CHARACTERISATION OF IMMUNOGLOBULIN-IgG RESPONSES DURING COMBINATION THERAPY OF CHRONIC HEPATITIS- C VIRUS INFECTION

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Abbreviations

HCV	Hepatitis C virus
ORF	open reading frame
NTR	nontranslated regions
IRES	internal ribosome entry site
ER	endoplasmic reticulum
RdRp	RNA-dependent RNA polymerase
HVR	hypervariable regions
SR-BI	scavenger receptor class B type I
GAGs	glycosaminoglycans
LDLR	Low density lipoprotein receptor
HCC	Hepatocellular carcinoma
IFN	interferon
NK cells	Natural Killer cells
DC	Dentritic cells
ALT	alanine amininotransferase
CTL	cytotoxic T cells
EIA	Enzyme immunoassay
PCR	polymerase chain reaction
bDNA	branched-chain DNA
SVR	sustained virological response
TMA	transcription-mediated amplification
PAMP	pathogen-associated molecular pattern
STATs	signal transducers and activators of transcription
GTP	guanosine triphosphate
IMPDH	inosine monophosphate dehydrogenase
RDP	ribavirin diphosphate
RMP	ribavirin monophosphate
RTP	ribavirin triphosphate
MS	Multiple Sclerosis
AICH	autoimmune chronic hepatitis
CFT	Complement Fixation Test
VB	veronal buffer
MEIA	microparticle enzyme immunoassay
MUP	4-Methylumbeliferyl phosphate
CMIA	Chemiluminescence microparticle immuno assay
RLUs	relative light units
S/CO	sample/cut off

ABSTRACT

Current recommended treatment of chronic hepatitis C is the combination of peg interferon and ribavirin. Ribavirin has shown that it poses both direct and indirect action mechanisms against several DNA and RNA viruses. Interferon is a potent immune modulating substance, but its effect on the immune system of HCV-infected patients during treatment is not known in detail. It has been shown that interferon induces a significant increase in total IgG and C1 inhibitor (INH) in patients with MS.

The primary aim of this work was to study the effects of the combination therapy on the immunoglobulin levels. More specifically to see whether, the increased IgG levels were primarily due to HCV antibodies or whether antibodies to other viruses such as adenovirus, measles virus, and rubella virus are also increased. Sera from 24 patients in the NORDynamIC study at Haukeland University Hospital between February 2004 and March 2006 were used. Combination therapy with ribavirin and peginterferon was given for 12 or 24 weeks. Total IgG level, anti-HCV level, and antibodies to rubella, adeno and morbilli viruses were studied at different time points over 48 weeks.

Total IgG level was fluctuating during the period of study. The highest level of IgG was found at day 8 whereas the lowest level was found at week 8 of treatment. Those who responded to the combination therapy showed a declining level of total IgG even after cessation of therapy while the non-responders showed an increased level og IgG after cessation of therapy. The relationship between IgG and anti-HCV levels showed the same pattern in responders whereas in non-responders total IgG show considerable variation and did not follow anti-HCV levels as observed in responders. All patients were anti-rubella IgG positive and there is a correspondence between the mean rubella antibody levels and mean IgG levels both in responders and non-responders. Most of the sera had no reactivity to either adeno virus or morbilli virus and there was no correspondence between antibody levels for morbilli or adeno viruses and total IgG.

The study concludes that there seems to be an effect of combination therapy on total IgG and total IgG levels corresponds well with the anti-HCV antibody levels in responders. Rubella antibody was increased in all chronic HCV patients in the study and seems to have relationship with the total IgG level. Antibodies to adeno and morbilli viruses were not influenced by the treatment.

1. INTRODUCTION

1.1 Hepatitis C virus

Hepatitis C virus (HCV) is a small, enveloped RNA virus belonging to the Flaviviridae family genus Hepacivirus. HCV infects only humans and chimpanzees and causes acute and chronic liver disease in humans, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

1.1.1 HCV structure and genome

The HCV genome is a single-stranded RNA molecule of positive polarity. It contains one single open reading frame (ORF) encoding a polyprotein of about 3,000 amino acids (Fig. 1). The ORF is flanked by 5´ and 3´ nontranslated regions (NTR) and contain highly conserved RNA structures essential for polyprotein translation and genome replication. The 5´ NTR contains an internal ribosome entry site (IRES) that binds the 40S ribosomal subunit and initiates polyprotein translation in a cap-independent manner. The polyprotein precursor is co-translationally and post-translationally processed by both cellular and viral proteases at the level of the endoplasmic reticulum (ER) membrane to yield 10 mature proteins [1]. The structural proteins include the core (C), which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2. They are released by host-cell signal peptidases. The structural proteins are separated from the nonstructural (NS) proteins by the short membrane peptide p7, which is a viroporin. The nonstructural proteins NS2 to NS5B are involved in polyprotein processing and viral replication. The proteolytic processing of NS part of polyprotein requires the NS2-NS3 zinc-dependent metalloproteinase and the NS3 serine proteinase. The NS2-NS3 proteinase appears to cleave at the NS2/NS3 site by a conformation-dependent, autocatalytic mechanism [2]. The NS3 proteinase with its cofactor NS4A releases the remaining NS proteins. The C-terminal region of NS3 protein includes RNA helicase and NTPase activities**.** Both NS4B and NS5A are membrane associated proteins. NS5A has three domains where domain I has cystein residues essential for RNA replication, domain II is involved in inhibition of the IFN induced double stranded RNA activated protein kinases PKR and domain III is less conserved where deletions and insertions can occur. NS5B is an RNA-dependent RNA polymerase (RdRp) and activity is modulated by interactions by NS3 and NS5A. [3-7]

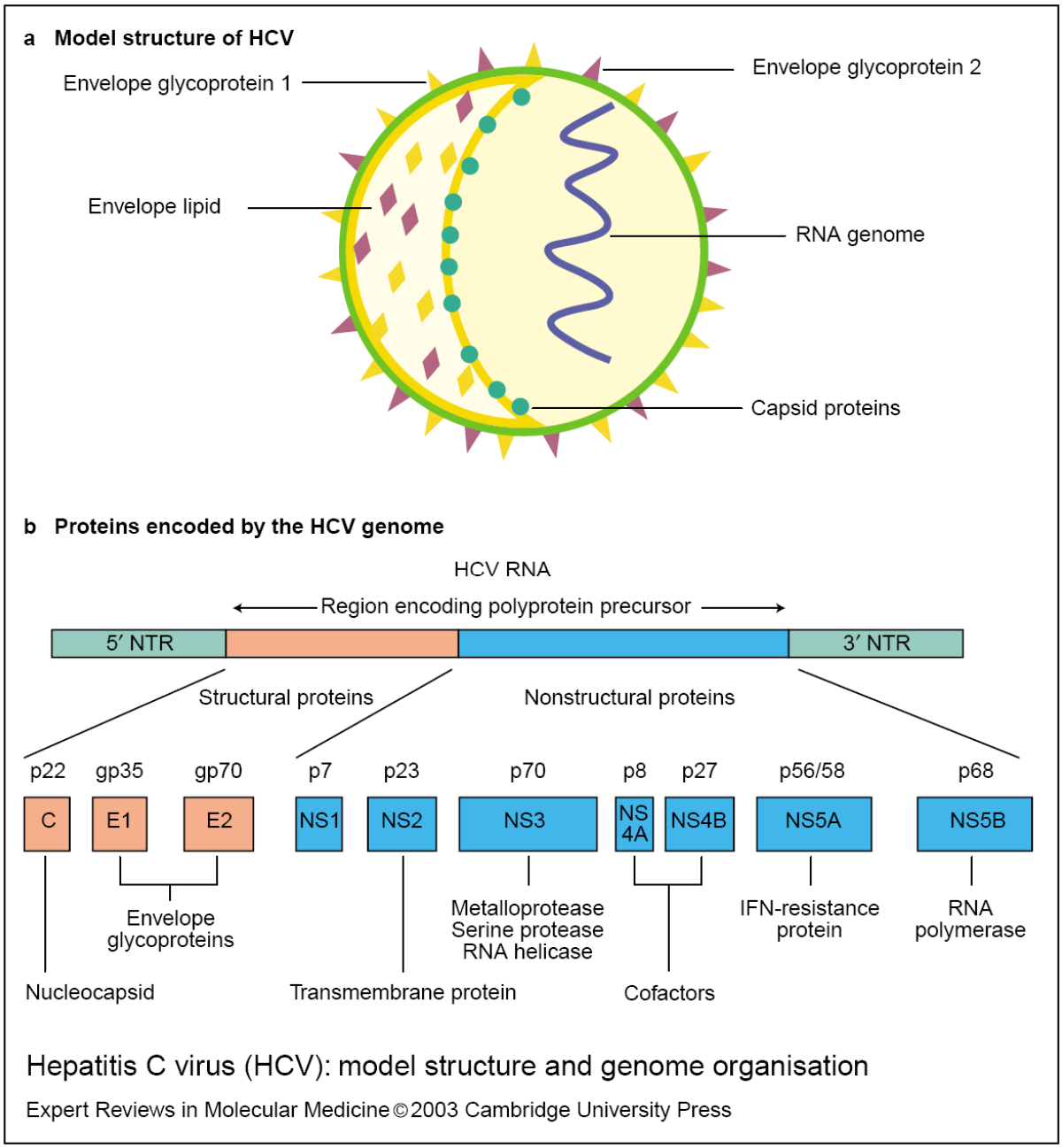


Figure1. Hepatitis C virus: model structure and genome organisation. (a) Model structure of HCV. The left-hand side of the illustration shows the viral surface of envelope lipids and glycoproteins; the right-hand side shows the RNA genome enveloped by capsid proteins. (b) Proteins encoded by the HCV genome. Putative functions of the cleavage products are shown [8].

1.1.2 Genotypes

Genetic differences between HCV isolates are used to classify HCV into genotypes and subtypes. There are six genotypes (1-6) and more than 70 subtypes (a,b, c, etc) termed as isotypes or quasispecies. The genotypes differ in 30-35% of neucleoside sites, of the genome and most of the variability occurs in the 'hypervariable' regions (HVRs) found in the E1 and

E2 glycoproteins. Sequences of the core gene and some of the non-structural protein genes, such as NS3, are more conserved. Between the six genotypes the lowest sequence variability is found in the 5´ NTR [9]. All HCV genotypes have a common ancestor virus. Distribution of HCV genotypes varies globally. For example, in North America, genotype 1a predominates followed by 1b, 2a, 2b, and 3a. In Europe, genotype 1b is predominant followed by 2a, 2b, 2c, and 3a. Genotypes 4 and 5 are found almost exclusively in Africa. The genotype is clinically important in determining potential response to interferon-based therapy and the required duration of such therapy. Furthermore, infection with one genotype does not confer immunity against others, and concurrent infection with two strains is possible. In most of these cases, one of the strains removes the other from the host in a short time [10].

1.1.3 Life cycle

The HCV life cycle is poorly known because of the lack of a productive culture system which is established very recently [11]. Hepatocytes are the main replication site for HCV, although studies have shown replication in B cells, dendritic cells (DC), and other peripheral blood mononuclear cells. [11].

HCV entry seems to be a complex multistep process that starts with virion attachment to its specific cell surface protein receptors. The recent studies have identified several of them such as, CD81, scavenger receptor class B type I (SR-BI) occulidin and claudin-1. The exact role of each molecule involved in HCV entry is not clear yet. Further additional accessory factors like glycosaminoglycans (GAGs) and the low density lipoprotein receptor (LDLR) are involved in the initial attachment. After the initial binding step, the particle interacts with SR-BI and CD81 (fig 2). Virions bound to SR-BI and CD81 are transported to the tight junction. Contact with the occludin and claudin-1 at tight junction is the final keys for viral entry. Then the HCV is internalized by clathrin-mediated endocytes and fusion probably occurs in early endosomes [12]. After the decapsidation, the genomic HCV RNA is replicated and translated in the cytoplasm. Replication and post-translational processing appear to take place in the"replication complex", located in close contact with perinuclear membranes. Genome encapsidation appears to take place in ER and nucleocapsids are enveloped and matured in the Golgi apparatus. Newly produced virions are released in the pericellular space by exocytosis [13, 14].

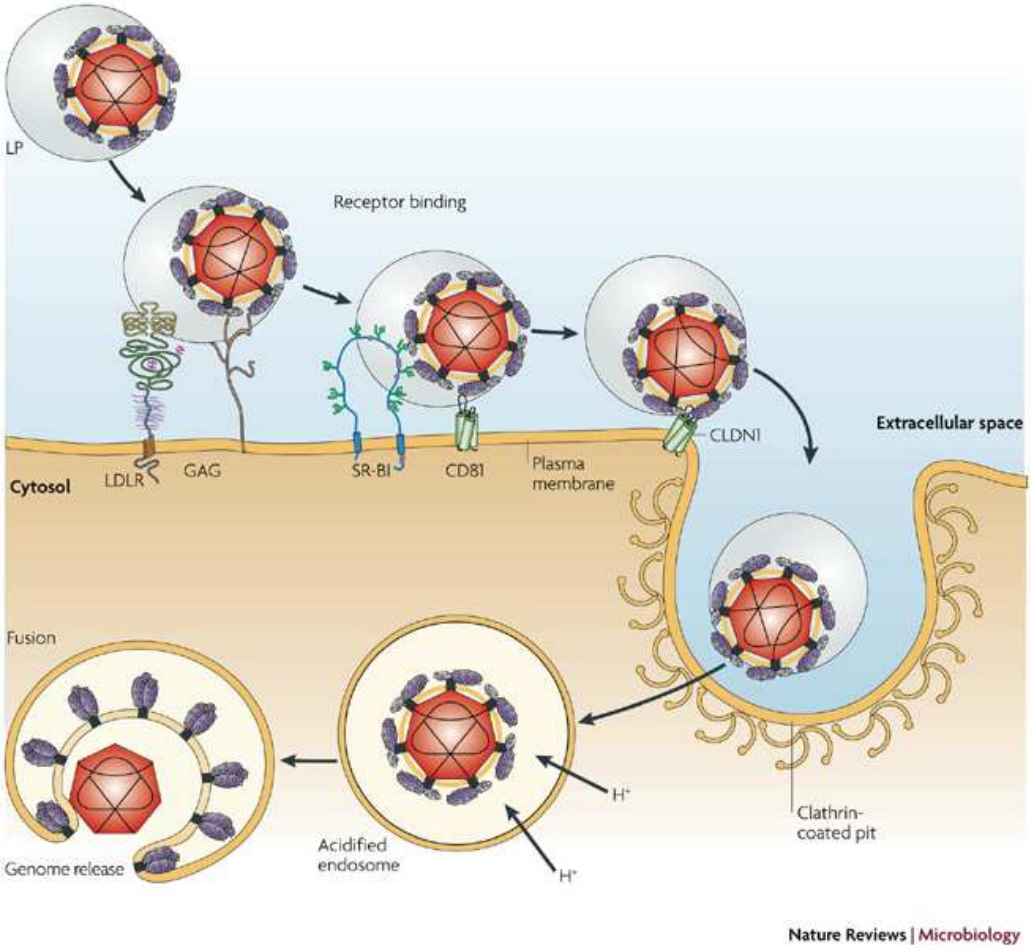


Figure 2: Circulating HCV particles can be associated with low- and very-low-density lipoproteins (LP). Virus binding to the cell surface and entry may involve the low density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1 [15].

1.2 Natural history of Heapatitis C infection

The World Health Organization estimates that about 3% of the world population is infected with HCV and 3-4 million new infections occur each year. A Norwegian survey showed 0,13% anti-HCV positivity among new blood donors, 0,7% in pregnant women and in the general adult population prevalence was 0,55% [16]. In high risk groups like active drug users and past drug users the prevalence is 70%. This means Norway can have 20 000-30 000 HCV infected persons and HCV has the largest share among the hepatotrophic viruses found in Norway.

The rate of chronic HCV infection is influenced by a person's age, gender, race, and viral immune response. Approximately 75%-85% of HCV infected persons progress further to chronic HCV infection with risk for the development of extrahepatic manifestations, compensated and decompensated cirrhosis, and hepatocellular carcinoma. The transmission of

HCV is primarily through exposure to blood. Other risk factors include intravenous drug use, high-risk sexual activity, solid organ transplantation from an infected donor, occupational exposure and haemodialysis [17].

1.2.1 Acute infection

Acute hepatitis C infection is infrequently diagnosed because the majority of acutely infected individuals are asymptomatic and these account for about 20% of acute hepatitis. Of adults with acute HCV infection 20-30% may develop clinical symptoms. Onset of symptoms ranges from 3 to 12 weeks after exposure. Symptoms may include malaise, weakness, anorexia, and jaundice. Incubation period is on average 50 days but it can vary from 15 to 150 days [17].

1.2.2 Chronic infection

Chronic hepatitis C infection is marked by the persistence of HCV RNA in the blood for at least 6 months after onset of acute infection. HCV is self-limiting in only 15%-25% of patients in whom HCV RNA in the serum becomes undetectable and alanine amino tranferase (ALT) levels return to normal. Approximately 75%-85% of HCV infected persons will progress to chronic HCV infection and are at risk for advanced liver fibrosis, HCV-related extrahepatic complications, cirrhosis and HCC. Risk factors for developing chronic infections are age >25 yrs, male gender, no symptoms during acute infection, African American race, HIV infection and immune suppression. Chronic infection is often asymptomatic. An estimated 10%-20% of chronic HCV infections advance to end-stage liver disease over one or two decades. Extrahepatic manifestations involving multiple organ systems can occur during chronic HCV infection or cirrhosis, but HCC appears to develop only after cirrhosis [17].

1.3 Immune response during HCV infection

Viral RNA appears in the peripheral blood within 1-2 weeks of HCV exposure, and triggers both the innate and the adaptive immune responses [11].

1.3.1 Early control of HCV infection

1.3.1.1 The Innate Immune Response

Rapid and efficient activation of the different components of the innate immune system is crucial not only for the initial containment of virus replication and spread, but also for a timely and efficient promotion of downstream adaptive responses. Adaptive responses require more time for their induction but are essential for complete and persistent control of infection.

HCV RNA triggers the production of type 1 interferon (IFN) α / β and activation of multiple IFN responsive genes in infected cells. IFN also inhibits replication of viruses and induce apoptosis in infected hapatocytes in addition to upregulate the expression of HCV antigens on the surface of infected hapatocytes. Natural Killer cells (NK cells) contribute to the control of viral replication through cytolysis of infected cells, induction of replication inhibitory cytokines like IFN and activation of both Dentritic (DC) and T cells. DCs play an important role as antigen presenting cells and link innate and adaptive immune response [18, 19].

1.3.1.2 Adaptive Immune Response

Development of neutralizing antibodies is a hallmark of clearance in many viral infections. HCV-specific antibodies usually become detectable in the serum within several weeks after the primary infection and they appear after cellular immune responses and ALT elevations. The first detectable antibodies against HCV antigens in serum usually target NS3 protein (anti-c33 Ab) and core protein (anti-capsid or anti-22c Ab). Later, the specific antibodies against NS4 protein and envelope glycoproteins (E1 and E2) appear [20].

A number of studies have indicated that successful cellular immune responses in recovered patients appear to be multispecific and sustained; with CD4+ T cells playing major roles. CD4+ T cells have antiviral responses, both via secreting antiviral cytokines from HCV-specific type 1 helper T cell and via activation of B cells and CD8+ T cells. Vigorous and sustained HCV-specific CD4+ and CD8+ T cell responses against multiple HCV epitopes are necessary for spontaneous viral clearance during the acute phase. The definitive role of CD4+ T cell responses in acute HCV infection was shown in a recent study where in absence of CD4+ T cell responses, HCV-specific CD8+ T cell and neutralizing antibodies may develop, but fail to control viremia [21]. CD8+ T cells could respond to HCV viral infection through two main mechanisms: the killing of infected hepatocytes or the secretion of antiviral cytokines. Studies have indicated that CD8+ T cells are primarily effector cells mediating protective immunity, while CD4+ T cell help is required for CD8+ T cells to keep pace with the evolution of viral escape mutants [11, 19].

1.3.2 Immune escape

HCV has developed several escape mechanisms to evade host´s immune systems. In addition to interference with the endogenous IFN system and DC dysfunction, there is evidence for viral evasion of the adaptive immune response. Loss of CD4+ T cells during early infection is one mechanism of persistence in HCV infection. Others have shown that HCV-specific CD8+

T cells in chronic infection are functionally impaired with respect to IFNγ production [22-24] and, it is consistent with loss of CD8+ function [25]. Mutational escape from adaptive immune response has been suggested to be one of the evasion strategies used by HCV. The mutation rate of HCV is high due to the lack of proofreading capacity of its polymerase. This property helps HCV to escape T- and B-cell surveillance by mutating residues relevant to T- and B-cell recognition. HCV is able to acquire an early survival advantage over the host immune system due to its fast replication kinetics and its capacity to interfere with the early intracellular pathways of antiviral defence. These intrinsic features of the virus may also impact on priming and maturation of adaptive responses through T-cell exhaustion, mutational escape from T- and B-cell surveillance, and negative modulation of the early cross-talk between innate and adaptive immunity, providing the virus with an additional survival advantage that can contribute to the high rate of HCV persistence. Classic cytotoxic T cells (CTL) may primarily damage the liver in chronic HCV, but there may be subpopulations of T cells that protect against liver inflammation [19].

