estimates, standard error and p-value for the final model is presented in table 6.

Table 6: Parameter Estimates, Standard Errors and p-values for Final Model

Effect	Parameter	Estimate	Standard Error	Pr > |t|
Intercept	β_0	5.0692	0.1151	<0.0001
Cmvpos (Not infected)	β_1	0.1879	0.0965	0.0531
Group (FluEld)	β_2	-0.3125	0.1392	0.0260
Group (FluNewEld)	β_3	-0.2977	0.1382	0.0326
Serotest (1320)	β_4	-0.7329	0.1207	<.0001
Serotest (1321)	β_5	-0.8566	0.1207	<.0001
Serotest(1321)*Group(FluEld)	β_6	0.3411	0.1571	0.0312
Serotest (1321)*Group(FluNewEld)	β_7	0.6433	0.1580	<.0001

From Table 6, it can be seen that group, serotest and the interaction between group and serotest is significant. This means that the treatment group to which a subject belongs in conjunction with the influenza strain will influence the response to influenza vaccine. The variable cmvpos is borderline significant. This is unclear whether response to influenza vaccine is affected by a subject's CMV status. The fold difference between CMV seropositive and seronegative subjects is $10^{0.1879}$=1.54 with a 95% confidence interval of 0.994 to 2.389.

Table A3 in the appendix contains results of type 3 test of fixed effects

3.2 Logistic Regression

Logistic regression models were fitted for the different HI serotests separately. The results of the final models are presented in table 8 below.

Table 7: Parameter Estimates, standard error and p-values based on Seroconversion

Effect	Parameter	Estimate	Standard Error	P-value
Serotest 1320				
Intercept	β_0	0.7362	0.5747	0.2002
Cmvpos (Not infected)	β_1	1.4313	0.7021	0.0415
Group (FluEld)	β_2	-0.8785	0.6497	0.1764
Group (FluNewEld)	β_3	-0.1401	0.6651	0.8332
Serotest 1321				
Intercept	β_0	1.2862	0.6926	0.0633
Cmvpos (Not infected)	β_1	1.7881	1.1205	0.1105
Group (FluEld)	β_2	-0.4578	0.7758	0.5551
Group (FluNewEld)	β_3	0.9145	0.9093	0.3145
Serotest 1322				
Intercept	β_0	0.9476	0.6139	0.1227
Cmvpos (Not infected)	β_1	1.6987	0.8133	0.0367
Group (FluEld)	β_2	-0.8749	0.6894	0.2044
Group (FluNewEld)	β_3	-0.2298	0.7081	0.7455

From the above table, the response to influenza vaccine based on the seroconversion seroponse is affected by the CMV status for serotests 1320 and 1322 while for serotest 1321, it seems that the CMV status does not affect the response to the vaccine. For serotest 1320, while controlling for all other variables, the odds of responding to influenza vaccine for CMV seronegative subjects is exp(1.4313)= 4.18 times the odds of responding to influenza vaccine for CMV seropositive subjects. Also, for serotest 1322, while controlling for all other variables, the odds of responding to influenza vaccine for CMV seronegative subjects is exp(1.6987)= 5.47 times the odds of responding to influenza vaccine for CMV seropositive subjects. Hence from the above analysis, it can be seen that CMV seronegative subjects seem to respond better to influenza vaccines for strains H1N1 and B (serotests 1320 and 1321 respectively) than CMV seropositive subjects.

Furthermore, from the above analysis, there seem to be no effect of treatment group on the response to the vaccine for all the HI serotests. Since there is no significant interaction between cmvpos and group, it is likely that there is no evidence that the response to influenza varies between groups.

Response to influenza vaccine based on the seroprotection seroresponse is presented in table 9 below.