1.4 Diagnostic tools for HCV infection

1.4.1 Serological assays

The detection of anti-HCV antibodies in plasma or serum is based on the use of third-generation EIAs (example: Axsym, Abbott systems), which detect mixtures of antibodies directed against various HCV epitopes. Recombinant antigens are used to capture circulating anti-HCV antibodies. The specificity of third-generation EIAs for anti-HCV is greater than 99%. Their sensitivity is more difficult to determine, given the lack of a gold standard method, but it is excellent in HCV-infected immunocompetent patients [26].

1.4.2 Detection of HCV RNA

1.4.2.1 Qualitative Tests

Qualitative detection assays are based on the principle of target amplification using either “classic” polymerase chain reaction (PCR), “real-time” PCR or transcription-mediated amplification (TMA) [27]. Qualitative tests are used to confirm viremia. Moreover, these tests also have clinically important applications in predicting patients at risk for virological relapse once therapy stops and in diagnosing acute HCV infection [28, 29].

1.4.2.2 Quantitative tests

There are 3 types of tests to quantify HCV RNA: quantitative reverse transcription–PCR (Monitor® Roche Diagnostics), real-time PCR (Cobas Amplicor® Roche Diagnostics, Abbott Diagnostic), and branched-chain DNA (bDNA) (Versant® Bayer Health care) [30].

1.4.3 Genotyping

The reference method for HCV genotype determination is direct sequencing of the NS5B or E1 regions of HCV genome. In clinical practice, HCV genotype can be determined by various commercial kits (Trugene® 5`NCH CV Genotyping kit, Bayer Health care, INNO-LiPA HCV II, Innogenetics, Versant® HCV genotyping assay, Bayer Health care). Genotype tests are important clinically because they predict most accurately the chance of antiviral response, and dictate the duration of therapy. Genotype is the strongest predictor of response to interferon and ribavirin; patients who had genotype 2 or 3 were 3 to 6 times more likely to achieve sustained virological response (SVR) in the 2 large registration trials of peginterferon [31, 32]. Therefore, genotyping is necessary before initiation of therapy.

1.4.4 Diagnosis of acute HCV infection

The presence of HCV RNA in the absence of anti-HCV antibodies is a strong indicator of acute HCV infection, which will be confirmed by seroconversion days to weeks later (fig 3). Acutely infected patients can also have both HCV RNA and anti-HCV antibodies at the time of diagnosis. It is difficult, in this case, to distinguish acute hepatitis C from an acute exacerbation of chronic hepatitis C. Acute hepatitis C is very unlikely if both anti-HCV antibodies and HCV RNA are absent or if anti-HCV antibodies are present without HCV RNA. The presence of anti-HCV antibodies in the absence of HCV RNA is generally seen in patients who have recovered from past infection.

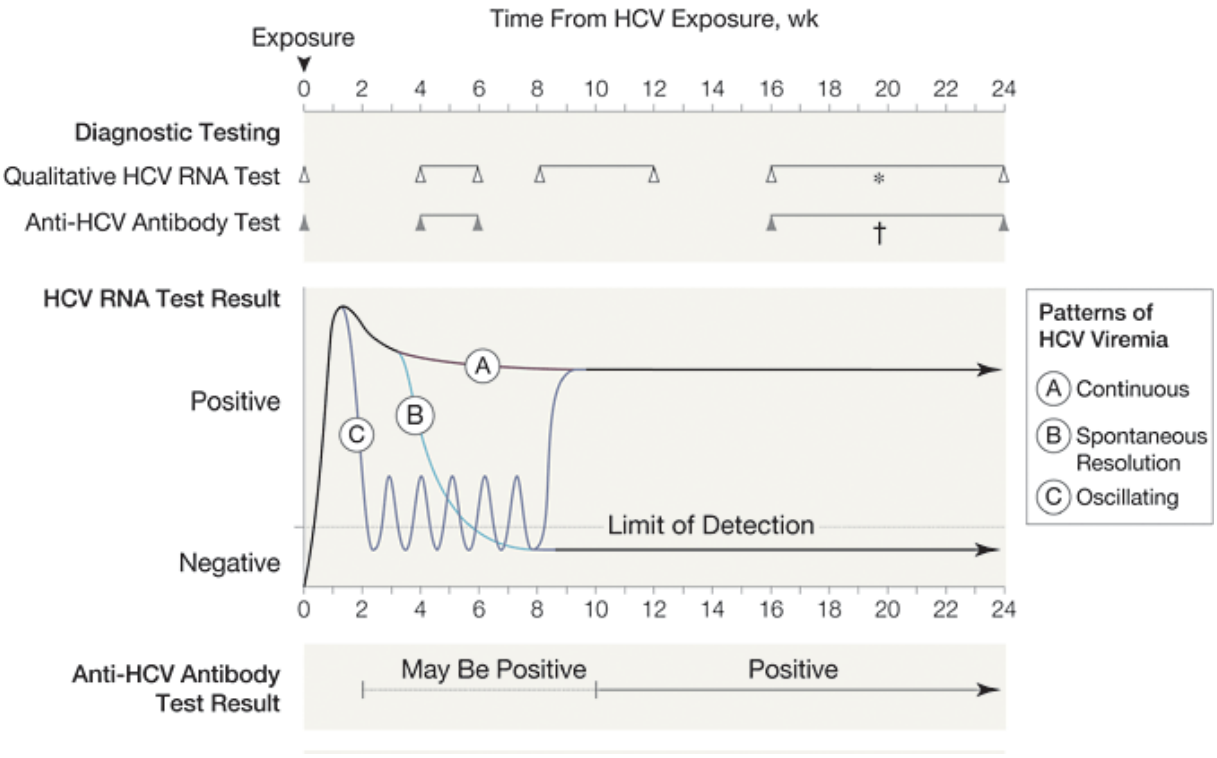


Figure 3: Use of Molecular Tests for Diagnosis of Acute Hepatitis C Virus Infection Following Known Exposure

*Repeat HCV RNA testing at 16 to 24 weeks if the patient had previous negative qualitative test results.

†Repeat anti-HCV antibody testing at 16 to 24 weeks if patient had a negative anti-HCV test result at 4 to 6 weeks after exposure [30].

1.4.5 Diagnosis of persistent hepatitis C

In patients with clinical or biological signs of chronic liver disease, chronic hepatitis C infection is certain when both anti-HCV antibodies and HCV RNA are present. Detectable HCV replication in absence of anti-HCV is exceptional but found in profoundly immunsuppressed patients and haemodialysis patients.

1.5 Treatment

1.5.1 Chronic hepatitis C virus infection

Interferon, used as monotherapy for HCV infection for many years, gives a SVR in < 20% of patients. The introduction of combination therapy with interferon-α and oral ribavirin was a major success [33]. This regimen shows a SVR, as defined by undetectable virus RNA in the peripheral blood at 24 weeks after stopping therapy, in 40% of previously untreated patients with chronic HCV infection. More recently, combination therapy using interferons modified by the addition of either a 12-kDa or a 40-kDa polyethylene glycol molecule (peginterferons)

was found to be superior to combination therapy involving standard interferon or treatment with peginterferon alone [31, 32]., The combination of peginterferon and ribavirin given for 48 weeks, which is the current best therapy for chronic hepatitis C, yields overall SVR rates of 50% to 60% [31, 32]. Importantly, response rates are higher among patients infected with genotypes 2 and 3 (75% to 80%) than with genotype 1 (46% to 52%) and can be achieved with 24 weeks of therapy and lower doses of ribavirin (800 mg rather than 1000-1200 mg daily) [34]. Analyses of HCV RNA levels during antiviral therapy have identified important factors associated with response. These factors include both viral (HCV genotype and initial HCV RNA level) and host factors such as age, sex, race, body weight, hepatic steatosis, insulin resistance, hepatic fibrosis, immunodeficiency, adherence, and presence of other comorbidities.

1.5.2 Mechanisms of interferon

The outcome of any viral infection depends on innate and adaptive immune responses. Immune reactions are first triggered by engagement of pathogen-associated molecular pattern (PAMP) receptors that sense the viral threat and induce an antiviral state through a myriad of pathways including activation of IFN. IFN-α/β acts to induce antiviral responses in cells via interaction with specific cell surface receptors, the type I IFN receptors. These in turn stimulate a number of pathways such as the phosphorylation of signal transducers and activators of transcription (STATs) which lead to gene expression [35]. These proteins are capable of disrupting viral gene expression at multiple levels, including viral mRNA stability, transcription and translation. In addition to its direct antiviral actions, IFN acts as an immunomodulator by promoting memory T-cell proliferation, preventing T-cell apoptosis, and stimulating NK cell activation and DC cell maturation [36, 37]. Exogenously supplied recombinant IFN-α binds to and activates cellular receptors, leading to the same response cascades that occur with endogenous production.

1.5.3 Ribavirin: Mechanism of action in chronic hepatitis C

Ribavirin was introduced into hepatitis C virus therapy in the early 1990s and monotherapy was associated with improvements in serum ALT levels in half of the patients but the viral level was persisting. The addition of ribavirin to IFN-α-based regimens produced dramatic improvement in SVR rates among patients with chronic hepatitis C. Ribavirin clinically appears both to increase the end-of-treatment response rate and to decrease the subsequent

relapse rate [36]. The mechanism by which this effect occurs has not been elucidated. Several hypotheses for the mechanism of action of ribavirin have been proposed (Figure 4): (1) a direct antiviral effect against the HCV RNA-dependent RNA polymerase; (2) depletion of intracellular guanosine triphosphate (GTP) pools through its action as an inhibitor of inosine monophosphate dehydrogenase (IMPDH); (3) induction of misincorporation of nucleotides by the viral RNA polymerase, leading to lethal mutagenesis and production of virus with diminished infectivity; and (4) enhancement of host T cell-mediated immunity against viral infection through switching the T cell phenotype from type 2 to type1.

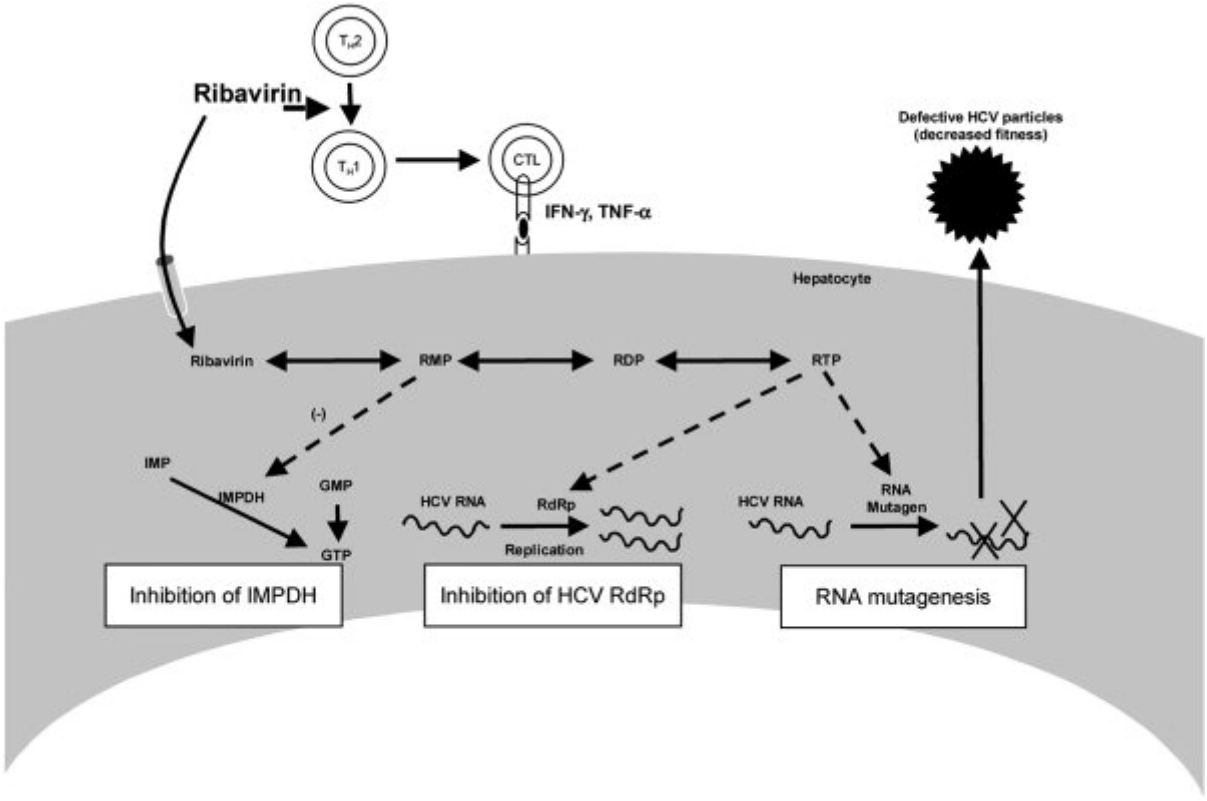


Figure 4: Ribavirin: proposed mechanisms of action. GMP guanosine monophosphate; GTP, guanosine triphosphate; IFN-, interferon; IMPDH, inosine monophosphate dehydrogenase; RDP, ribavirin diphosphate; RdRp, RNA-dependent RNA polymerase; RMP, ribavirin monophosphate; RTP, ribavirin triphosphate; Th1, T helper 1; and Th2, T helper 2 [37]

Viral kinetic studies during IFN- therapy of chronic hepatitis C have defined two phases of viral decline: a rapid, initial first phase, which is believed to reflect antiviral efficacy of IFN-α, and a more delayed and slower second phase, which is believed to reflect eradication of virus-infected hepatocytes. Using kinetic models, it has been proposed that ribavirin exerts its effect predominantly in the second phase of viral decay, through increased mutagenesis,

resulting in a lower rate of new productively infected hepatocytes [38]. These findings support a theoretical model wherein ribavirin, through its mutagenic actions, lowers HCV fitness. Ribavirin might reduce the ability of HCV to escape immune and antiviral pressures, and thereby increase the effectiveness of IFN by decreasing replicative fitness and narrowing the genomic diversity of HCV [37].

1.6 Rational for the study

Interferons are known to modulate immune responses. Moreover the biologic effects of various lFN are quite heterogenous and IFN has been shown to inhibit cell growth, to activate natural killer cells and to affect leukocyte migration. From studies in mainly animal models it has also been demonstrated a modulating effect of IFN on B cell functions, particularly measured as immunoglobulin IgG production in vivo and in vitro [39-43]. The experimental data of these studies indicate that IFN induces enhanced production of immunoglobulins indirectly via effects on CD4+ T cells and dendritic antigen-presenting cells [42, 43]. More specifically, IFN enhances the primary antibody response, stimulates the production of all IgG subclasses and induces long-lived antibody production against the stimulating antigen. Multi model activities of IFN have made use of IFN in various medical conditions like malignancies, viral and immune-mediated diseases. Currently, IFN has shown clinical benefit in Multiple Sclerosis (MS) and in chronic hepatits C infection. Ulvestad et.al have shown that interferon induces a significant increase in total IgG and C1 inhibitor (INH) in patients with MS, thus verifying that interferon modulates and activates both innate and adaptive immune system in these patients [44]. A question is whether IFN also induces the production of Igs in a more unspecific manner, thereby enhancing the total concentration of serum Igs.

It has previously been shown that in chronic liver disease there is a marked increase in morbilli and/or rubella antibodies [45-47]. This finding was prominent in autoimmune chronic hepatitis (AICH) and the authors tried to explain this as a reactivation of these viruses and or a general hyper reactive B-lymphocyte system since reactivation without clinical laboratory verification of viruses may influence the total IgG. Therefore, the aim of the project was to see more specifically whether the increased IgG levels were primarily due to HCV antibodies or whether antibodies against other viruses such as adenovirus, morbilli virus, and rubella virus were also increased.

2. AIM OF THE STUDY

The primary aim of this work was to study the effects of the combination therapy on the immunoglobulin levels. More specifically to see whether, the increased IgG levels were primarily due to HCV antibodies or whether antibodies to other viruses such as adenovirus, measles virus, and rubella virus are also increased.

The specific tasks were:

- To analyse the variation in IgG by quantification of total IgG in chronic HCV patients during combination therapy.
- To see whether antibody titers to other viruses like adenovirus, measles virus, and rubella virus are influenced by the increase in total IgG.
- To quantitate total anti-HCV antibody titers in responders and non responders.
- To establish an in-house Elisa assay for detection of anti-C22 antibodies.

3. MATERIALS AND METHODS

3.1 Materials

NorDynamIC was a Randomized, Open-label, Parallel Group, Multi-centre Study of 12 weeks treatment and follow-up period of 36 weeks (Group1) versus 24 weeks treatment and follow-up period of 24 weeks (Group2). The patients were untreated, and chronically infected with HCV genotype 2 or 3. The treatment consisted of Peginterferon alfa-2a 40kD (PEGASYS®) combined with Ribavirin (Copegus®) [48]. NorDynamic and the amendments for our study were clarified by RKK (Regional komite for med.forskningsetikk) and NSD (Personvernombudet for forskning).The sera were collected in the period from February 2004 till March 2006. The samples were stored frozen at -70 ^{o} C.

Our study included the 24 patients enrolled in the study at Haukeland University Hospital.

These patients' sera were collected at the following time points: day 0 (before start of treatment), day 3, 7, 8, 14, 29, and week 8, 12, 16, 20, 24, 36 and 48. See table-1.

Table-1: Schedule of serum collection

Study points	1	2	3	4	5	6	7	8	9	10	11	12	13
day/week	d0	d3	d7	d8	d14	d29	w8	w12	w16	w20	w24	w36	w48
Group 1	x	x	x	x	x	x	x	x	na	na	x	x	x
Group 2	x	x	x	x	x	x	x	x	x	x	x	x	x

(na: not applicable to group 1)

In addition there were some sera missing from the study points. These are shown as missing (m) in the respective tables.

3.2 Methods

3.2.1 Nephelometer

Total IgG in all the sera was quantified by using nephelometer.

The quantification in nephelometer is based upon the specific reaction of an anti-IgG specific antiserum with the human IgG to be determined. The generated immune complexes are quantified by measuring the side-scattered light. It is an automated assay with relatively short incubation time. The samples were diluted and standard and control sera were introduced in the reaction tubes of the nephelometer. Anti-IgG reagent and reaction buffer were then added

and side-scattered light was recorded. Total IgG concentration in the test samples were then calculated relative to the calibration curve, which was obtained with the IgG standard serum. A control serum was assayed to check the validity of the calibration curve.
We have used kit from Dade Behring Inc. See table-2.

Table-2: Reagents and instruments in total IgG quantification

Instrument- Dade Behring BN II
Antiserum for human IgG - Rabbit anti-human IgG
Protein Standard SL (human)
Protein Control SL/M (human)
Reaction Buffer
Diluent Buffer
Distilled water with 25% Tween

3.2.2 Complement Fixation Test (CFT)

Complement fixation test was used to detect antibodies (IgG) against adeno virus and morbilli virus. The serum samples were diluted 1:4 with veronal buffer (VB) (see table-3). Diluted patient sera, positive and negative controls were incubated in a water bath at 56^{o} C for 30 minutes to inactivate endogenous complement. Antigens, run control antibodies, complement and VB were prepared according to the procedure from Serion Immunodiagnostica GmbH.
Microtiter plates (96 wells, U bottom) were used for titration. A pre-programmed pipetting instrument (Micro Lab® AT Plus, Hamilton Company, USA) was used for titration. Titration started with addition of 25µl VB to each well. Then 25µl of 1:4 diluted serum was added to well number one and from there two-fold titrations were done up to well number seven which results in dilutions from 1:8 up to 1:512. In well number eight, 25µl of 1:4 diluted serum was added without serum antigens to serve as control for detection of anti-complementarity. Thereafter 25 µl of antigen dilution was added to wells 1-7 and 25 µl of VB was added to well number eight (serum control). Finally, 25 µl of complement solution was added and plates were incubated overnight at 2-8 ^{o}C.
Indicator system was prepared by mixing amboceptor (haemolytic anti-sheep erythrocyte serum) and sheep erythrocytes. Packed sheep erythrocytes (Dade Behring) were washed by

centrifugation at 2000 rpm for 20 minutes twice in physiological salt water and three times in VB. Working solution of sheep erythrocytes (0.8%) was prepared by diluting in VB.

Amboceptor working solution was prepared by diluting the stock solution to 1:100 with VB (350µl amboceptor diluted in 9, 4 ml VB and 300 µl 5% phenol).

The haemolytic system was prepared by mixing amboceptor working dilution and erythrocyte suspension in equal parts and incubated at room temperature for a minimum of 30 minutes to allow the erythrocytes to be sensitized and kept at 2-8 C^o overnight.

The second day the plates with sera were incubated for 30 minutes at 37^o C and 50 µl haemolytic system was added to each well and incubated for 30 minutes at 37^o C. Then plates were incubated at 4-8 C^o for about 1 hour before reading. Total haemolysis was recorded as negative reaction and total inhibition of haemolysis recorded as positive reaction and the highest titer recorded as result. See table-3, for materials used in CFT. .The explanation for recording the results from CFT is shown in appendix-1

Table-3: Reagents and instruments used in CFT

Instrument for titration: Micro Lab® AT Plus, Hamilton Company.
Reagents from Serion Immunodiagnostica GmbH
Antigens: Lyophilized adeno virus and measles virus antigens isolated from infected cells.
Positive/Negative control sera: Lyophilized adeno virus and morbilli virus human control sera.
Complement: Lyophilized pig serum.
Veronal Buffer: Dissolve vial in distilled water, pH 7,3 +/- 0,1
Haemolytic system from Dade Behring Inc.
Amboceptor: Haemolytic anti-sheep serum.
Erythrocytes: Packed sheep erythrocytes in solution.
Other Reagents
Physiological salt water: To wash packed sheep erythrocytes, pH 7,3 +/- 0,1

3.2.3 Microparticle Enzyme Immunoassay

Abbott semiautomatic Axsym system was used for Rubella IgG antibody detection. The Axsym system is a microparticle enzyme immunoassay (MEIA) for the qualitative and

quantitative detection of IgG antibodies. Diluted serum samples and microparticles coated with partially purified rubella virus (strain HPV77) antigen were mixed and incubated in the processing centre according to the manufacturer's instructions. Microparticles coated with rubella virus antigen would then bind to rubella antibodies forming an antigen-antibody complex.

An aliquot of the antigen-antibody complex was transferred to the glass fibre matrix where microparticles bound irreversibly to the matrix. Unbound materials were then removed by washing. An alkaline phosphatase conjugated anti-human IgG was dispensed onto the matrix cell and then bound to the antigen-antibody complex. Unbound materials were removed by washing the matrix cell. Then 4-Methylumbeliferyl phosphate (MUP) substrate was added to the matrix cell and emitted fluorescence was measured in the Axsym instrument. Results are given in IU/ml which is calculated from the master calibration curve. See table-4, for Rubella IgG quantification in Abbott Axsym system.

Table-4: Reagents and instruments in quantification of rubella IgG

Instrument: Abbott Axsym System®
Reagents for Rubella IgG
Antigen: Partially purified Rubella Virus (strain HPV77) coated on microparticles
Conjugate: Goat anti-Human IgG conjugated with alkaline phosphatase
Assay Diluents: For serum dilution
Run Controls
Negative: Non reactive pooled human serum for anti-rubella IgG
Positive: Reactive pooled human sera to anti-rubella IgG
Substrate: 4-Methylumbeliferyl phosphate (MUP)
Matrix cell wash: 0,3M Sodium Chloride

Both positive and negative controls are non reactive to anti-HIV1/2, HIV-1Ag, HBsAg, and to anti-HCV.

3.2.4 Chemiluminiscence Microparticle Immuno Assay

The ARCHITECT Anti-HCV assay from Abbott Laboratories is a two-step immunoassay using the Chemiluminescence microparticle immuno assay (CMIA) technology with recombinant HCV antigen-coated paramagnetic microparticles and murine anti-IgG/anti-IgM acridinium-labeled conjugate. All sera were tested in 1:50 dilution. Testing was done according to the manufacturer's instructions. Briefly, the diluted serum samples, recombinant HCV antigen coated paramagnetic microparticles and assay diluent were mixed. Anti-HCV antibodies present in the samples would then bind to HCV coated microparticles. After washing, anti-human acridinium-labeled conjugate was added. Pre-Trigger and Trigger solutions were added after another wash cycle. The resulting chemiluminescent reaction was measured as relative light units (RLUs) by the ARCHITECT optic system. Results were calculated automatically and presented as sample/cut off (S/CO). See table-5, for reagents and instrument to detect anti-HCV.

Table-5: Reagents and instruments in detection of anti-HCV

Instrument : Abbott Architect System®
Reagents for anti-HCV
Antigen: Recombinant HCV antigen coated on paramagnetic microparticles.
Conjugate: Acridinium labelled Murine anti-Human IgG/anti-IgM conjugate.
Assay Diluents: For serum dilution
Run Controls
Negative: Non reactive pooled human serum for anti-HCV
Positive: Reactive pooled human sera to anti-HCV
Pre-Trigger: 1,32% (w/v) hydrogen peroxide solution
Trigger: 0,35 N Sodium hydroxide
Wash buffer: Phosphate buffered saline solution

3.2.5 ELISA

An in-house anti-HCV EIA was designed to detect anti-HCV core IgG using recombinant C22 core antigen from Feldan Bio Inc (Quebec, Canada). In brief, the wells of microtiter plates (Maxi Sorp, Nunc) were coated with different concentrations of antigen in 100 µl antigen coating buffer. The plates were then incubated overnight at 4^{o}C or at room temperature. The coating buffer was removed and 200 µl of different blocking buffers were added. All the blocking buffers (except Super-Block) were incubated for 2 hours at 37^{o}C in a humid chamber. Then the plates were washed 3 times using PBS with 0, 05% Tween 20. For Super-Block, the buffer was removed immediately after addition by inversion of the plate and this procedure was repeated three times. The plates were then packed in plastic bags and stored at 4^{o}C. For the ELISA assay, 100 µl patient sera in different dilutions (1:50, 1:100, 1:500, 1:1000, 1:10000) were added to the wells and incubated for one hour at 37^{o}C. After three washes with PBS with 0, 05% Tween 20, 100 µl conjugate of different types was added in different dilutions (1:25, 1:50, and 1:100) and the plates were incubated for 30 minutes at 37 ^{o} C. Plates were then washed four times. Finally, 100 µl of the appropriate substrate was added according to the enzyme conjugate, and incubated at 18-25^{o}C for 30 minutes. The reaction was stopped by adding 100 µl 0, 5 N sulphuric acid and absorbance was read in a micro-titer plate reader at a wavelength of 450nm. See table-6 for details of reagents and instruments used for in-house Elisa.

Table-6: Reagents and instruments for in-house Elisa assay

Instruments				
Microplate washer: Athos company				
Microplate Reader: Athos company				
Data Program Microplate Reader: Megallan company				
Antigen				
Recombinant Hepatitis C Virus Core - rHCV				
Catalogue #	*Clone*	*Concentration*	*Purity*	*Supplier*
FB01-80-1203	rHCV	1 mg/ml	>95%	Feldan Bio Inc, Canada.
Blocking Buffer				
Non-fat Dried Milk: Gene Labs Diagnostics (From the HIV 1/2 Western Blot Kit), Singapore.				
Foetal Bovine Serum: PAA Laboratories GmbH, Austria.				
Super Block Blocking Buffer in PBS: Pierce Biotechnology, USA.				
Conjugates				
Rabbit anti-human IgG HRP: Parvo IgG Elisa kit, Biotrin International Ltd, Ireland.				
Goat anti-human IgG HRP: EBV VCA IgG Elisa kit, Novitec, HISS Diagnostics GmbH, Germany				
F(ab) Rabbit anti-human IgG HRP: Enzygnost® VZV IgG kit, Dade Behring				
Substrates				
Tetramethylbenzidene (TMB)				
Stop solution				
1) 0, 5 mol/l Sulphuric acid from Parvo kit.				
2) 0, 5 N Hydrochloric acid from EBV kit or the Enzygnost® kit.				
Coating Buffer				
15 nM Na_2CO_3, 35 mM $NaHCO_3$ and 3 mM NaN_3 at pH 9,6				

4. RESULTS

4.1 Total IgG Quantification

Total IgG was analysed in serum at the different time points and data are presented in tables-7a and -7b in appendix-2.

Graphically data from all sera are illustrated in figure 5a (see below). Data points from weeks 16 and 20 are excluded because not all patients were included at those dates. It can be seen that there is a marked increase in IgG levels from day 0 to day 8, and a sharp drop till week 8. Most of the sera had the lowest levels of total IgG at week 8 and mean IgG value almost 1g/l lower than at start of the therapy. From week 8, total IgG levels increase and return to baseline. During the first 24 weeks, values for total IgG at day 0 and week 24 were similar whereas day 8 has the highest values and week 8, the lowest values. These time points (d 0, d 8, w 8 and w 24) give clear differences in the IgG levels and were selected to explore the antibody patterns to other viruses such as adeno virus, morbilli virus and rubella virus.

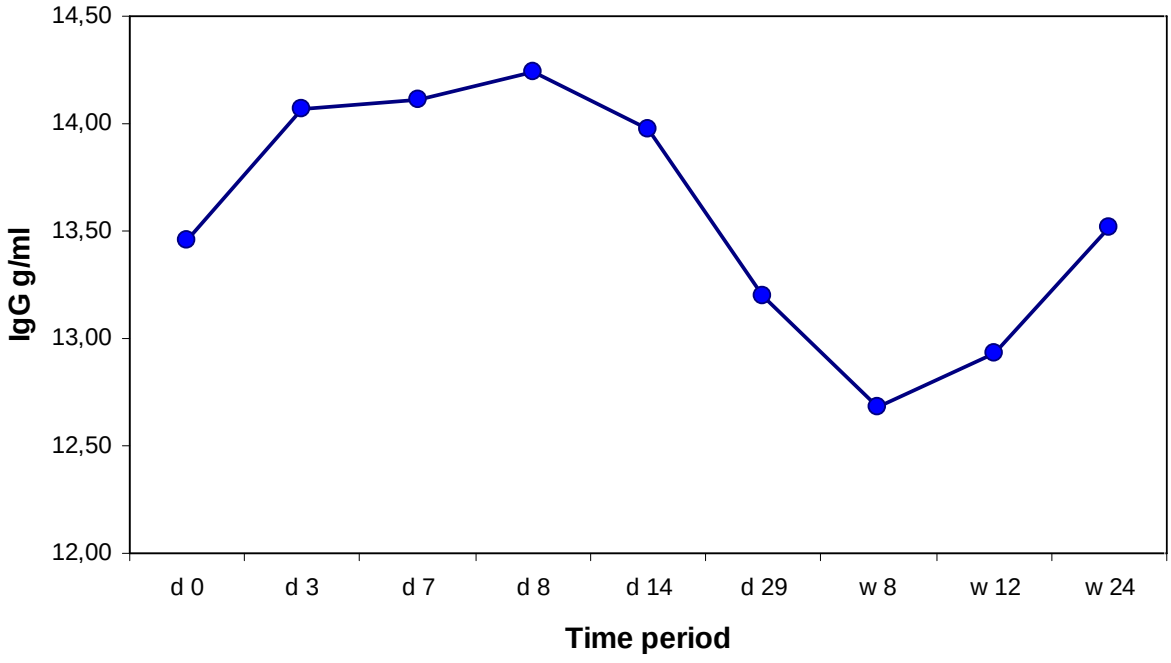


Figure 5a: Mean total IgG over 24 weeks for all patients

Of the 24 patients in the study, 19 were responders and 5 were non-responders. Interestingly, it turned out to be that all the non-responders were in the 12 week therapy group. Figure 5b

shows the total IgG curves of mean values for responders and non-responders. In responders mean total IgG values continue to decline after the cessation of therapy at week 24. However in non-responders there is an increase of total IgG level from week 24 to 36 and it falls back to level lower than baseline at week 48. Figure 5b further clearly shows that the mean total IgG level at each time point studied is higher in non-responders than in responders.

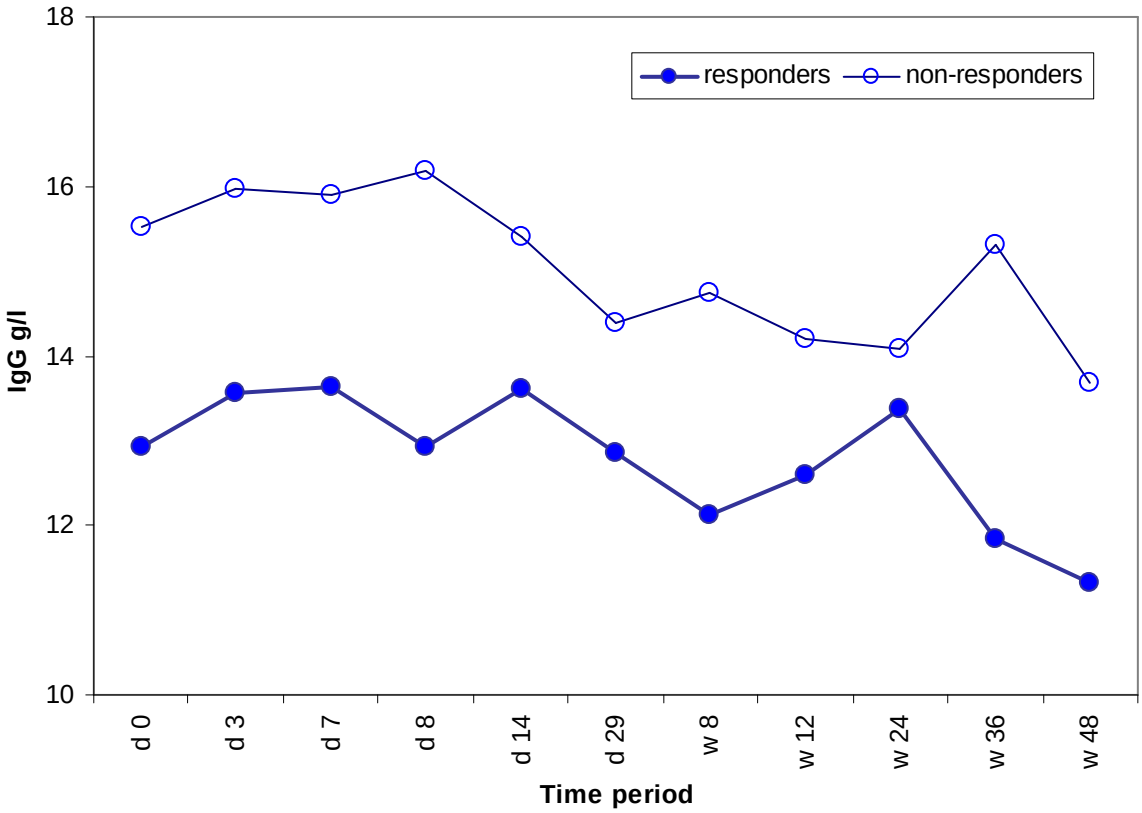


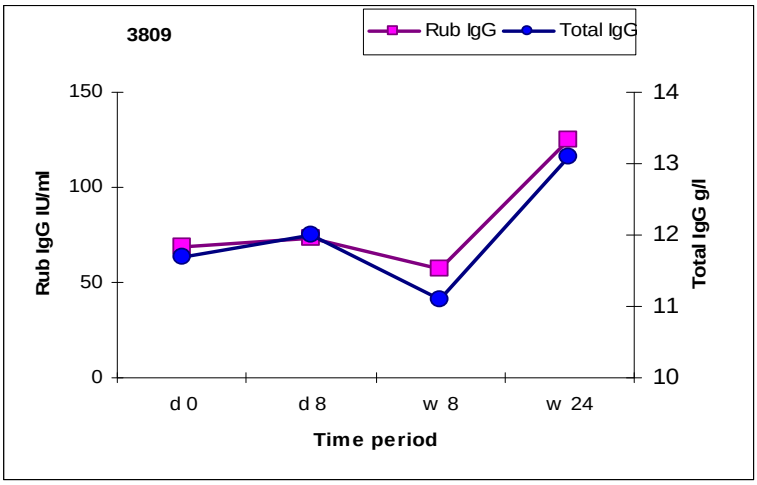
Figure 5b: Mean total IgG for responders and non-responders

4.2 Anti-rubella antibody levels and relationship with total IgG

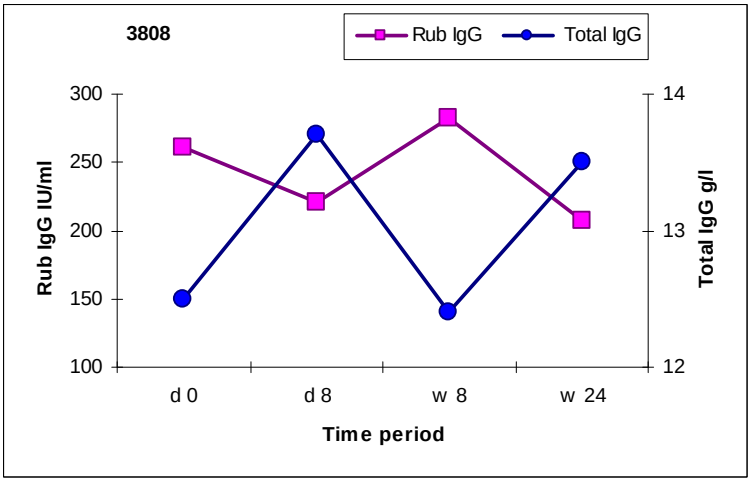
All patients were anti-rubella IgG positive (> 8 IU/ml) at baseline see table-8 (Appendix-3). Anti-rubella IgG levels varied considerably and showed 100 fold difference in range. To see whether the changes in the total IgG were reflected in changes in anti-rubella titers, the data were analysed separately for each patient and are presented in Figure 6a (Appendix-4).

Thirteen sera (3803, 3804, 3805, 3809, 3811, 3814, 3815, 3816, 3817, 3819, 3820, 3824, and 3825) showed the same patterns for rubella IgG and total IgG. Eight sera (3806, 3808, 3810, 3812, 3813, 3818, 3822, and 3826) tended to show the opposite pattern and three sera (3807, 3812, and 3823) had no clear pattern. Figure 6b gives an illustration of these patterns.

SAME Pattern



OPPOSITE Pattern



No Pattern

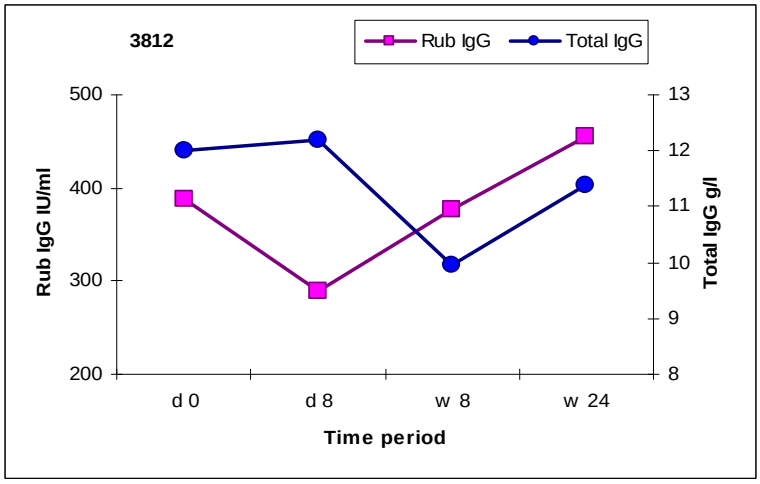


Figure 6b: Patterns of reactivity between rubella IgG and total IgG

4.2.1 Rubella IgG and total IgG in responders and non-responders

Figure 6c shows the pattern for rubella and corresponding total IgG in responders and non-responders. Even though the patterns differ between the two groups with respect to IgG levels after week 8, there was a relationship between rubella IgG and total IgG. The curves are more or less parallel.

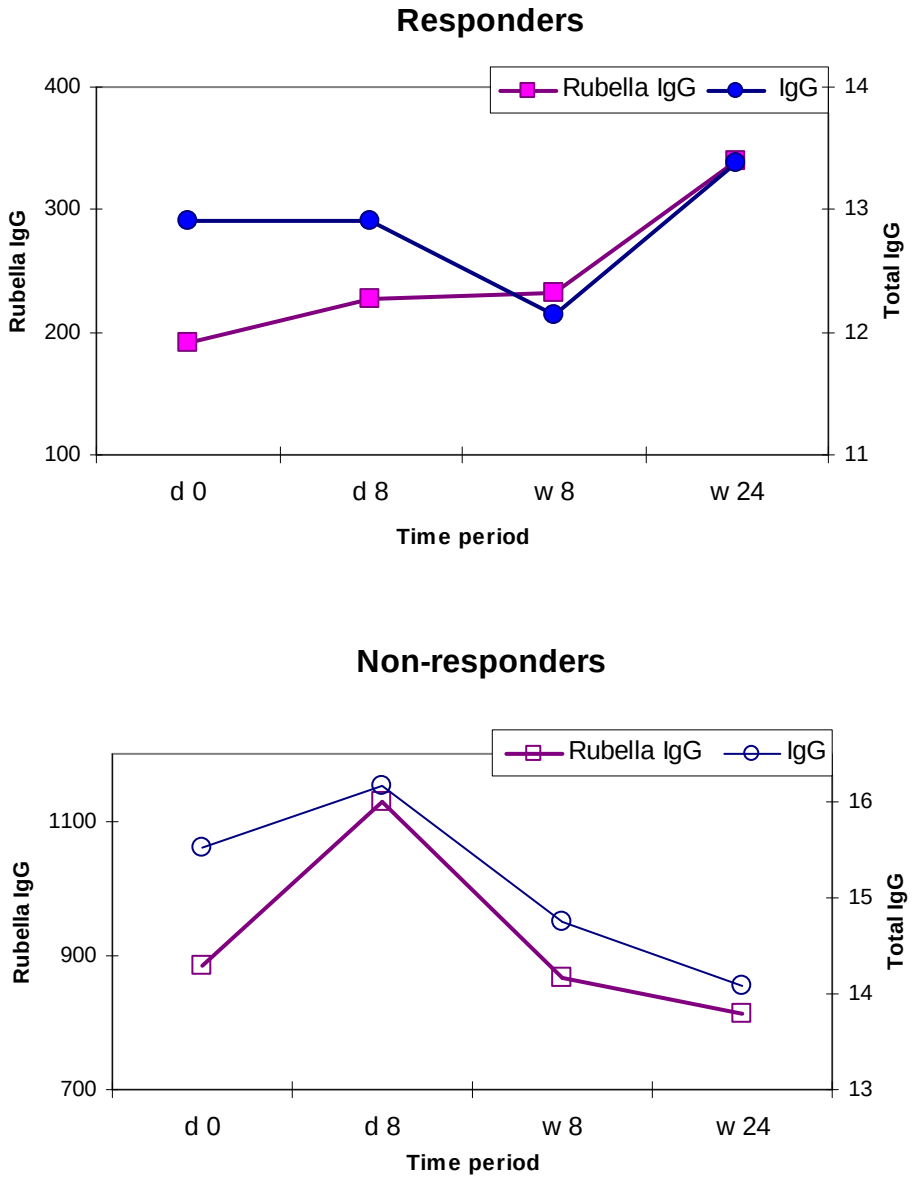


Figure 6c: Rubella IgG and total IgG in responders and non-responders

4.3 Reactivity to adeno virus and morbilli virus and relationships to total IgG and rubella antibodies

The results from CFT to adeno virus, morbilli virus and corresponding total IgG values are shown in table-9a (Appendix-5). It can be seen that, there are fewer patients with antibodies to adeno virus than to morbilli virus. For some of the patients a two fold increase in titer can be seen during therapy, which is not significant. There is thus no evidence that adeno virus and morbilli virus antibodies have influence on the total IgG levels.