Table 8: *Parameter Estimates, standard error and p-values based on Seroprotection*

Effect	Parameter	Estimate	Standard Error	P-value
Serotest 1320				
Intercept	β_0	2.3026	1.0488	0.0281
Cmvpos (Not infected)	β_1	24.0627	1.2537	<.0001
Group (FluEld)	β_2	-1.0296	1.1326	0.3633
Group (FluNewEld)	β_3	-0.1431	1.2132	0.9061
Cmvpos(not infected)*group(FluEld)	β_4	-24.2371	1.7573	<.0001
Serotest 1321				
Intercept	β_0	1.5041	0.7817	0.0544
Cmvpos (Not infected)	β_1	26.4715	278830.0	0.99
Group (FluEld)	β_2	-0.0377	0.9035	0.97
Group (FluNewEld)	β_3	26.6010	235363.7	0.99

From Table 8 above, for serotest 1320, cmvpos is highly significant and the interaction between cmvpos and FluEld. The main effect for treatment group is not significant. Hence there seem to be an association between the CMV status and response to influenza vaccine based on the seroprotection seroresponse and this association varies between the treatment group. The log odds ratio for cmvpos for CMV seronegative subjects is 24.0627, which is extremely high.

For serotest 1321, there seem to be no association between cmvpos and seroprotection seroresponse. Also, no association seems to exist between treatment group and seroprotection. The standard errors are extremely large for cmvpos (not infected) and group (FluNewEld) with p-values approximately 1, indicating independence. This implies a large variability between subjects within these groups.

Definite conclusions should not be made on the models based on seroprotection seroresponse because the model fit is questionable. This may be because there were no CMV seronegative subjects who did not respond to influenza vaccine.

4. CONCLUSION AND DISCUSSION

Influenza viruses, which circulate worldwide, are the etiological agents of seasonal flu. There are three types: A, B and C. Types A and B viruses cause seasonal epidemics while type C infections cause a mild respiratory illness, reason why type C is not included in influenza vaccines. Subtypes of these viruses emerge when they undergo point mutation and antigenic variations and the difference between these subtypes are based on the different kinds and combinations of virus surface proteins. These subtypes include Influenza A|H1N1 and A|H3N2 which are the predominant subtypes of Influenza A viruses circulating among humans.

Influenza virus infection is a public health concern since it is worldwide, highly contagious and also life–threatening in immunocompromised subjects and the elderly population.

To avoid influenza infection, repetitive vaccination has been known to be the most effective way. However, the efficacy is consistently lower in the elderly, especially the frail, as compared to the younger subjects. There are indications that this lower vaccine efficacy in the elderly is as a result of immunosenescene. A number of studies suggest that infection with the Cytomegalovirus (CMV) contribute to the age-related changes in immunity and possibly indirectly affect the response to influenza vaccine. Two of such studies were carried out by Trzonkowski et al (2003) and den Elzen et al (2011).

Trzonkowski et al (2003) indicated that infection with CMV accelerates aging of the immune system, thus contributing to poor responsiveness to influenza vaccine in the elderly, but the study by den Elzen et al (2011) failed to confirm this. It is in this context that a clinical trial was carried out in young adults and older subjects, using a standard vaccine versus a new adjuvanted influenza vaccine with the objective of assessing the impact of CMV infection on the response to influenza vaccine. Another objective of this study is to assess if the response to the vaccine varies between the treatment groups. Response to the influenza vaccines was based on seroconversion and seroprotection rates. In addition to these seroresponses, the Geometric Mean Titer (GMT) which is a surrogate for the response to influenza vaccine was also used because in addition to showing that a subject has responded to the vaccine or not, it also gives the average immune response to the vaccine.

A number of statistical analyses were carried out to meet the objectives of the research. Exploratory data analyses using two-sample t-tests for the continuous seroresponse GMT and chi square tests for the categorical responses seroconversion and seroprotection were carried out to investigate the possibility of an association between CMV and the response to influenza vaccine. From these analyses, there seemed to be a difference between CMV seropositive and

seronegative subjects. However, conclusions could not be made from these analyses because of issues like multiple testing and the presence of sparse tables as mentioned earlier. Furthermore, graphical techniques using boxplots and evolutionary graphs were also implemented. Still, there seemed to be evidence of a difference in the response to influenza vaccine for the CMV seronegative and seropositive subjects.