In order to analyse the results of adeno virus and morbilli virus and comparing these with rubella virus and total IgG, the data was sorted according to the reactivity in the CFT. See table-9b. (Appendix-6)

4.3.1 No antibody reactivity to adeno virus and morbilli virus

Sera with no reactivity to adeno virus and morbilli virus combined with rubella and total IgG are shown in figure 7a (Appendix-7). Two sera (3811, 3822) have more or less the same patterns to total IgG and to rubella virus IgG whereas the other three (3808, 3818, 3826) had opposite curve patterns.

4.3.2 No antibody reactivity to morbilli virus

The sera which did not show any antibody reactivity or insignificant titers to morbilli virus showed also low or insignificant reactivity to adeno virus (figure 7b, Appendix-8). One exception is patient 3816 that had detectable anti-adeno antibodies and a slight but not significant increase between week 8 and week 24. Several sera (3803, 3807, 3809, 3814, 38 16, 38 17 and 3825) showed the same pattern for rubella antibodies and total IgG but there was no relationship observed between adeno virus antibodies and total IgG in this group.

4.3.3 No antibody reactivity to adeno virus

The sera that did not show any or insignificant antibody titers to adeno virus, showed various reactivity to morbilli virus. See figure 7c (Appendix-9). Sera 3804, 3819, 3821, 3823 and 3824 show a similar pattern for total IgG and rubella virus antibody. However there was no relation between morbilli virus antibodies and total IgG observed in this group either. The results showed that when there is high reactivity (>1:128) to morbilli virus, there is a tendency to get high amount of total IgG, especially in sera 3820 and 3824. Moreover in these two cases, there is also a very high reactivity to rubella virus. However, sera 3803, 3814, and 3815 showed relatively high total IgG without any or little antibody reactivity to morbilli virus.

4.4 Anti-HCV titers and its relationship to total IgG

4.4.1 Anti-HCV

Table-10 (Appendix-10) shows the individual values for antibodies to HCV for all patients whereas figure 8a illustrates curves with mean values for responders and non-responders. In responders the antibodies to HCV declined continuously even after cessation of the treatment. Mean anti-HCV levels dropped from a value of 9 day 0 to 4,5 at week 48.

It so happened that all five non-responders were in group 1, that had only 12 weeks of treatment and antibodies to HCV declined to week 12 but increased after treatment was stopped. Thus, once the treatment is stopped, levels of antibodies increased and returned to levels before treatment, indicating only a temporary response to treatment.

4.4.2 Relationship between anti-HCV and total IgG

The relationship between total IgG and antibodies to HCV for individual patients are shown in figure 8b (responders) and 8c (non-responders) (Appendix-11 and 12). In general shapes of the total IgG and anti-HCV curves follow each other quite well, both in responders and non-responders. For some of the patients the two curves run in parallel whereas for others there is more variation. To get a better appreciation of the profiles, figure 8d was made to show mean values for total IgG and corresponding mean values for antibodies to HCV in responders and non-responders.

For responders both curves are more or less parallel up to week 8. From week 8 to 24 there is an increase in total IgG whereas anti-HCV declines continuously. After 24 weeks both curves become parallel again.

Interestingly the curves for antibodies to HCV and total IgG show remarkably similar profiles for the non-responders. HCV antibodies and total IgG increased up to day 8 of treatment, and then levels were declining to week 12 for anti-HCV whereas total IgG had a fluctuation between day 29 and week 24. After cessation of therapy at week 12 the anti-HCV level increased above the baseline level. Total IgG showed a transient and delayed increase from week 24 to 36 and thereafter declined below the baseline level at week 48.

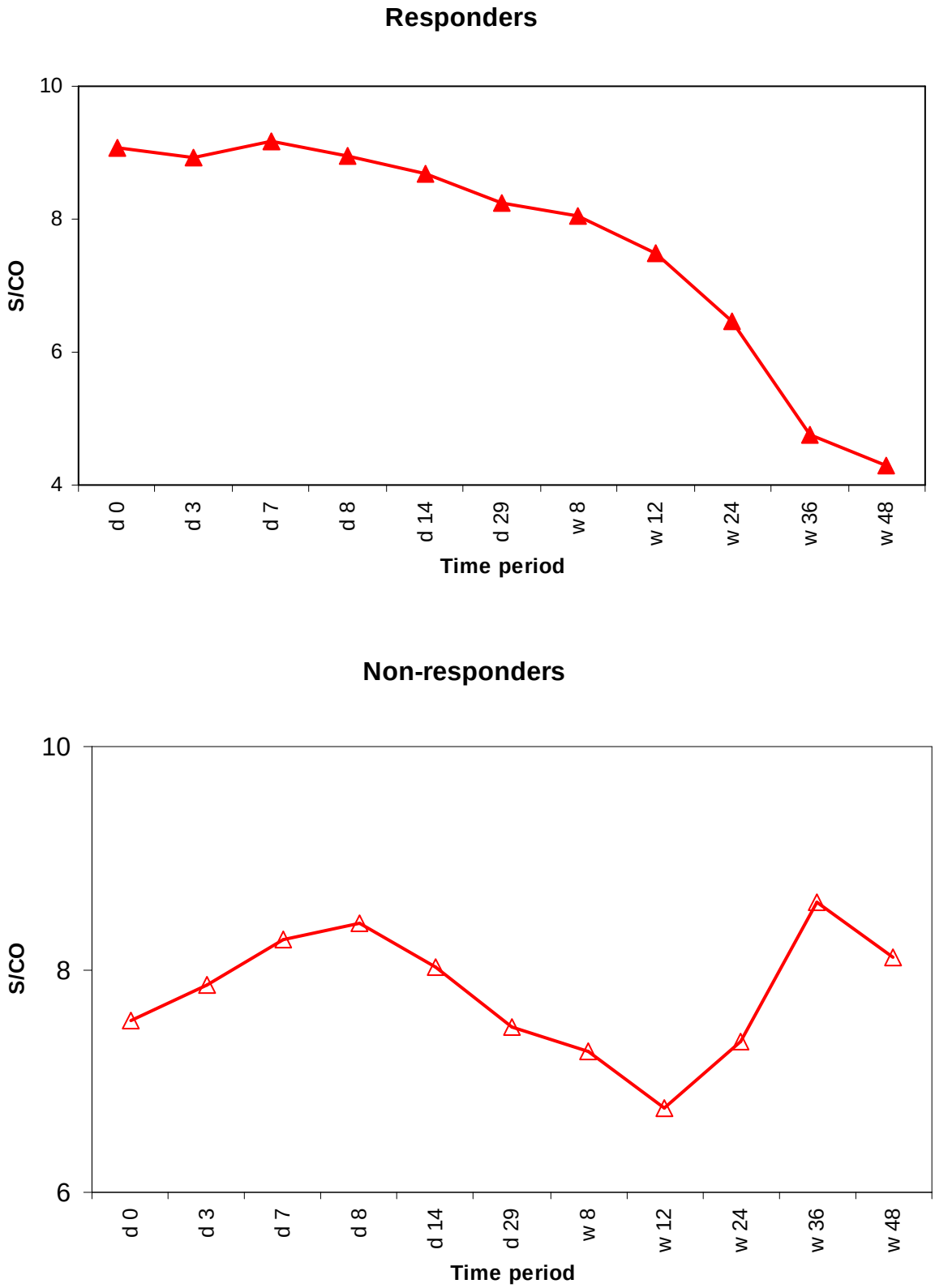


Figure 8a: Mean antibody levels to HCV in responders and non-responders

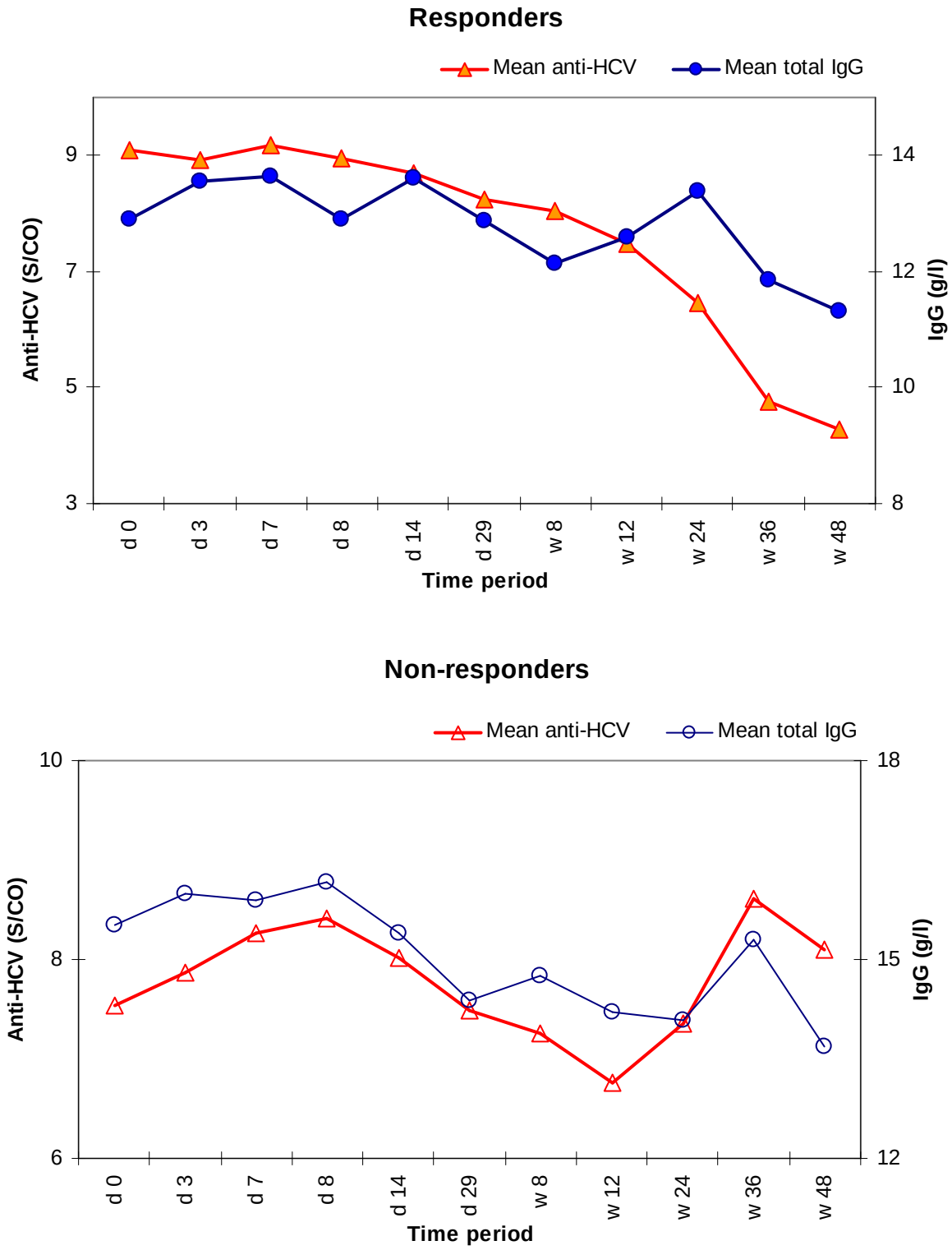


Figure 8d: Mean values of antibodies to HCV and total IgG in responders and non-responders

4.5 In-house ELISA

Several different combinations of conjugates and blocking buffers were used to establish the in-house Elisa with the commercially available antigen. Table-11 summarizes the different combinations used in the efforts to establish the test. All in all, sera from five HCV positive patients with high and four patients with low anti-HCV antibody levels were included. Negative control sera were obtained from six blood donors.

Table-11: Different combinations used to establish in-house ELISA.

Plate nr	Antigen (ng/well)	Conjugate	Blocking Buffer
1	100, 200	F(ab) Rabbit anti-human IgG Enzygnost® VZV IgG kit, Dade Behring	5 % Nonfat dried milk
2	100	F(ab) Rabbit anti-human IgG Enzygnost® VZV IgG kit, Dade Behring	20 % FBS
3	50	F(ab) Rabbit anti-human IgG Enzygnost® VZV IgG kit, Dade Behring	5 % Nonfat dried milk
4	100	Rabbit anti-human IgG HRP Parvo IgG Elisa kit, Biotrin International Ltd	5 % Nonfat driedmilk
5	50	Goat anti-human IgG HRP EBV IgG Elisa kit, Novitec, HISS Diagnostics	20 % FBS
6	100	F(ab) Rabbit anti-human IgG Enzygnost® VZV IgG kit, Dade Behring	Super Block
7	50	F(ab) Rabbit anti-human IgG Enzygnost® VZV IgG kit, Dade Behring	Super Block

Plate 1

OD values from Plate 1 are shown in table-12a (Appendix-13). Half of the plate was coated with 100 ng antigen per well and the other half coated with 200 ng. Only one dilution (1: 50) of conjugate was used. Four low positive anti-HCV sera (columns1-4 and 7-10) and one high positive serum (columns 5 and 11) were used. PBS was used as blank in the first four wells of the columns 6 and 12 and the remaining wells were used for undiluted negative sera from two blood donors in duplicate. Nonfat dried milk (5%) in PBS was used as blocking buffer.

As seen from the table, there is no difference in OD values between sera with high or low anti-HCV antibody levels. Also, the anti-HCV negative sera show the same reactivity as the

positive sera. The use of higher or lower antigen concentration made no difference in OD values.

Plate 2

One antigen concentration (100 ng/well) and two conjugate concentrations (1:25, 1:50) were used. Three high positive (columns 1-3 and 7-9) and two negative sera were used (columns 4-5 and 10- 11). PBS was used as blank in the first four wells of columns 6 and 12 and the remaining four wells were used for undiluted negative sera. All sera were tested in duplicate. Wells were blocked by 20% FBS in PBS.

OD values are shown in table-12b (Appendix-13). Highest OD values can be seen for undiluted negative control sera whereas there is no difference in OD values between diluted positive sera. Since the PBS blanks have very low OD values, this indicates non-specific binding throughout the plate in presence of human serum. OD values from wells with 1:25 conjugate dilution is approximately twice as high as those with 1:50 dilution.

There is no difference or improvement in using the FBS as blocking buffer.

Plate 3

The design for plate 3 is the same as for plate 2 but here the wells were coated with 50 ng antigen, and 5% nonfat dried milk was used for blocking.

Similarly to plate 2, OD values are higher with higher conjugate concentration. Again, it was not possible to distinguish between the positive and negative sera though blocking was done with 5% nonfat dried milk. See table-12c (Appendix-13).

Plate 4

In plate 4, a new conjugate was tested undiluted (ready to use from the Parvo-kit) and diluted 1:2. The plate was coated with 100 ng antigen per well and blocked by 5% nonfat dried milk. Even though OD values were almost doubled with high concentration of conjugate, it does not differentiate between positive and negative sera. Background is high except for blanks.

This shows that different conjugate does not solve the problem with the high background. See table-12d (Appendix-13)

Plate 5

Plate 5 was coated with 50 ng antigen per well. Another new conjugate from the EBV-kit was used undiluted (ready to use) and diluted1:2. The other parameters were the same as for plate 4. Again, the OD values were very high and gave no difference with the two concentrations of conjugates used, and do not differentiate between positive and negative serum. Blanks had very low OD values and were comparable to the other plates. See table-12e (Appendix-13).

Plate 6

At this point, the commercial antigen supplier (Feldan Bio Inc, Canada) was contacted in order to discuss the problem. However, the supplier could only give the information that the product was used for ELISA tests by three large diagnostic companies, and by four others used in rapid diagnostic kits. Due to confidentiality agreements between the companies, Feldan Bio Inc could not disclose any procedures or customer references, but they suggested lowering the antigen concentration to 100 ng, which was already tried in different combinations (Plates 1, 2 and 4).The choice was to use a new blocking buffer (Super Block) since nonfat dried milk and FBS could not discriminate positive sera from negative in the previous tests.

Plate 6 was coated with100 ng antigen/well and two dilutions of the conjugate (1:50 and 1:100) were used. Five positive (wells A1 to G3) and five negative sera (wells A3 to G4) were used in three dilutions (1:50, 1:100 and 1:1000) and added to 4 single wells as shown in table-12f (Appendix-13). A high positive serum in two-fold dilutions (starting from dilution 1:50 to 1:6400) was added in column 5. A negative serum titrated in two fold (1:50 – 1:200) in first four wells in column 6 and the remaining wells were used for PBS as blanks. The same setup applied for the other half of the plate with a different conjugate concentration.

Table-12f (Appendix-13), shows that the OD values are lower with Super Block as compared to the other blocking buffers. Still there is no discrimination between the positive and the negative sera. PBS blanks continue to show very low OD values. Thus, with the use of Super Block, the OD values are considerably lower. Higher or lower concentrations of the conjugate also did not make any difference.

Plate 7

The wells were coated with 50 ng and all other conditions were the same for plate 6 (Table-12f). OD values for plate 7, shown in table-12g (Appendix-13) were very low. Thus lowering the antigen concentration shows no improvement in OD values compared to plate 6.

5. DISCUSSION

Current guidelines for chronic Hepatitis C recommend peginterferon-alpha and ribavirin combination therapy for 24 or 48 weeks, based on viral factors (genotype, viral load), host factors (stage of liver disease) and virological response during treatment. The main goal of the treatment is eradication of HCV infection which is defined by HCV RNA negativity 24 weeks after end of treatment (i.e. sustained virological response). It has long been recognized that patients with HCV genotype 2 or 3 respond better to IFN treatment than patients infected with HCV genotype 1 [49]. Roughly 80% of patients with HCV genotype 2/3 are cured from their disease after the combination treatment.

Although immune mediated mechanisms are likely to be involved in the pathophysiology of chronic HCV-infection little is known about the effects of interferon on immunoglobulin synthesis in these patients and in particular in combination with Ribavirin. This study was conducted in order to study the effects of the combination therapy on the total immunoglobulin levels in HCV patients.

The sera included in this study are from the 24 patients included in the NorDynamIC study at Haukeland University Hospital. The patents had confirmed chronic HCV hepatitis with compensated chronic liver disease and were infected with genotype 2 or 3 and had no history of other immunomodulating treatment. Approximately 80 % (19 patients) showed good response to the combination therapy that is in line with previous findings [49]. All five non-responders were in group 1 with treatment for 12 weeks whereas only 4 patients in the responding group had 12 weeks of treatment.

The main aim of this work was to study whether the combination therapy with IFN and ribavirin would affect total IgG in chronic HCV patients like it has been found for IFN-treatment for MS patients [44]. Interestingly the results showed that total IgG is increased already three days after start of therapy for most of the patients. The increase continued up to day eight. After that the mean total IgG curves showed a clear decline with a minimum value at week 8, almost 1 g/l lower than the level at the start of therapy. There is thus an early and clear effect on total IgG levels by the combination therapy in patients with chronic HCV infection. This was not observed for the MS patients, possibly because the earliest observation was at three months of therapy. However the variation in the IgG levels during treatment and

a clear decline to levels even below the pretreatment total IgG levels differed from the elevated total IgG values during IFN treatment in MS patients which lasted even six months after termination of treatment [44].

Also in contrast to findings for the MS patients total IgG decline to levels even below those for pretreatment in the HCV patients [44]. The same finding was observed even for the five non-responders. It has been shown previously that in chronic HCV infections there is an increase in total IgG and some subclasses (IgG1, IgG2, and IgM) but no difference in IgG level when the extent of liver disease was considered [50-52]. However, Ulvestad et al showed in their study that there was elevated IgG even after the termination of IFN treatment [44]. Although IFN-α and -β are best known for their role in promoting innate anti-viral immunity, numerous studies have recently indicated a more pervasive role for these cytokines in the adaptive immune response. Emphasis has also been put on the capability of type I IFNs to connect and integrate the functioning of the innate and adaptive immune systems [43].

The anti-HCV antibodies showed a gradual decline in the responding group, whereas total IgG showed variation during the treatment period up to 24 weeks. The underlying mechanism is not known but can be speculated to be due to the variation in activation of immunogobulins by immunomodulating treatment. Tamura et el, showed that total anti-HCV core (anti-HCc) IgG titre correlated well with the IgG1 titre, indicating that IgG1 was the main virus-specific IgG and the changes of IgG1 production mainly contributed to fluctuations of the anti-HCc IgG titre and was related to positivity for HCV-RNA during and after IFN therapy. To our knowledge, there is no other study which shows the relationship between total IgG and anti HCV during combination therapy and the possible reasons for the elevated total IgGs.
Furthermore, the analysis of non-responders revealed that total IgG decreased while patients were on therapy and tended to increase after the cessation of therapy. Interestingly for the non-responders there was a correspondence between antibodies to HCV and total IgG. The increase in total IgG after the termination of therapy is difficult to explain only by IFN therapy. The possible explanation for this may be the persistent activity of HCV that result in antibody synthesis which may contribute to the increase in the total IgG level. This issue has to be studied further.

In this material reactivity to either adeno virus or morbilli virus was not found for the majority of sera. Moreover, there was no correspondence between antibody levels for morbilli or adeno

viruses and total IgG. This is in contrast to previous studies where it has been shown that in chronic liver disease there is a marked increase in morbilli and / or rubella virus antibody titers [45-47]. In the present study our results indicate that there was no increase in antibodies to either adeno or morbilli viruses which could have influenced the total IgG levels in chronic HCV patients.

Interestingly, all the sera in this study showed good antibody reactivity to rubella virus which may indicate a non-specific effect or production of rubella antibodies due to immunomodulatory effect of IFN therapy. The results further show that there is a correspondence between the mean rubella antibody levels and mean IgG levels both in responders and non-responders, which may indicate that the total IgG level may be influenced by the rubella antibodies. Several studies have previously shown an increase in rubella antibodies in chronic liver disease but the exact mechanisms for this finding have yet to be clarified [45-47, 53]. Similarly, further studies are needed to clarify the issue of the production of rubella antibodies due to IFN therapy in chronic HCV patients.

One of the aims of the study was to establish an in-house Elisa for detection of anti-C22 antibodies to see whether the level of antibody has any relationship to total IgG, anti-HCV and to viremia in responders and non-responders. We have tried different combinations of conjugates, conjugate dilutions, different concentrations of antigen per well, different dilutions of sera and different types of blocking buffers. Every attempt of the different combinations did not give satisfactory results. We were informed by the antigen supplier that three big diagnostic companies were using the particular antigen in Elisa methods and four others were used in rapid test. We could not find any references either from Medline search or from the supplier because of the confidentiality agreement with the customers. Our attemps in establishing an Elisa method have so far failed and we could not find any specific reasons for this. A possible explanation can be related to the antigen. Recently, we have found out that the antigen supplier have changed the catalogue number of the product and thus possibly the characteristics of the antigen.

The study concludes that there seems to be an effect of combination therapy on total IgG and total IgG levels corresponds well with the anti-HCV antibody levels in responders. Rubella antibody was increased in all chronic HCV patients in the study and seems to have relationship with the total IgG level. Antibodies to adeno and morbilli viruses were not influenced by the treatment.

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APPENDIX-1

	P1	P2	P3	P4	P5	P6	P7	P8	P9	+ cont	- cont
Serum control											
>512											
1:256											
1:128											
1:64											
1:32											
1:16											
1:8											
Titer	<8	1:8	1:16	1:32	1:64	1:128	1:256	>512	AC	1:64	<8

Explanation for recording of results of CFT. P1- P9; Patient number, AC – Anti complementarity. Antigen and sera mixed together in the presence of complement. Any complex formed will fix the complement remaining in the system. Addition of haemolytic indicator system (sheep erythrocytes coated with antibodies) demonstrates the presence or absence of a specific reaction: when added to a system containing complement, this indicator system will be attacked by the, activated complement and the erythrocytes lysed. If the complement has been used up by antigen-antibody complexes in the serum, the erythrocytes will stay intact. After incubation a “button” of erythrocytes show the positivity of CFT. P1 shows total lysis of erythroctes and results was read as negative or titer <8. From P2 to P8 titers were read as 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and >512. Anticomplementary (AC) activity may result in false positive results. To identify AC serum control (test run without antigen) is included for each serum. P9 has a titer of 1:64 but shows anticomplementarity and has to be retested by pretreating the serum with undiluted complement. Positive and negative controls are also shown in the figure.

APPENDIX – 2

Table-7a: Total IgG in responders

Pt. id	D 1	D 3	D 7	D 8	D 14	D 29	W 8	W 12	W 16	W 20	W 24	W 36	W 48
3803	16,7	18,9	20,3	18,9	18,1	15,6	16,5	15,9	16,2	14,8	16,9	14,9	m
3805	12,7	16,2	15,5	16,5	15,2	16,4	13,9	14	11,6	14,8	14,6	12,9	12,2
3806	13,4	14	13,9	13,6	14,4	14,1	12,6	13,1	na	na	12,6	m	11,3
3807	14,3	15,2	16,5	15,6	15,5	16,8	15,3	18	na	na	15,8	15,3	16,3
3808	12,5	15,3	13,5	13,7	12,9	14,7	12,4	14,5	na	na	13,5	11,6	12,7
3809	11,7	12,3	11,9	12	12,1	11,1	11,1	11	11,4	11,9	13,1	11,1	10,3
3811	7,8	8,51	7,56	7,83	8,9	8,07	6,06	6,39	6,11	5,37	6,39	7,82	6,68
3812	12	11,2	12,4	12,2	11	9,69	9,95	9,69	10,6	10,9	11,4	11,5	9,59
3813	11,4	12,2	11,5	11,4	11	11,3	11,3	11	na	na	13	10,9	10,5
3814	17,3	16,9	18,4	18,5	19,3	17,5	16,4	16,4	na	na	16,1	13,3	10,6
3815	16,5	16,6	16,2	17,8	18	17,2	16,9	18,2	19	17,6	20,1	16,3	13,2
3816	13,7	14,2	14,8	14,1	13,9	11,8	10,1	10,9	na	na	13,1	m	12,3
3817	9,79	12,6	12,8	13,9	12,9	11,1	10	11,2	na	na	12,7	11,6	10,8
3818	10,6	11,4	11,9	11,6	11,3	12,2	11,6	11,4	11,6	12	10,8	10,8	9,86
3819	11,7	11,4	11,9	12,2	11,8	10,9	11	11,2	na	na	13	10,5	13,7
3822	14,1	15,2	15,3	15	14,1	14,3	12,3	12,7	11,9	12,8	13,4	m	12,2
3823	10,7	11,3	10,7	11,3	10,8	11,6	10,9	11,3	12,2	12,9	14	10,8	8,93
3825	12,4	10,7	9,13	11,1	12,3	7,25	7,16	9,82	na	na	10,6	8,33	m
3826	16,1	13,6	14,9	15,6	14,9	m	15,1	12,7	na	na	13	m	m
Mean	**12,92**	**13,56**	**13,64**	**13,83**	**13,60**	**12,87**	**12,14**	**12,60**	**12,29**	**12,56**	**13,37**	**11,84**	**11,32**

Table-7b: Total IgG for non-responders

Pt.id	D 0	D 3	D 7	D 8	D 14	D 29	W 8	W 12	W 24	W 36	W 48
3804	14,2	14	14,1	15,7	14,1	14,4	12,7	12,9	12	13	14,2
3810	11,9	11,2	11	10,9	10,5	9,16	10,3	11,3	11,8	12	9,13
3820	16,9	17,7	17,6	17,4	18,2	16,6	18	15,4	17,3	m	17,7
3821	14,9	14,9	15,6	m	16,1	16,1	15,9	16,2	14,4	m	m
3824	19,7	22,1	21,2	20,7	18,1	15,7	16,8	15,2	14,9	20,9	m
Mean	**15,52**	**15,98**	**15,9**	**16,18**	**15,4**	**14,39**	**14,74**	**14,2**	**14,08**	**15,3**	**13,68**

na - not applicable m - missing serum

APPENDIX - 3

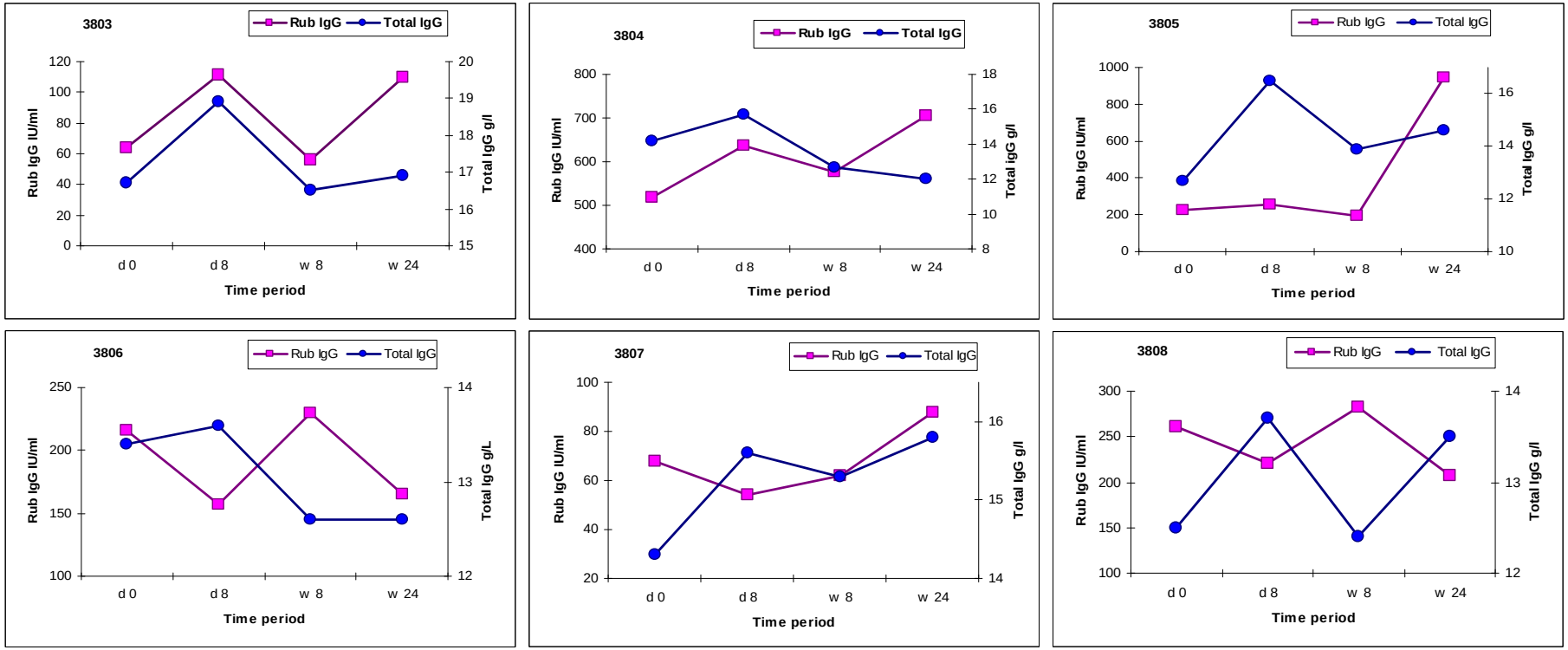
Table-8: Rubella IgG (IU/ml) and total IgG (g/l) for day 0, 8 and week 8 and 24.

RUBELLA IgG - IU/ml				
Pt.id	Day 0	Day 8	Week 8	Week 24
3803	63,7	111,6	56,4	109,9
3804	518,6	635,6	576,9	706,4
3805	224,9	256,5	193	947,7
3806	215,5	157,4	229,6	165,4
3807	67,6	54,1	62,1	88
3808	260,5	220,7	281,9	206,9
3809	68,8	72,9	56,8	124,8
3810	120,8	137,5	150,3	140,3
3811	32,3	29,6	28,3	31,6
3812	388,7	289,4	377,2	454,9
3813	269,6	334	374,4	309,1
3814	229,2	254,1	203,6	115,2
3815	80,3	170,5	128,5	318,7
3816	101,2	100,4	61	123,7
3817	168,6	161,9	106,7	471,3
3818	254,4	211,6	217,7	284,3
3819	136,3	140,3	121,5	156,3
3820	1142,8	1167,9	1162,1	1199,1
3821	357,8	m	466,8	350,1
3822	39,7	34,9	36,9	204,6
3823	47,2	53,2	51,1	45,7
3824	2286,2	2578,7	1977,7	1663,2
3825	791,1	721	655,5	1092
3826	209,9	945,9	1166,1	1196,2

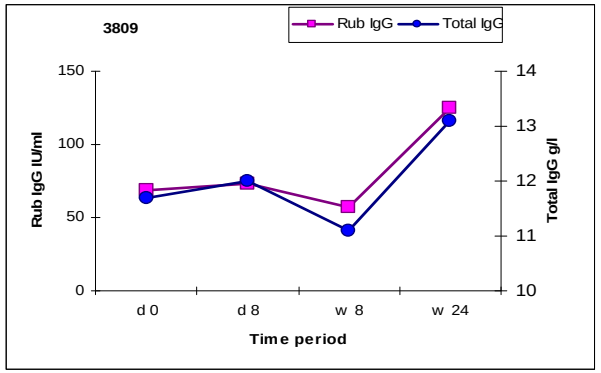
Total IgG - g/l				
Pt.id	Day 0	Day 8	Week 8	Week 24
3803	16,7	18,9	16,5	16,9
3804	14,2	15,7	12,7	12
3805	12,7	16,5	13,9	14,6
3806	13,4	13,6	12,6	12,6
3807	14,3	15,6	15,3	15,8
3808	12,5	13,7	12,4	13,5
3809	11,7	12	11,1	13,1
3810	11,9	10,9	10,3	11,8
3811	7,8	7,83	6,06	6,39
3812	12	12,2	9,95	11,4
3813	11,4	11,4	11,3	13
3814	17,3	18,5	16,4	16,1
3815	16,5	17,8	16,9	20,1
3816	13,7	14,1	10,1	13,1
3817	9,79	13,9	10	12,7
3818	10,6	11,6	11,6	10,8
3819	11,7	12,2	11	13
3820	16,9	17,4	18	17,3
3821	14,9	m	15,9	14,4
3822	14,1	15	12,3	13,4
3823	10,7	11,3	10,9	14
3824	19,7	20,7	16,8	14,9
3825	12,4	11,1	7,16	10,6
3826	16,1	15,6	15,1	13

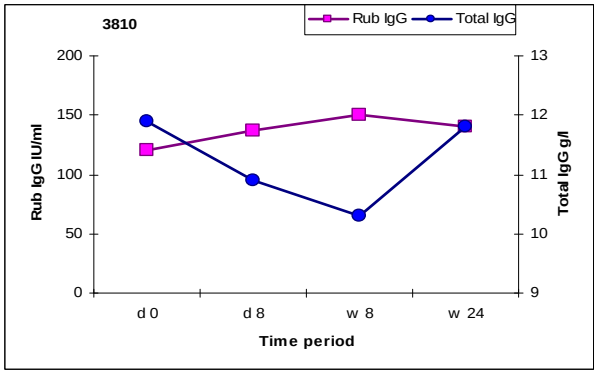
APPENDIX - 4

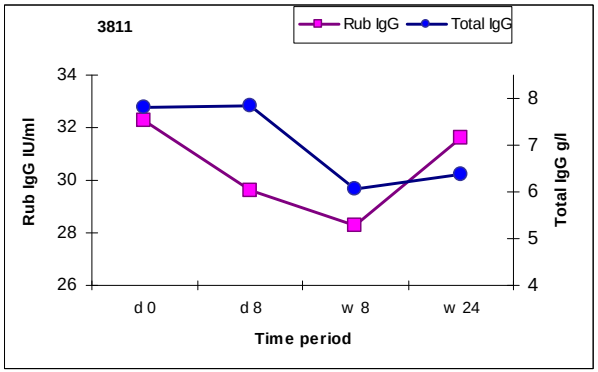
Figure 6a: Rubella IgG and Total IgG for day 0, day 8, week 8, and week 24