Further investigations were carried out using other statistical tools. Since interest in the study was not on the evolution of the response to influenza vaccine, the Area Under the Curve (AUC) technique was used to summarize the data from longitudinal such that for every serotest, a subject had just one value for the response. The log of the AUC values was then used to fit a linear mixed model. Based on the GMT seroresponse, results for the linear mixed model did not expressly indicate whether infection with CMV affects the response to the influenza vaccine as can be seen on table 7. However, it shows that the treatment group to which a subject belongs varies with the influenza strain and influences the response to influenza vaccine.

Logistic regression models for each HI serotests were fitted to investigate whether there is an association between CMV infection and response to influenza vaccine based on the seroconversion and seroprotection seroresponses. For seroconversion, infection with CMV was shown to affect the response to influenza for serotests 1320 and 1322 which are the serotests for strains H1N1 and B respectively. It indicated that CMV seronegative subjects responded better to the vaccine than seropositive subjects. These results confirmed results from the graphical techniques. However, for serotest 1321 (serological test for H3N2), there was no evidence of CMV infection affecting the response to influenza vaccine.

These results are partially in line with the results of Trzonkowski et al who also showed that CMV infections affects response to influenza vaccine. However, for the current study, for serotest 1321, CMV infection did not seem to affect the response to influenza vaccine while the study by Trzonkowski et al showed that it did.

Also, the results for serotest 1321 is in line with the results of den Elzen et al., both showing that CMV infection does not affect the response to influenza vaccine.

The models based on seroconversion did not provide evidence that the response to treatment varies between groups.

For the seroprotection seroresponse, the model for serotest 1320 showed a very high significance in the association between CMV infection and response to influenza vaccine as well as the interaction between CMV and the FluEld group. For the serotest 1321, there was

no evidence of an association. The standard errors were very large. The model fit for the seroprotection seroresponse was questionable. Hence valid inference could not be made from the results based on seroprotection. A possible solution may be to use models suitable for zero counts since there were no CMV seronegative subjects who did not respond to influenza vaccine.

It can be concluded from this study that CMV seronegative subjects respond better to influenza vaccines than CMV seropositive subjects for strains H1N1 and B based on the seroconversion seroresponse. Response to the influenza vaccine varies between treatment groups based on the GMT seroresponse.

Further research options may consider experiments to investigating the difference between the influenza strains, why subjects respond to influenza vaccine for strains H1N1 and B but not strain H3N2.

A recommendation is to use larger samples with the equal number of CMV seropositive and seronegative subjects which may be a possible reason for the difference in the results from the linear mixed model and the logistic regression model based on seroconversion. This may also avoid issues of sampling zeroes.

5. REFERENCES

Agresti, A. (2002). *Categorical Data Analysis* (2nd ed.). New Jersey: John Wiley & Sons.

Bernstein ED, Gardner EM, Abrutyn E, Gross P, Murasko DM. Cytokine production after influenza vaccination in a healthy elderly population. Vaccine 1998;16:1722.

Bush, R. M., C. A. Bender, K. Subbarao, N. J. Cox, and W. M. Fitch. 1999. Predicting the evolution of human influenza A. Science 286:1921–1925.

Cox, N. J., and K. Subbarao. 1999. Influenza. Lancet 354:1277–1282.

den Elzen, W.P., Vossen, A.C., Cools, H.J., et al. Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities. Vaccine 2011;29:4869-4874

Govaert, T.M., Thijs, C.T., Masurel, N., Sprenger, M.J., Dinant, G.J., Knottnerus, J.A. The efficiacy of influenza vaccination in elderly individuals. A randomised double-blind placebo-controlled trial. J Am Med Assoc 1999;272:1661–5.