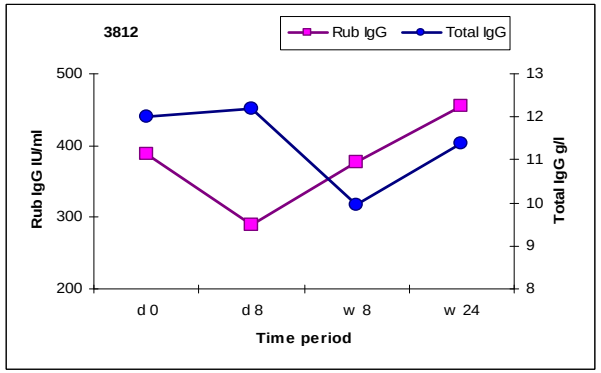


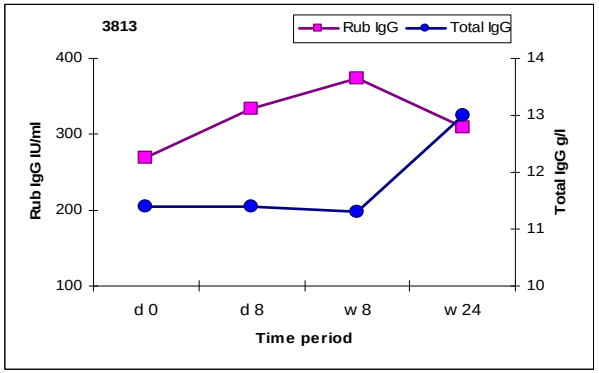
Appendix – 4 Continued

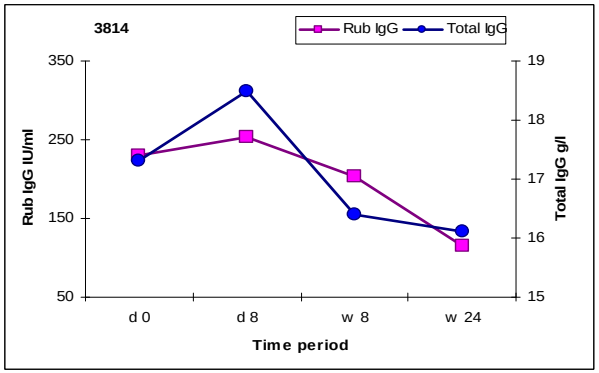


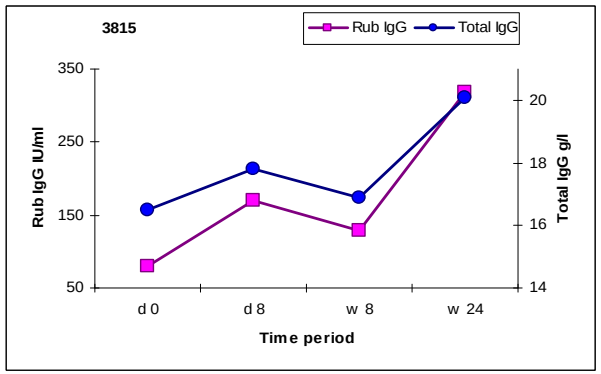


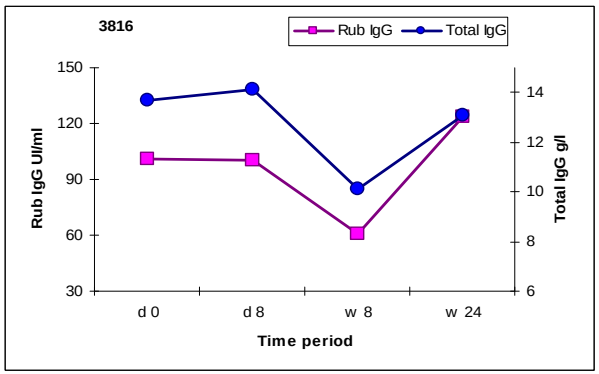


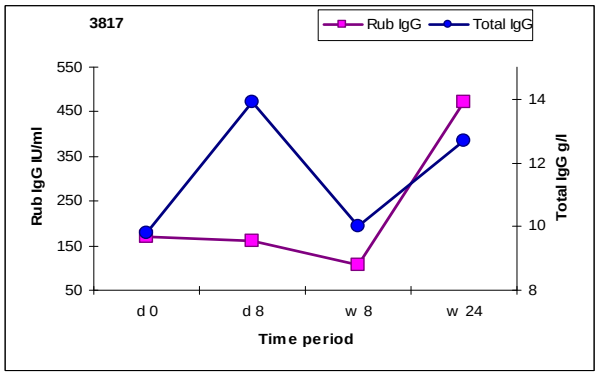




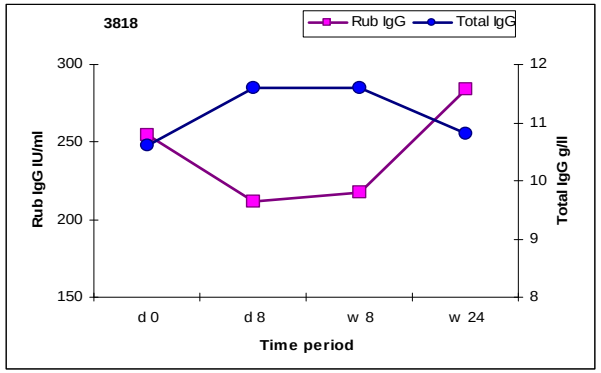


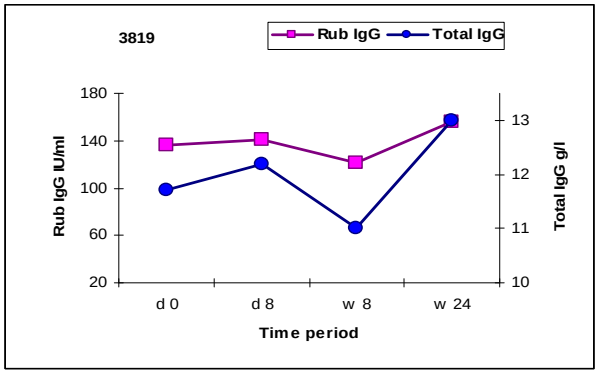


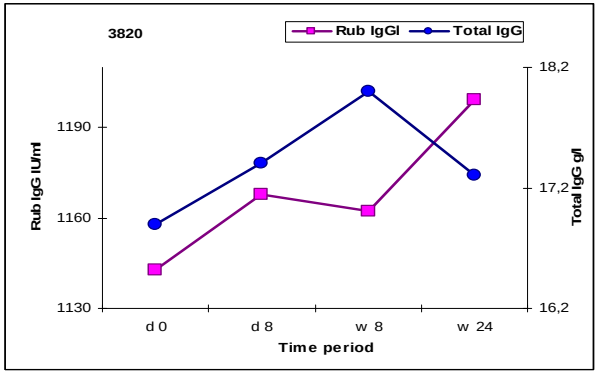


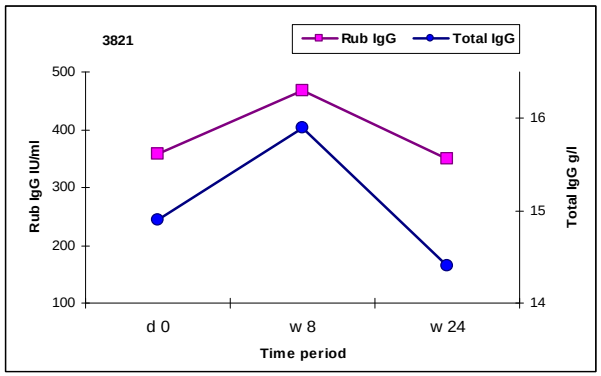


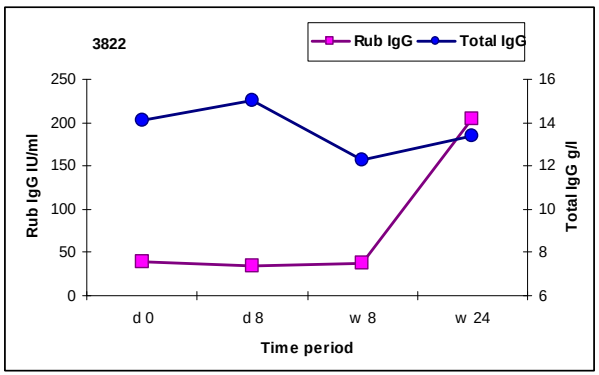
Appendix – 4 Continued

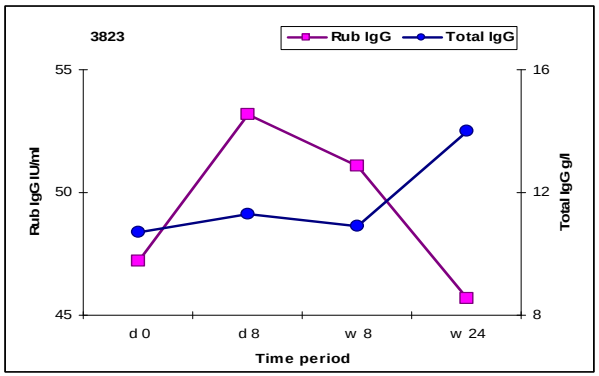


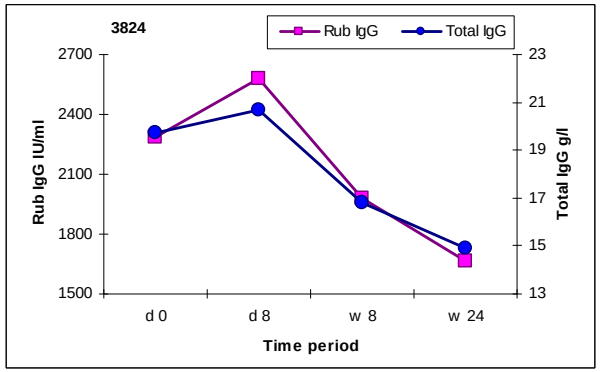


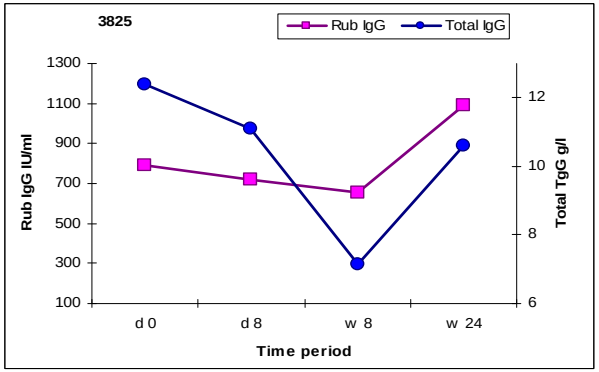


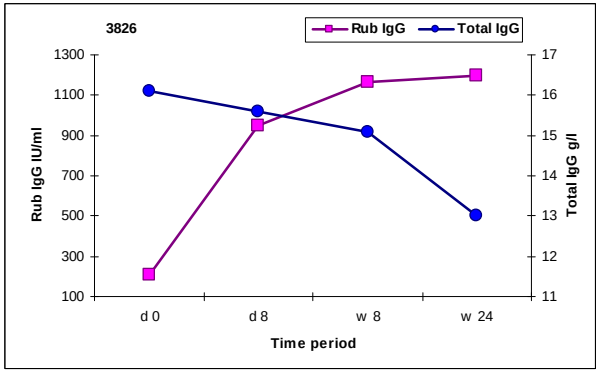












APPENDIX - 5

Table-9a: CFT results for adeno virus and morbilli virus and total IgG

Day/week	d 0	d 3	d 7	d 8	d 14	d 29	w 8	w 12	w 16	w 20	w 24	w 36	w 48
Pt. id.3803													
Adeno	1:32	1:16	1:16	1:16	1:16	1:16	1:16	1:8	1:8	1:8	1:8	1:8	m
Morbilli	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	m
IgG	16,7	18,9	20,3	18,9	18,1	15,6	16,5	15,9	16,2	14,8	16,9	14,9	m
Pt. id.3804													
Adeno	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	na	na	< 8	< 8	< 8
Morbilli	1:128	1:128	1.128	1:128	1:128	1:128	1:128	1:256	na	na	1:256	1:256	1:256
IgG	14,2	14	14,1	15,7	14,1	14,4	12,7	12,9	na	na	12	13	14,2
Pt. id.3805													
Adeno	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:128	1:64
Morbilli	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:64	1:128	1:128	1:128	1:64
IgG	12,7	16,2	15,5	16,5	15,2	16,4	13,9	14	11,6	14,8	14,6	12,9	12,2
Pt. id.3806													
Adeno	1:8	1:8	1:8	1:8	1:16	1:16	1:16	1:12	na	na	1:16	m	1:16
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	na	na	<8	m	<8
IgG	13,4	14	13,9	13,6	14,4	14,1	12,6	13,1	na	na	12,6	m	11,3
Pt. id.3807													
Adeno	1:8	1:16	1:16	1:32	1:32	1:32	1:32	1:32	na	na	1:32	1:32	1:32
Morbilli	1:8	1:8	1:8	1:8	1:8	1:8	1:16	1:16	na	na	1:16	1:8	1:8
IgG	14,3	15,2	16,5	15,6	15,5	16,8	15,3	18	na	na	15,8	15,3	16,3
Pt. id.3808													
Adeno	<8	<8	<8	<8	<8	<8	<8	<8	na	na	< 8	< 8	< 8
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	na	na	< 8	< 8	< 8
IgG	12,5	15,3	13,5	13,7	12,9	14,7	12,4	14,5	na	na	13,5	11,6	12,7
Pt. id.3809													
Adeno	1:8	1:8	1:16	1:8	1:8	1:8	1:8	1:8	1:32	1:8	1:8	1:8	1:8
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
IgG	11,7	12,3	11,9	12	12,1	11,1	11,1	11	11,4	11,9	13,1	11,1	10,3
Pt. id.3810													
Adeno	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	na	na	1:64	1:64	1:64
Morbilli	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	na	na	1:16	1:16	1:16
IgG	11,9	11,2	11	10,9	10,5	9,16	10,3	11,3	na	na	11,8	12	9,13
Pt. id.3811													
Adeno	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
IgG	7,8	8,51	7,56	7,83	8,9	8,07	6,06	6,39	6,11	5,37	6,39	7,82	6,68
Pt. id.3812													
Adeno	1:16	1:16	1:16	1:16	1:16	1:16	1:24	1:32	1:32	1:32	1:32	1:32	1:24
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
IgG	12	11,2	12,4	12,2	11	9,69	9,95	9,69	10,6	10,9	11,4	11,5	9,59
Pt. id.3813													
Adeno	1:8	1:8	1:8	1:8	1:8	1:16	1:16	1:16	na	na	1:16	1:16	1:16
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	na	na	<8	<8	<8
IgG	11,4	12,2	11,5	11,4	11	11,3	11,3	11	na	na	13	10,9	10,5
Pt. id.3814													
Adeno	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	na	na	1:16	1:16	1:16
Morbilli	<8	<8	<8	<8	<8	<8	<8	<8	na	na	<8	<8	<8
IgG	17,3	16,9	18,4	18,5	19,3	17,5	16,4	16,4	na	na	16,1	13,3	10,6

Appendix -5 continued

Pt. id.3815													
Adeno	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**1:64**	**1:64**	**1:64**	**1:64**	**1:128**	**1:64**	**1:64**
Morbilli	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**1:64**	**1:64**	**1:32**
IgG	**16,5**	**16,6**	**16,2**	**17,8**	**18**	**17,2**	**16,9**	**18,2**	**19**	**17,6**	**20,1**	**16,3**	**13,2**
Pt. id.3816													
Adeno	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:64**	**na**	**na**	**1:64**	**m**	**1:64**
Morbilli	**1.8**	**1:8**	**1:8**	**1:8**	**1:8**	**<8**	**<8**	**<8**	**na**	**na**	**<8**	**m**	**<8**
IgG	**13,7**	**14,2**	**14,8**	**14,1**	**13,9**	**11,8**	**10,1**	**10,9**	**na**	**na**	**13,1**	**m**	**12,3**
Pt. id.3817													
Adeno	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:32**	**na**	**na**	**1:32**	**1:32**	**1:32**
Morbilli	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**na**	**na**	**1:16**	**1:16**	**1:16**
IgG	**9,79**	**12,6**	**12,8**	**13,9**	**12,9**	**11,1**	**10**	**11,2**	**na**	**na**	**12,7**	**11,6**	**10,8**
Pt. id.3818													
Adeno	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**
Morbilli	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**
IgG	**10,6**	**11,4**	**11,9**	**11,6**	**11,3**	**12,2**	**11,6**	**11,4**	**11,6**	**12**	**10,8**	**10,8**	**9,86**
Pt. id.3819													
Adeno	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**na**	**na**	**8**	**8**	**8**
Morbilli	**1:64**	**1:64**	**1:64**	**1:128**	**1:128**	**1:128**	**1:128**	**1:128**	**na**	**na**	**1:128**	**1:128**	**1:128**
IgG	**11,7**	**11,4**	**11,9**	**12,2**	**11,8**	**10,9**	**11**	**11,2**	**na**	**na**	**13**	**10,5**	**13,7**
Pt. id.3820													
Adeno	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:32**	**1:64**	**na**	**na**	**1:32**	**m**	**1:32**
Morbilli	**1:256**	**1:256**	**1:256**	**1:256**	**1:512**	**1:512**	**1:256**	**1:256**	**na**	**na**	**1:256**	**m**	**1:256**
IgG	**16,9**	**17,7**	**17,6**	**17,4**	**18,2**	**16,6**	**18**	**15,4**	**na**	**na**	**17,3**	**m**	**17,7**
Pt. id.3821													
Adeno	**<8**	**<8**	**<8**	**m**	**<8**	**<8**	**<8**	**<8**	**na**	**na**	**<8**	**m**	**m**
Morbilli	**1:128**	**1:128**	**1:128**	**m**	**1:128**	**1:128**	**1:128**	**1:256**	**na**	**na**	**1:128**	**m**	**m**
IgG	**14,9**	**14,9**	**15,6**	**m**	**16,1**	**16,1**	**15,9**	**16,2**	**na**	**na**	**14,4**	**m**	**m**
Pt. id.3822													
Adeno	**8**	**8**	**8**	**8**	**8**	**8**	**<8**	**<8**	**<8**	**<8**	**<8**	**m**	**<8**
Morbilli	**8**	**8**	**8**	**8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**m**	**<8**
IgG	**14,1**	**15,2**	**15,3**	**15**	**14,1**	**14,3**	**12,3**	**12,7**	**11,9**	**12,8**	**13,4**	**m**	**12,2**
Pt. id.3823													
Adeno	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**	**<8**
Morbilli	**1:32**	**1:32**	**1:32**	**1:32**	**1:32**	**1:64**	**1:64**	**1:64**	**1:128**	**1:128**	**1:256**	**1:128**	**1:64**
IgG	**10,7**	**11,3**	**10,7**	**11,3**	**10,8**	**11,6**	**10,9**	**11,3**	**12,2**	**12,9**	**14**	**10,8**	**8,93**
Pt. id.3824													
Adeno	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**
Morbilli	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**	**1:512**
IgG	**19,7**	**22,1**	**21,2**	**20,7**	**18,1**	**15,7**	**16,8**	**15,2**	**m**	**m**	**14,9**	**20,9**	**m**
Pt. id.3825													
Adeno	**1:32**	**1:16**	**1:16**	**1:16**	**1:16**	**1:16**	**1:32**	**1:32**	**na**	**na**	**1:32**	**1:32**	**m**
Morbilli	**1:16**	**1:8**	**1:8**	**1:8**	**1:8**	**1:8**	**1:16**	**1:16**	**na**	**na**	**1:16**	**1:8**	**m**
IgG	**12,4**	**10,7**	**9,13**	**11,1**	**12,3**	**7,25**	**7,16**	**9,82**	**na**	**na**	**10,6**	**8,33**	**m**
Pt. id.3826													
Adeno	**<8**	**<8**	**<8**	**<8**	**<8**	**m**	**<8**	**<8**	**na**	**na**	**<8**	**m**	**m**
Morbilli	**<8**	**<8**	**<8**	**<8**	**<8**	**m**	**<8**	**<8**	**na**	**na**	**<8**	**m**	**m**
IgG	**16,1**	**13,6**	**14,9**	**15,6**	**14,9**	**m**	**15,1**	**12,7**	**na**	**na**	**13**	**m**	**m**

APPENDIX - 6

Table-9b: Combined table for CFT, rubella IgG and total IgG

Pt.id	Day 0	Day 8	Week 8	Week 24
3803				
Adeno	1:32	1:16	1:16	1:8
Morbilli	< 8	< 8	< 8	< 8
Rubella	63,7	111,6	56,4	109,9
IgG	16,7	18,9	16,5	16,9
3804				
Adeno	< 8	< 8	< 8	< 8
Morbilli	1:128	1:128	1:128	1:256
Rubella	518,6	635,6	576,9	706,4
IgG	14,2	15,7	12,7	12
3805				
Adeno	1:64	1:64	1:64	1:64
Morbilli	1:32	1:32	1:32	1:128
Rubella	224,9	256,5	193	947,7
IgG	12,7	16,5	13,9	14,6
3806				
Adeno	1:8	1:8	1:8	1:16
Morbilli	< 8	< 8	< 8	< 8
Rubella	215,5	157,4	229,6	165,4
IgG	13,4	13,6	12,6	12,6
3807				
Adeno	1:8	1:32	1:32	1:32
Morbilli	1:8	1:8	1:16	1:16
Rubella	67,6	54,1	62,1	88
IgG	14,3	15,6	15,3	15,8
3808				
Adeno	< 8	< 8	< 8	< 8
Morbilli	< 8	< 8	< 8	< 8
Rubella	260,5	220,7	281,9	206,9
IgG	12,5	13,7	12,4	13,5

Pt.id	Day 0	Day 8	Week 8	Week 24
3809				
Adeno	1:8	1:8	1:8	1:8
Morbilli	< 8	< 8	< 8	< 8
Rubella	68,8	72,9	56,8	124,8
IgG	11,7	12	11,1	13,1
3810				
Adeno	1:64	1:64	1:64	1:64
Morbilli	1:16	1:16	1:16	1:16
Rubella	120,8	137,5	150,3	140,3
IgG	11,9	10,9	10,3	11,8
3811				
Adeno	< 8	< 8	< 8	< 8
Morbilli	< 8	< 8	< 8	< 8
Rubella	32,3	29,6	28,3	31,6
IgG	7,8	7,83	6,06	6,39
3812				
Adeno	1:16	1:16	1:24	1:32
Morbilli	0	0	0	0
Rubella	388,7	289,4	377,2	454,9
IgG	12	12,2	9,95	11,4
3813				
Adeno	1:8	1:8	1:16	1:16
Morbilli	< 8	< 8	< 8	< 8
Rubella	269,6	334	374,4	309,1
IgG	11,4	11,4	11,3	13
3814				
Adeno	1:16	1:16	1:16	1:16
Morbilli	< 8	< 8	< 8	< 8
Rubella	229,2	254,1	203,6	115,2
IgG	17,3	18,5	16,4	16,1

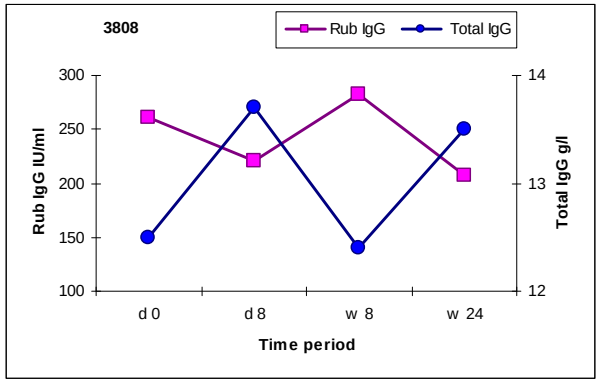
Appendix – 6 continued

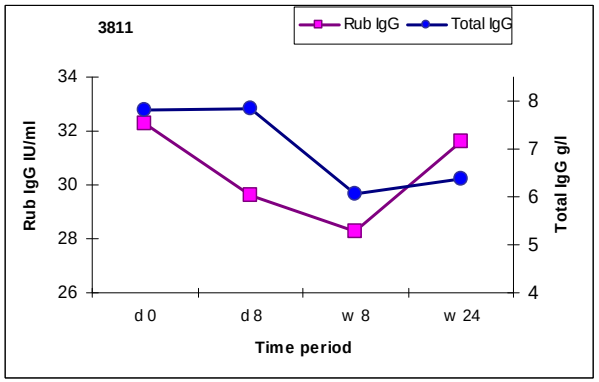
Pt.id	Day 0	Day 8	Week 8	Week 24
3815				
Adeno	1:32	1:64	1:64	1:128
Morbilli	1:32	1:32	1:32	1:64
Rubella	80,3	170,5	128,5	318,7
IgG	16,5	17,8	16,9	20,1
3816				
Adeno	1:32	1:32	1:32	1:64
Morbilli	1:8	1:8	< 8	< 8
Rubella	101,2	100,4	61	123,7
IgG	13,7	14,1	10,1	13,1
3817				
Adeno	1:16	1:16	1:16	1:32
Morbilli	1:16	1:16	1:16	1:16
Rubella	168,6	161,9	106,7	471,3
IgG	9,79	13,9	10	12,7
3818				
Adeno	< 8	< 8	< 8	< 8
Morbilli	< 8	< 8	< 8	< 8
Rubella	254,4	211,6	217,7	284,3
IgG	10,6	11,6	11,6	10,8
3819				
Adeno	< 8	< 8	< 8	1:8
Morbilli	1:64	1:128	1:128	1:128
Rubella	136,3	140,3	121,5	156,3
IgG	11,7	12,2	11	13
3820				
Adeno	1:16	1:16	1:32	1:32
Morbilli	256	256	256	256
Rubella	1142,8	1167,9	1162,1	1199,1
IgG	16,9	17,4	18	17,3