Kovaiou, R.D., Herndler-Brandstetter, D., Grubeck-Loebenstein, B. Age-related changes in immunity: implications for vaccination in the elderly. Expert Rev Mol Med 2007;9(3):1–17.

Kutner, M.H., Nachtsheim, C.J., Neter, J., and Li, W. (2005). *Applied Linear Statistical Models* (5th ed.). McGraw-Hill.

Laerd Statistics, (2012). Independent T-Test for Two Samples. Laerd Statistics website. Date Accessed: 2 July, 2012, from https://statistics.laerd.com/statistical-guides/independent-t-test-statistical-guide.php

Looney, R.J., Falsey, A., Campbell, D., et al. Role of cytomegalovirus in the T cell changes seen in elderly individuals. Clin Immunol 1999;90(2):213–9.

Molenberghs, G. and Verbeke, G. (2005). *Linear Mixed Models for Longitudinal Data*. NewYork: Springer.

Olsson, J., Wikby, A., Johannson, B., Löfgren, S., Nilson, B.O., Ferguson, F.G. Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the swedish longitudinal OCTO immune study. Mech Ageing Dev 2000;121:187–201

Pawelec, G., Derhovanessian, E., Larbi, A., Strindhall, J., Wikby, A. Cytomegalovirus and human immunosenescence. Rev Med Virol 2009;19(1):47–56.

SAS help and Manual documentation, SAS 9.2. Date Accessed: 17 August, 2012.

Sia, I. G., and R. Patel. 2000. New strategies for prevention and therapy of cytomegalovirus infection and disease in solid-organ transplant recipients. Clin. Microbiol. Rev. 13:83–121

Staras, S. A., S. C. Dollard, K. W. Radford, W. D. Flanders, R. F. Pass, and M. J. Cannon. 2006. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clin. Infect. Dis. 43:1143–1151.

Twisk , J. W. R. (2003). *Applied Longitudinal Data Analysis for Epidemiology: A Practical Guide.* Cambridge University Press.

Trzonkowski, P., Mysliwska, J., Szmit, E., et al. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination—an impact of immunosenescence. Vaccine 2003;21(25–26):3826–36.

Verbeke, G. and Molenberghs, G. (2000). *Linear Mixed Models for Longitudinal Data*. New York: Springer.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. Microbiol.Rev. 56:152–179.

Wells, J. E., Degenhardt, L., Bohnert, K., Anthony, J. and Scott, K. (2009). Geographical Clustering of Cannabis Use: Results from the New Zealand Mental Health Survey 2003-2004. *National Institute of Health Public Access,* **99**(1-3): 309–316. Available at: http://ukpmc.ac.uk/articlerender.cgi?tool=pubmed&pubmedid=18990513. Date accessed:29 August, 2012

WHO (2009). Influenza - Seasonal. WHO website, fact sheet N^{o} 211, Aprill 2009. Date accessed 28th April 2012, from http://www.who.int/mediacentre/factsheets/fs211/en/index.html

Wijma, G., Lighart, G.J. Influenza vaccination for all elderly. Gerontology 1996;42:270–3.

Wu, L. (2010). *Mixed Effects Models for Complex Data*. Chapman and Hall, New York.

6. APPENDIX

Table A1: Sample size with respect to treatment groups, Activity and CMV status

Sample size, N=96			
Day	Treatment Groups		
	FluNewEld	FluEld	FluYng
0	n = 35	n = 36	n = 25
	cmvpos 0=6	cmvpos 0=4	cmvpos 0= 14
	cmvpos 1=29	cmvpos 1=32	cmvpos 1= 11
21	n = 35	n = 36	n = 25
42	n = 35	n = 36	n = 25
	cmvpos 0= 6	cmvpos 0= 4	cmvpos 0= 14
	cmvpos 1= 29	cmvpos 1= 32	cmvpos 1= 11
180	n = 35	n = 36	n = 25
	cmvpos 0= 2	cmvpos 0= 2	cmvpos 0= 6
	cmvpos 1= 11	cmvpos 1= 15	cmvpos 1= 7