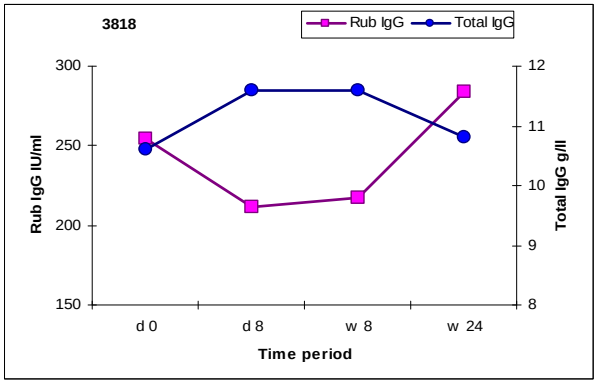
Pt.id	Day 0	Day 8	Week 8	Week 24
3821				
Adeno	< 8	m	< 8	< 8
Morbilli	1:128	m	1:128	1:128
Rubella	357,8	m	466,8	350,1
IgG	14,9	m	15,9	14,4
3822				
Adeno	1:8	1:8	< 8	< 8
Morbilli	1:8	1:8	< 8	< 8
Rubella	39,7	34,9	36,9	204,6
IgG	14,1	15	12,3	13,4
3823				
Adeno	< 8	< 8	< 8	< 8
Morbilli	1:32	1:32	1:64	1:256
Rubella	47,2	53,2	51,1	45,7
IgG	10,7	11,3	10,9	14
3824				
Adeno	1:16	1:16	1:16	1:16
Morbilli	1:512	1:512	1:512	1:512
Rubella	2286,2	2578,7	1977,7	1663,2
IgG	19,7	20,7	16,8	14,9
3825				
Adeno	1:32	1:16	1:32	1:32
Morbilli	1:16	1:8	1:16	1:16
Rubella	791,1	721	655,5	1092
IgG	12,4	11,1	7,16	10,6
3826				
Adeno	< 8	< 8	< 8	< 8
Morbilli	< 8	< 8	< 8	< 8
Rubella	209,9	945,9	1166,1	1196,2
IgG	16,1	15,6	15,1	13

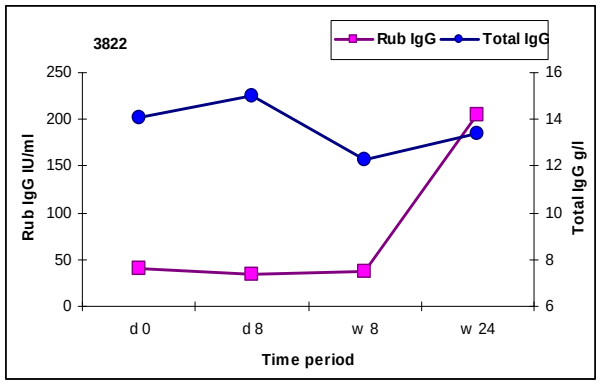
APPENDIX - 7

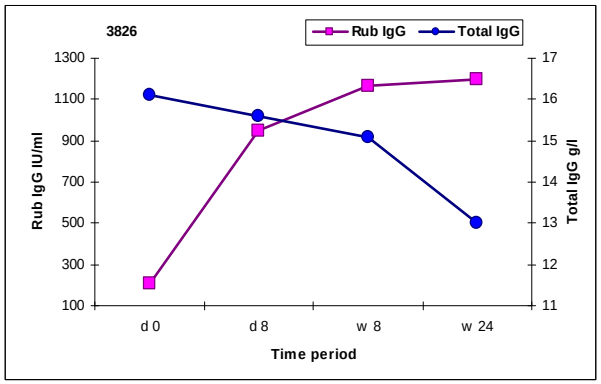
Figure 7a: Sera where there is no antibody reactivity to morbilli and adeno viruses





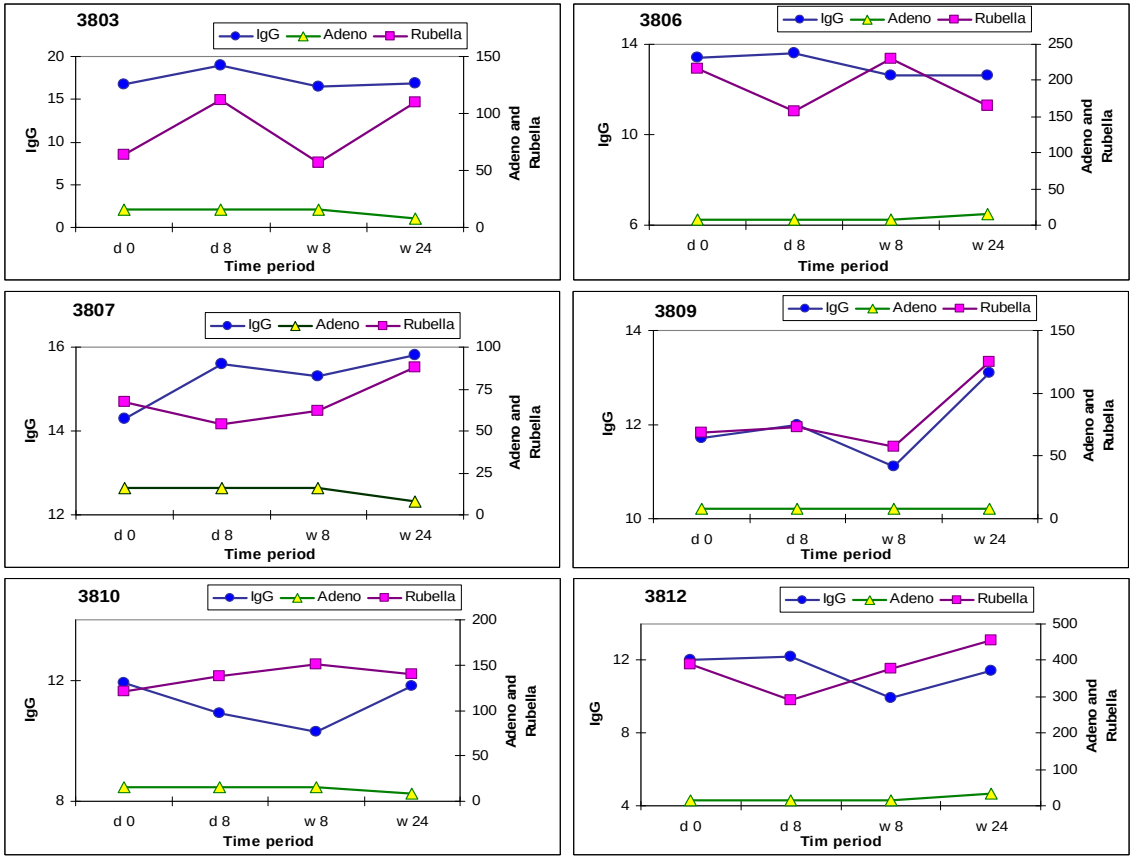




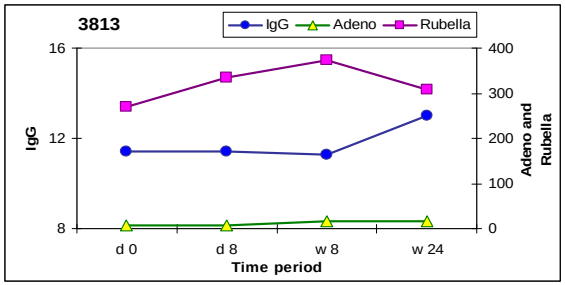


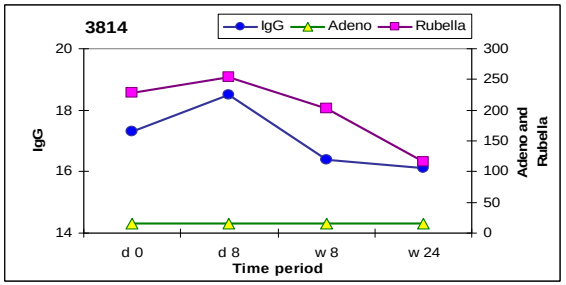
APPENDIX – 8

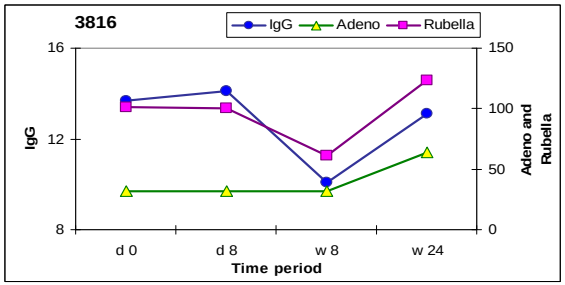
Figure 7b: Sera which did not show any or insignificant reactivity to morbilli virus

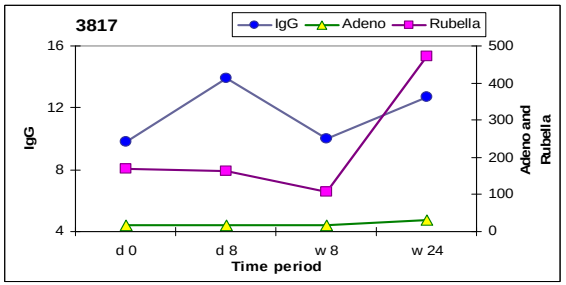


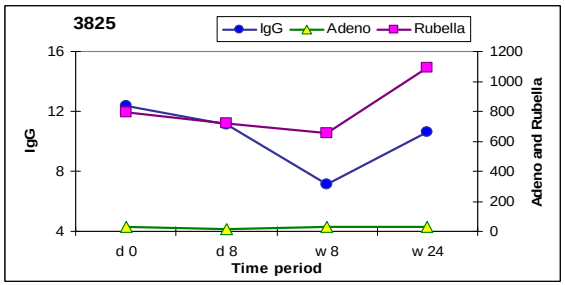
Appendix-8 continued





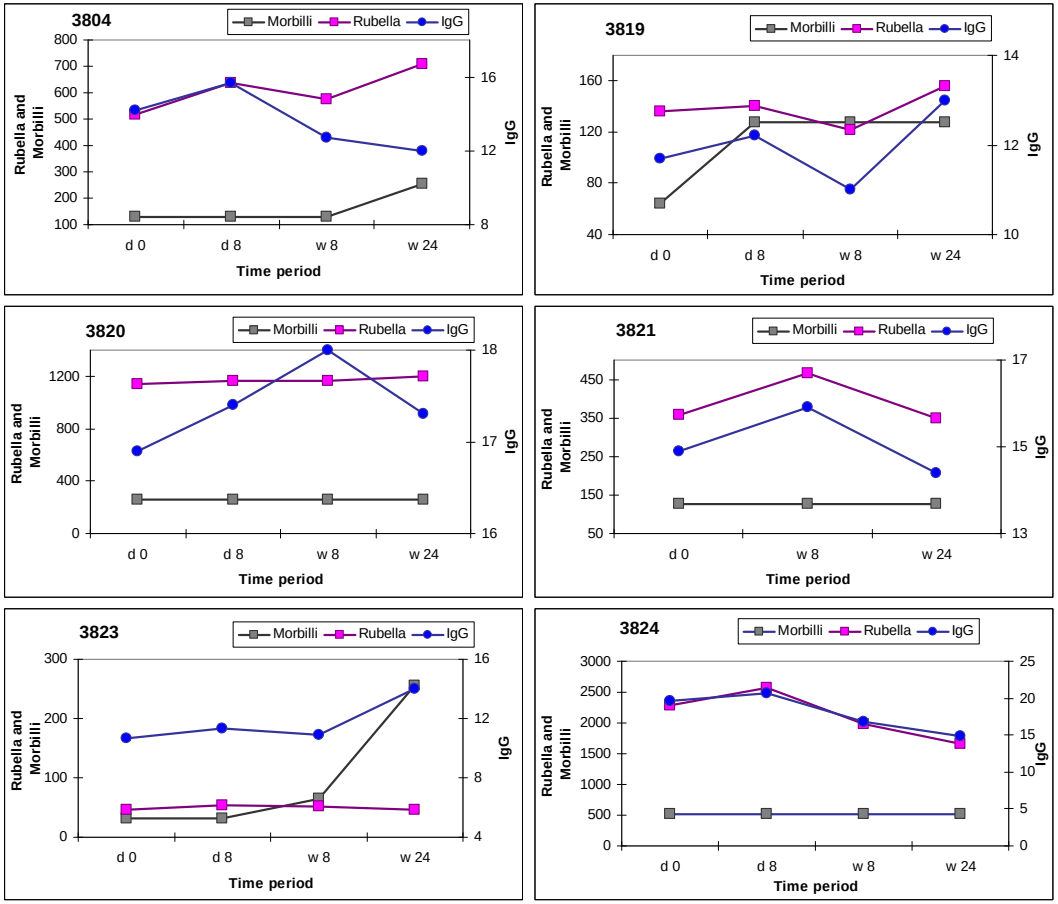






APPENDIX – 9

Figure 7c: Sera which did not show any or insignificant reactivity to adeno virus



APPENDIX - 10

Table-10: S/CO for Total anti-HCV, Dilution 1:50 in Abbott Architect system

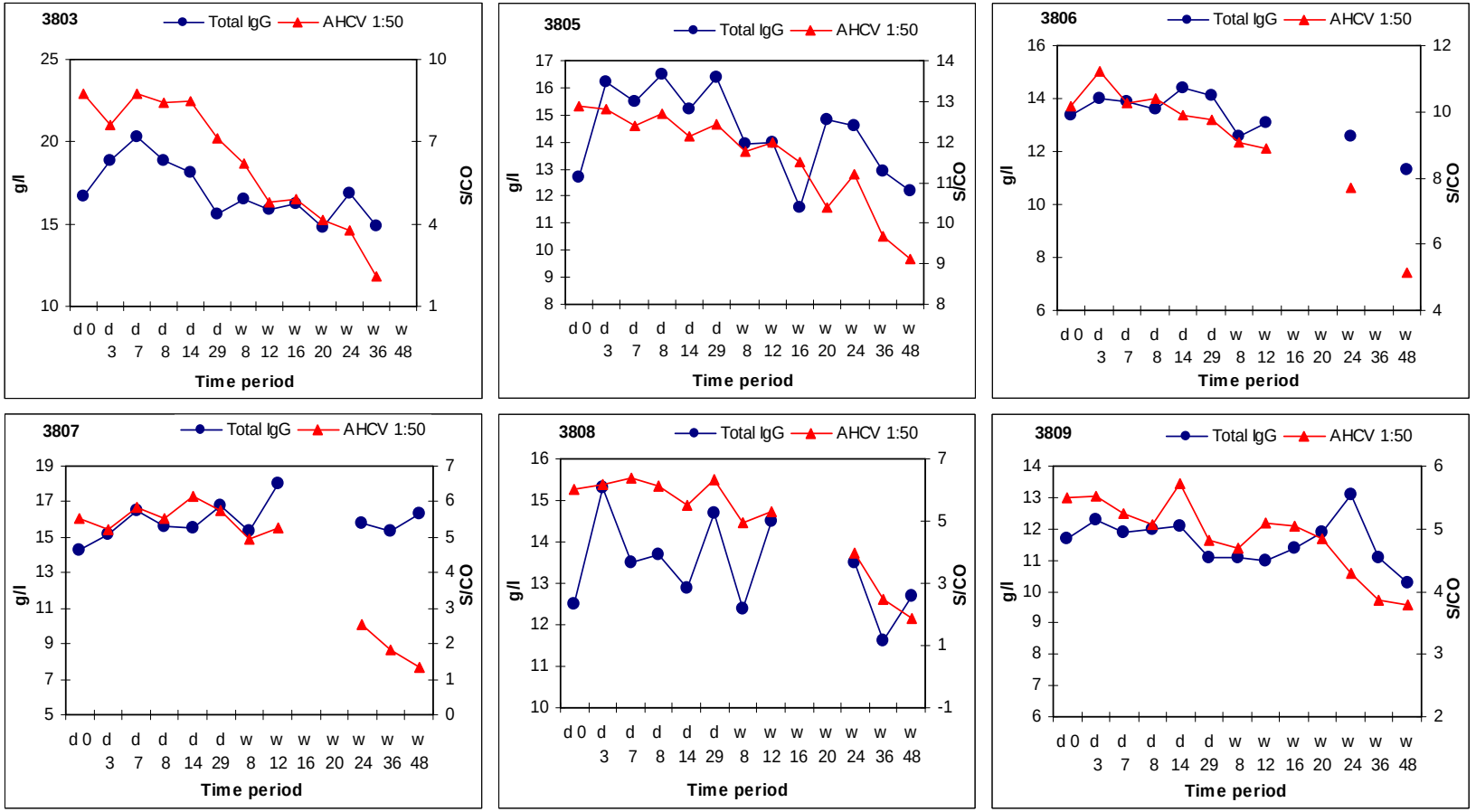
Responders													
Patient id	**D 1**	**D 3**	**D 7**	**D 8**	**D 14**	**D 29**	**W 8**	**W 12**	**W 16**	**W 20**	**W 24**	**W 36**	**W 48**
3803	8,73	7,6	8,75	8,42	8,49	7,1	6,19	4,82	4,89	4,14	3,77	2,08	m
3805	12,89	12,82	12,39	12,7	12,15	12,44	11,77	11,98	11,52	10,4	11,2	9,68	9,13
3806	10,19	11,23	10,25	10,39	9,89	9,75	9,09	8,88	na	na	7,71	m	5,15
3807	5,55	5,21	5,84	5,55	6,17	5,76	4,94	5,27	na	na	2,53	1,84	1,32
3808	6,01	6,18	6,41	6,13	5,51	6,32	4,94	5,31	na	na	na	2,48	1,86
3809	5,5	5,51	5,25	5,06	5,72	4,81	4,69	5,1	5,04	4,84	4,28	3,85	3,79
3811	7,18	7,74	6,51	6,54	6,83	5,39	5,13	4,57	3,96	3,66	3,62	2,69	1,71
3812	9,95	9,53	10,25	9,68	8,7	8,34	6,71	7,09	5,99	5,6	5,98	3,99	2,96
3813	6,04	6,41	5,48	5,59	4,7	5,48	5,5	5,31	na	na	5,07	3,98	2,8
3814	12,96	12,08	12,82	13,05	12,0	12,15	11,56	11,01	na	na	9,19	7,19	4,81
3815	11,87	12,83	13,39	13,18	12,24	12,18	12,09	8,95	10,36	9,9	10,26	9,09	7,22
3816	8,61	9,27	9,76	8,1	8,27	6,75	8,59	7,42	na	na	6,24	m	4,2
3817	10,33	9,07	8,77	9,3	8,71	8,96	7,82	7,93	na	na	6,6	5,64	4,91
3818	7,76	7,94	8,11	8,16	8,48	8,85	10,4	7,51	7,36	6,72	6,5	5,01	4,42
3819	8,44	7,83	9,01	8,78	8,75	7,87	7,87	7,6	na	na	7,52	6,16	6,29
3822	9,31	8,85	10,33	10,06	9,78	8,62	8,57	8,58	7,75	7,91	7,07	m	6,39
3823	5,74	5,52	5,61	5,79	5,58	5,82	5,11	4,09	4,37	4,86	4,03	2,96	1,64
3825	13,48	12,55	13,49	12,48	12,43	11,94	11,49	9,86	na	na	8,16	4,8	m
3826	12,06	11,41	11,84	11,3	10,57	m	10,57	10,93	na	na	9,2	m	m
Mean	**9,08**	**8,93**	**9,17**	**8,96**	**8,68**	**8,25**	**8,05**	**7,48**	**6,80**	**6,45**	**6,47**	**4,76**	**4,29**

Non-Responders											
Patient id	**D 1**	**D 3**	**D 7**	**D 8**	**D 14**	**D 29**	**W 8**	**W 12**	**W 24**	**W 36**	**W 48**
3804	11,16	10,87	11,37	12,07	11,03	10,56	9,56	9,25	9,98	10,2	9,85
3810	3,47	3,7	3,53	3,4	3,18	2,57	3,15	2,97	2,65	3,18	2,81
3820	3,41	5,02	5,53	6,07	5,1	5,17	4,94	4,28	4,87	m	7,75
3821	7,38	7,48	7,83	m	8,08	7,14	7,17	6,73	7,3	m	m
3824	12,29	12,26	13,08	12,14	12,7	11,98	11,48	10,56	11,98	12,43	12,01
Mean	**7,54**	**7,87**	**8,27**	**8,42**	**8,02**	**7,48**	**7,26**	**6,76**	**7,36**	**8,60**	**8,11**

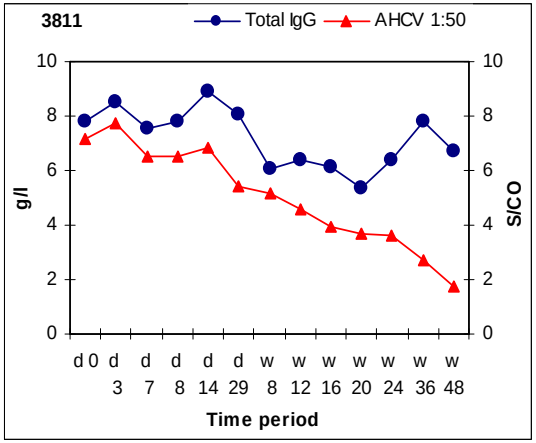
na – not applicable m – missing serum

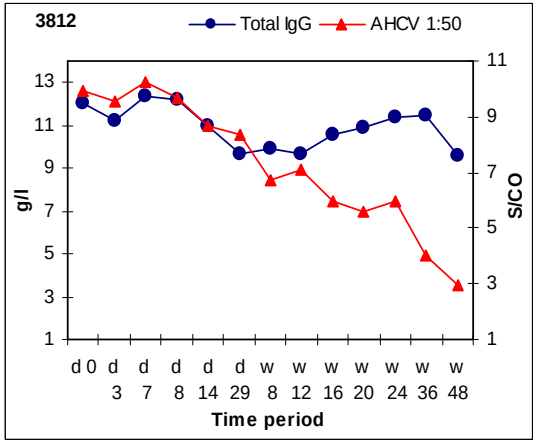
APPENDIX – 11

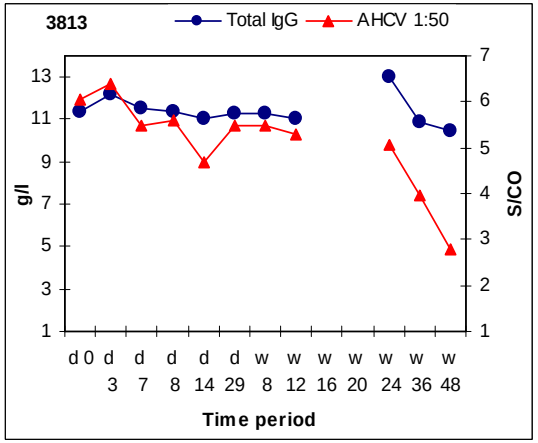
Figure 8b: Total IgG and anti-HCV for responders