Table A2: Non-seroconverted and Non-seroprotected subjects with percentages in brackets

	FluNewEld		FluEld		FluYng	
	cmvpos		cmvpos		cmvpos	
	0	1	0	1	0	1
n*	6	29	4	32	14	11
<4-fold increase						
Serotest 1320						
Day 21	0 (0%)	12 (41.38%)	0 (0%)	18 (56.25%)	3 (21.43%)	2 (18.18%)
42	1 (16.67%)	12 (41.38%)	0 (0%)	21 (65.63%)	3 (21.43%)	2 (18.18%)
180	2 (33.33%)	21 (72.41%)	2 (50%)	25 (78.13%)	5 (35.71%)	4 (36.36%)
Serotest 1321						
Day 21	0 (0%)	3 (10.34%)	0 (0%)	10 (31.25%)	2 (14.29%)	2 (18.18%)
42	0 (0%)	3 (10.34%)	0 (0%)	13 (40.63%)	2 (14.29%)	3 (27.27%)
180	1 (16.67%)	6 (20.69%)	0 (0%)	20 (62.5%)	4 (28.57%)	5 (45.45%)
Serotest 1322						
Day 21	1 (16.67%)	9 (31.03%)	1 (25%)	16 (50%)	1 (7.14%)	3 (27.27%)
42	1 (16.67%)	10 (34.48%)	0 (0%)	18 (56.25%)	3 (21.43%)	6 (54.55%)
180	2 (33.33%)	19 (65.52%)	1 (25%)	24 (75%)	4 (28.57%)	7 (63.64%)
Titer > 40						
Serotest 1320						
Day 21	1 (16.67%)	3 (10.34%)	1 (25%)	7 (21.88%)	0 (0%)	1 (9.09%)
42	2 (33.33%)	3 (10.34%)	1 (25%)	10 (31.25%)	1 (7.14%)	1 (9.09%)
180	3 (50%)	14 (48.28%)	2 (50%)	5 (15.63%)	3 (21.43%)	3 (27.27%)
Serotest 1321						
Day 21	0 (0%)	0(0%)	0 (0%)	6 (18.75%)	1 (7.14%)	2 (18.18%)
42	1 (16.67%)	0 (0%)	0 (0%)	7 (21.88%)	2 (14.29%)	2 (18.18%)
180	3 (50%)	5 (17.24%)	1 (25%)	10 (31.25%)	3 (21.43%)	3 (27.27%)
Serotest 1322						
Day 21	0 (0%)	0 (0%)	0 (0%)	1 (3.13%)	0 (0%)	0 (0%)
42	0 (0%)	0 (0%)	0 (0%)	1 (3.13%)	0 (0%)	0 (0%)
180	0 (0%)	1 (3.45%)	0 (0%)	1 (3.13%)	0 (0%)	1 (9.09%)

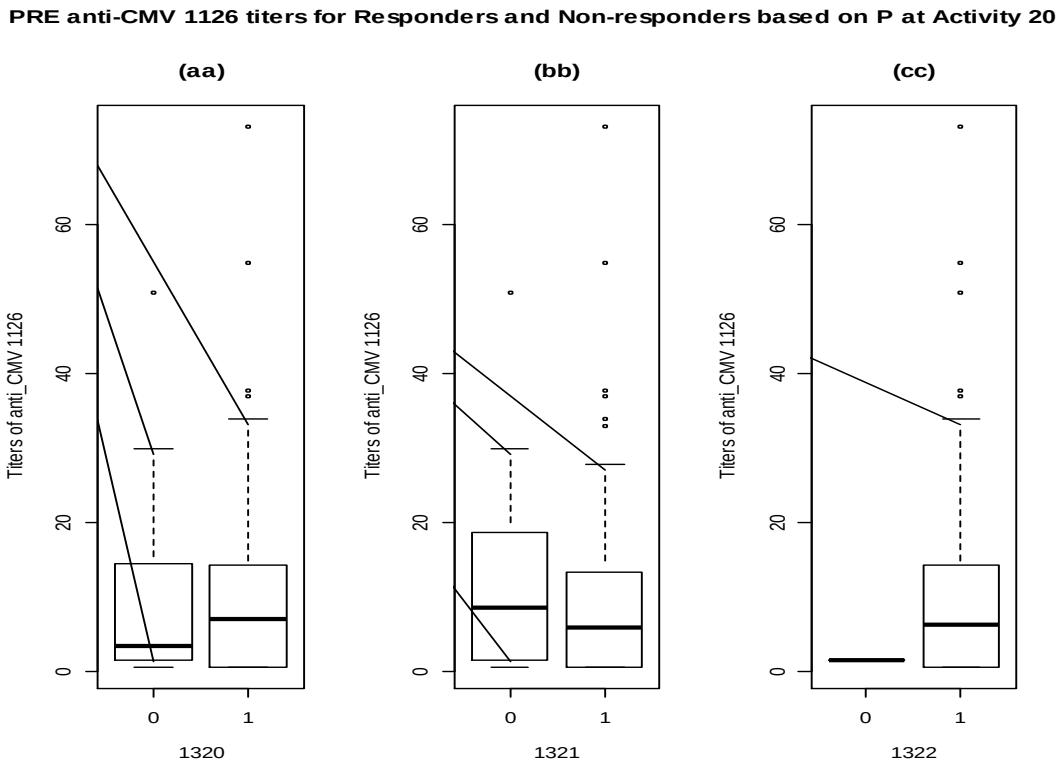


Figure A1: PRE Anti-CMV titers for Responders and Non-Responders at Activity day 21

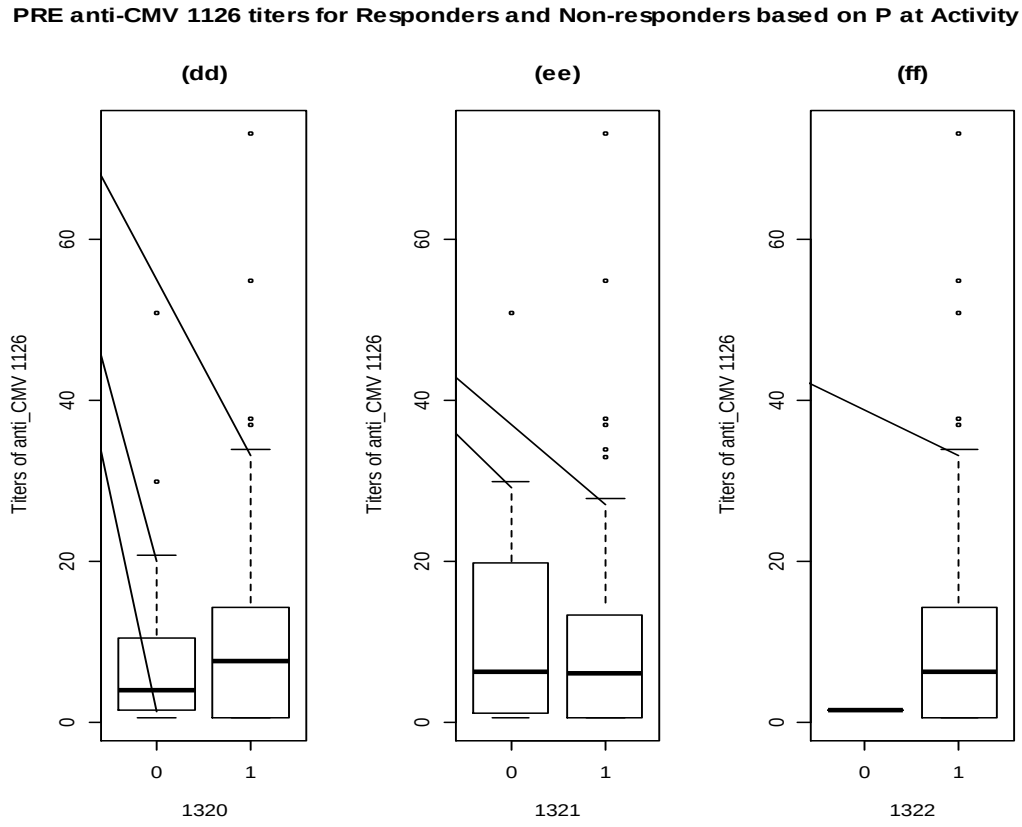


Figure A2: PRE Anti-CMV titers for Responders and Non-Responders at day 42

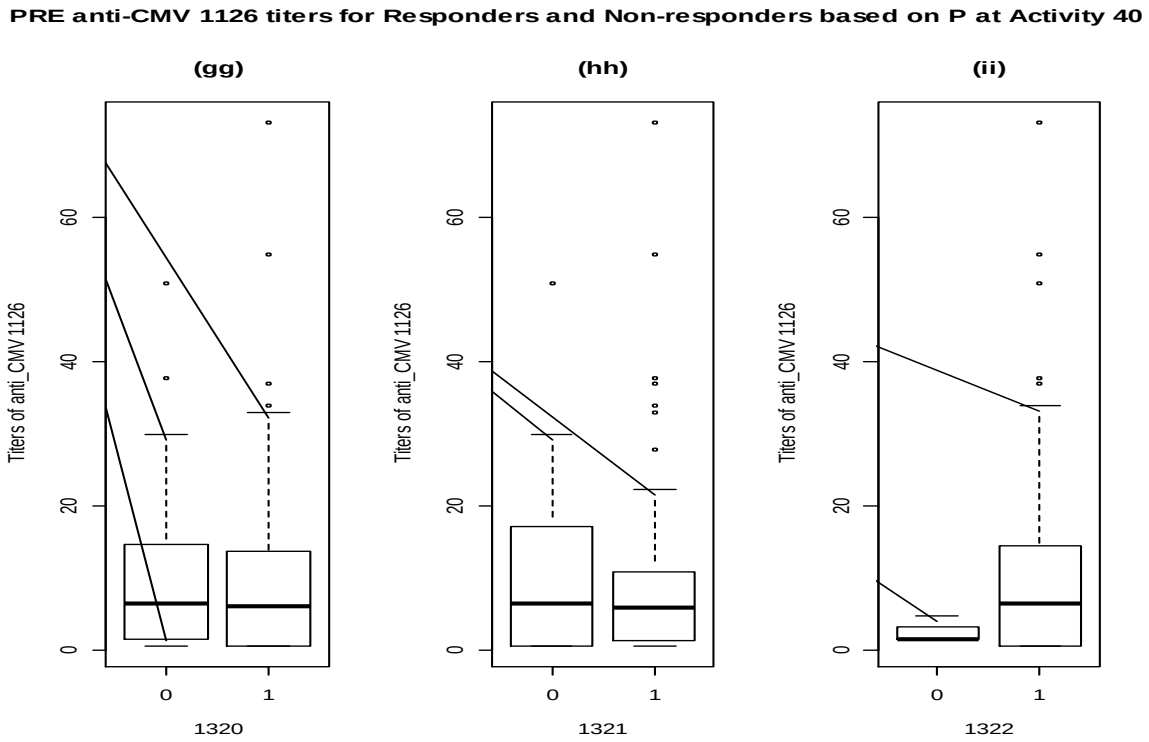


Figure A3: PRE Anti-CMV titers for Responders and Non-Responders at day 180

Table A3: Type 3 fixed effects

Effect	Num DF	Den DF	F Value	Pr > F
cmvpos	1	186	3.79	0.0531
group	2	186	2.82	0.0623
Serotest	2	186	82.47	<.0001
Serotest*group	4	186	6.04	0.0001

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Richting: **Master of Statistics-Biostatistics**
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