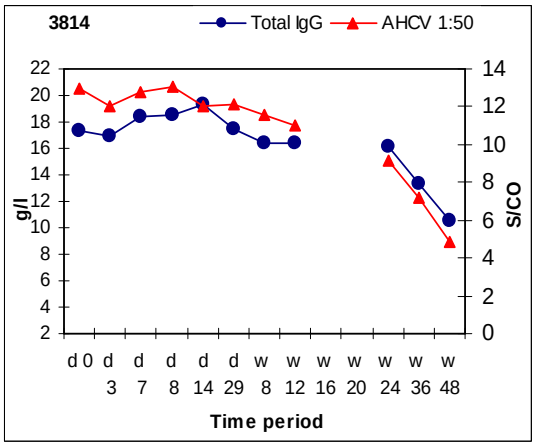


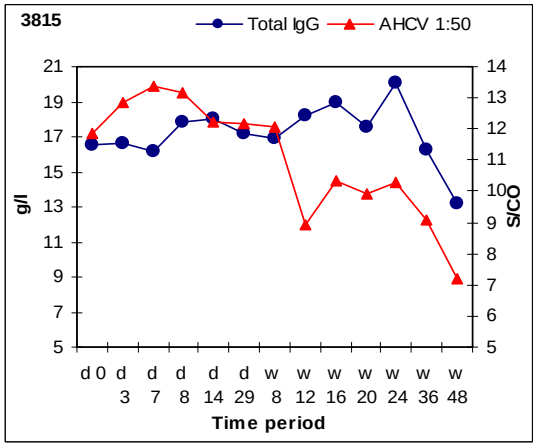
Appendix – 11 continued

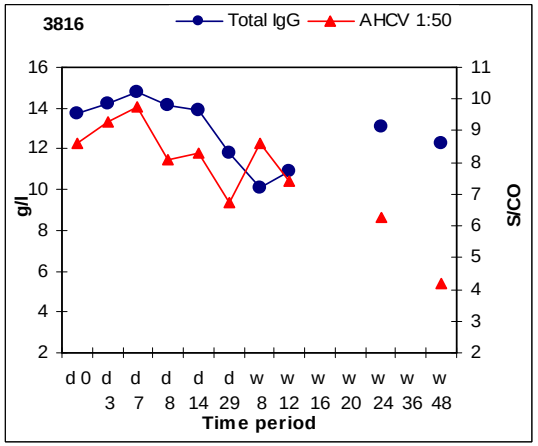




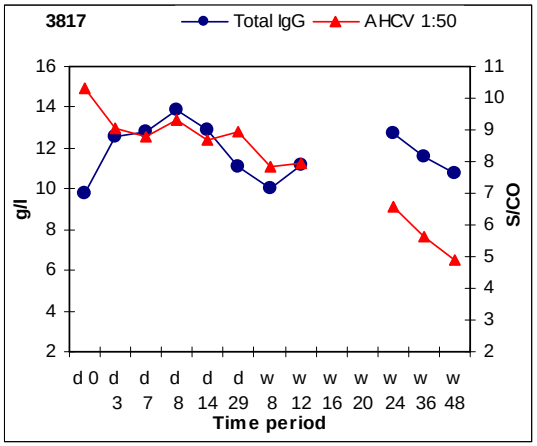


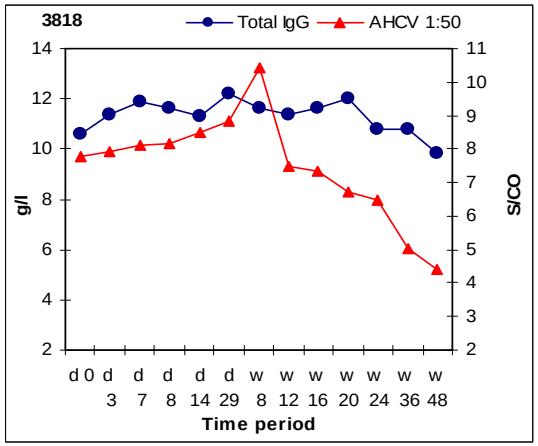


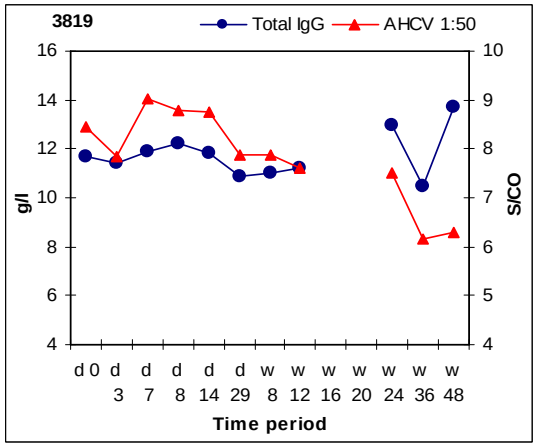


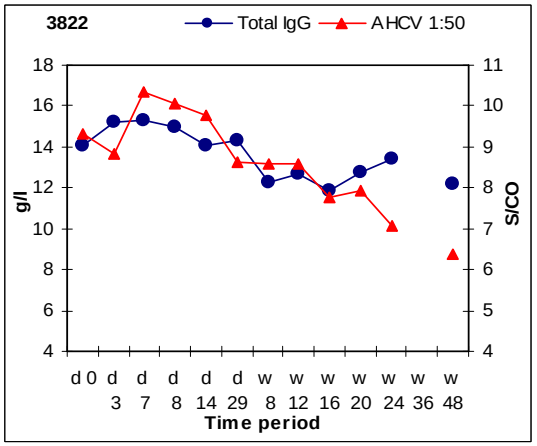


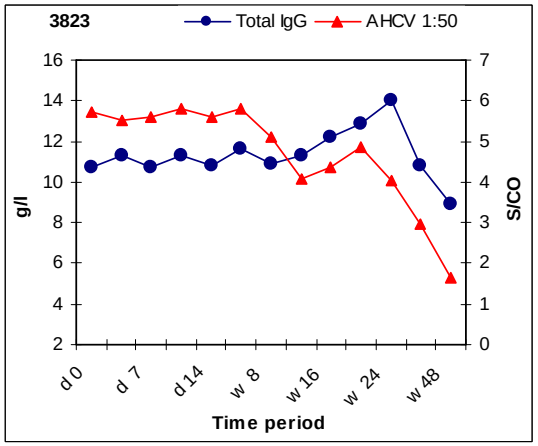
Appendix – 11 continued

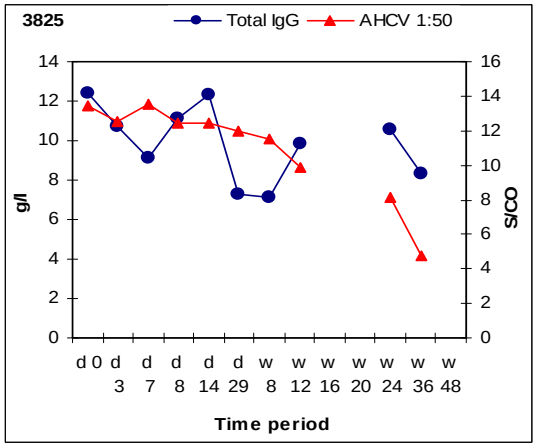




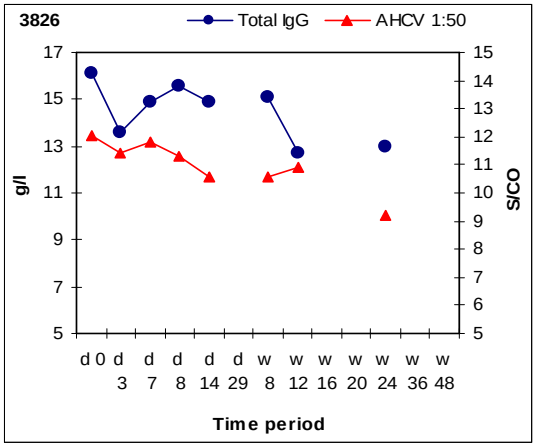






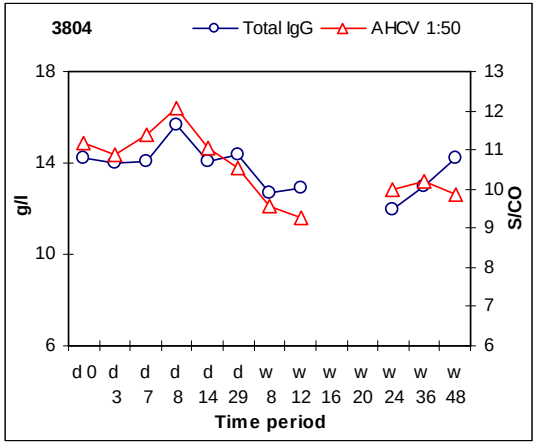


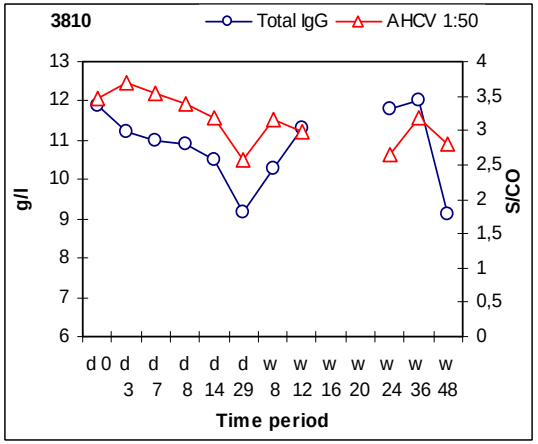
Appendix – 11 continued

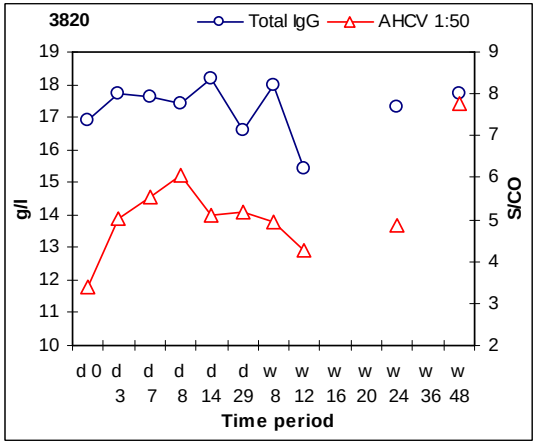


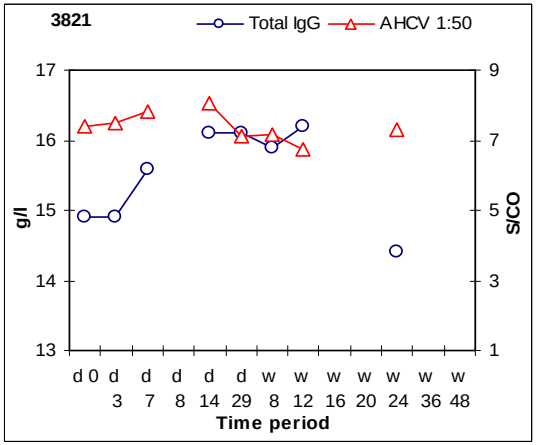
APPENDIX - 12

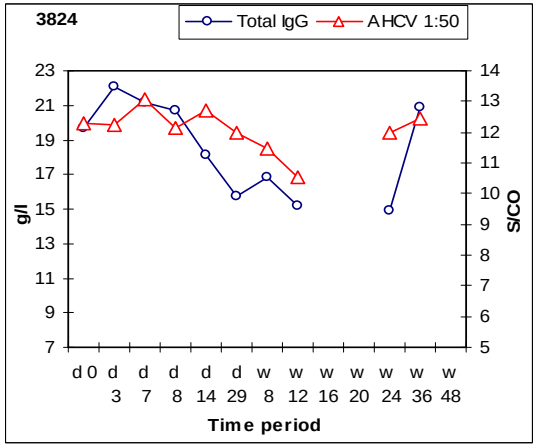
Figure 8c: Total IgG and anti-HCV for non-responders











APPENDIX - 13

Table-12a:

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	0,84	1.00	0,30	0,12	0,60	0,06	0,86	1,03	0,26	0,15	0,53	0,07
B	1,03	1,03	0,27	0,12	0.64	0,05	1,02	1,04	0,30	0,16	0,66	0,07
C	0,24	0,25	0.10	0,08	0,14	0,05	0,21	0,25	0,10	0,10	0,17	0,06
D	0,30	0,24	0,09	0,08	0,15	0,05	0,24	0,27	0,10	0,09	0,17	0,06
E	0,10	0,09	0,06	0,06	0,07	0,56	0,10	0,09	0,08	0,06	0,08	0,39
F	0.10	0,09	0,05	0,05	0,07	0,15	0,10	0,10	0,06	0,06	0,08	0,14
G	0,07	0,07	0,05	0,05	0,07	0,07	0,07	0,07	0,06	0,06	0,08	0,08
H	0,07	0,06	0,05	0,05	0,07	0,06	0,06	0,07	0,06	0,07	0,06	0,08
Antigen	50 ng per well						100 ng per well					
Conjugate	Dilution 1:50											
5 % nonfat dried milk used as blocking buffer												

Table-12b:

Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	0,68	1,21	0,45	0,60	0,76	0,06	0,43	0,65	0.22	0.33	0,35	0.07
B	0,76	1,28	0,44	0,43	0,71	0,06	0,41	0,7	0,24	0,32	0,41	0,07
C	0,26	0,58	0,2	0,19	0,25	0,06	0,15	0,29	0,13	0,13	0,16	0,08
D	0,27	0.55	0,18	0,18	0,27	0,06	0,14	0,28	0,12	0,12	0,14	0,07
E	0,16	0,31	0,13	0,12	0,13	3,40	0,09	0,16	0,10	0,09	0,09	1,50
F	0,13	0,34	0,13	0,11	0,13	3,34	0,09	0,15	0,10	0,09	0,09	1,54
G	0,08	0,13	0,09	0,07	0,08	2,57	0,06	0,08	0,07	0,07	0,08	1,54
H	0,07	0,1	0,09	0,07	0,07	2,31	0,06	0,07	0,06	0,07	0,07	1,52
Antigen	100 ng per well											
Conjugate	Dilution 1:25						Dilution 1:50					
20 % FBS used as blocking buffer												

Appendix – 13 continued

Table-12c:

Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	0,70	1,10	0.50	0,42	0,45	0,06	0,32	0,55	0,29	0,25	0,33	0,07
B	0,63	1,14	0,58	0.50	0,52	0,06	0,24	0,58	0,32	0,29	0,35	0,08
C	0,28	0,52	0,38	0,15	0,18	0,06	0,17	0,30	0,22	0,13	0,14	0,07
D	0,29	0,53	0,37	0,13	0,17	0,05	0,14	0.29	0,20	0,14	0,12	0,07
E	0,21	0.38	0,32	0.09	0.10	0,55	0,12	0,22	0,18	0,09	0,12	0,26
F	0,19	0,49	0,28	0,11	0.09	0,92	0,11	0,23	0,18	0,09	0,10	0,47
G	0,2	0,17	0,16	0,07	0,05	3,07	0,07	0,13	0,13	0,09	0,08	1,28
H	0,11	0,19	0,16	0,09	0,07	2,98	0,07	0,1	0,10	0,07	0,06	1,45
Antigen	50 ng per well											
Conjugate	Dilution 1:25						Dilution 1:50					
5 % nonfat dried milk used as blocking buffer												

Table-12d:

Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	0,27	0,69	0,37	0,27	0,27	0,06	0,54	1,37	0,81	0,49	0.65	0.08
B	0.26	0,72	0,38	0,25	0,26	0,06	0,53	1,34	0,81	0,53	0,63	0,08
C	0,18	0,46	0,26	0,13	0,13	0,06	0,34	0,77	0,50	0,20	0,26	0,08
D	0,18	0,46	0,26	0,13	0,14	0,06	0,33	0.82	0,51	0,21	0,29	0,08
E	0,13	0,35	0,21	0,10	0,1	0,30	0,25	0,63	0,33	0,17	0,17	0,59
F	0,14	0,33	0,21	0,09	0,09	0,20	0,25	0,64	0,40	0,15	0,17	0,36
G	0,09	0,22	0,16	0,07	0,07	0,15	0,15	0,41	0,31	0,11	0,12	0,26
H	0,09	0,23	0,17	0,07	0,07	0,11	0,13	0,37	0,29	0,11	0,12	0,16
Antigen	100 ng per well											
Conjugate	Undiluted conjugate-Parvo-kit						Diluted 1:2, conjugate-Parvo-kit					
5 % nonfat dried milk used as blocking buffer												

Appendix – 13 continued

Table-12e:

Plate 5	1	2	3	4	5	6	7	8	9	10	3,89	12
A	3,35	3,88	3,53	3,76	3,78	0,06	2,83	3,87	2,51	3,02	3,89	0.08
B	3,68	4,02	3,48	3,83	3,99	0,06	2,78	4,02	2,46	2,99	4,03	0,07
C	1,71	3,72	1,73	1,76	3,02	0,06	1,27	2,97	1,13	1,2	2,23	0,07
D	1,66	3,57	1,63	1,65	2,97	0,06	1,2	2,94	1,19	1,18	2,27	0,07
E	1,38	2,5	1,19	0,99	1,81	0,55	0,75	2,02	0,81	0,71	1,34	0,43
F	1,39	2,64	1,17	0,92	1,85	0,96	0,76	1,99	0,76	0,5	1,36	0,7
G	0.54	0,93	0,56	0,29	o,33	0,62	0,22	0,77	0,39	0,18	0,35	0,42
H	0,41	1,15	0,62	0,39	0,46	0,25	0,30	0,76	0,40	0,19	0,40	0,24
Antigen	50 ng per well											
Conjugate	Undiluted conjugate-EBV-kit						Diluted 1:2, conjugate-EBV-kit					
5 % nonfat dried milk used as blocking buffer												

Table-12f:

Plate 6	1	2	3	4	5	6	7	8	9	10	11	12
	0,22	0,06	0,72	0,08	0,49	0,42	0,12	0,06	0,39	0,08	0,27	0,27
A	P1,1:50	P3,1:1000	N1,1:50	N3,1:1000	P5,1:50	N5,1:50	P1,1:50	P3,1:1000	N1,1:50	N3,1:1000	P5,1:50	N5,1:50
	0,08	0,19	0,27	0,2	0,41	0,19	0,06	0,09	0,16	0,14	0,25	0,14
B	P1,1:100	P4,1:50	N1,1:100	N4,1:50	P5,1:100	N5,1:100	P1,1:100	P4,1:50	N1,1:100	N4,1:50	P5,1:100	N5,1:100
	0,06	0,1	0,17	0,11	0,35	0,13	0,06	0,06	0,11	0,09	0,23	0,10
C	P1,1:1000	P4,1:100	N1,1:1000	N4,1:100	P5,1:200	N5,1:200	P1,1:1000	P4,1:100	N1,1:1000	N4,1:100	P5,1:200	N5,1:200
	0,17	0,07	0,17	0,08	0,30	0,07	0,11	0,05	0,12	0,07	0,19	0,08
D	P2,1:50	P4,1:1000	N2,1:50	N4,1:1000	P5,1:400	N5,1:400	P2,1:50	P4,1:1000	N2,1:50	N4,1:1000	P5,1:400	N5,1:400
	0,09	0,30	0,09	0,40	0,24	0,05	0,07	0,07	0,08	0,25	0,16	0,07
E	P2,1:100	P5,1:50	N2,1:100	N5,1:50	P5,1:800	Blank	P2,1:100	P5,1:50	N2,1:100	N5,1:50	P5,1:800	Blank
	0,07	0,16	0,07	0,18	0,16	0,05	0,06	0,05	0,07	0,13	0,13	0,07
F	P2,1:1000	P5,1:100	N2,1:1000	N5,1:100	P5,1:1600	Blank	P2,1:1000	P5,1:100	N2,1:1000	N5,1:100	P5,1:1600	Blank
	0,10	0,11	0,20	0,12	0,13	0,05	0,07	0,05	0,14	0,10	0,11	0,07
G	P3,1:50	P5,1:1000	N3,1:50	N5,1:1000	P5,1:3200	Blank	P3,1:50	P5,1:1000	N3,1:50	N5,1:1000	P5,1:3200	Blank
	0,07	0,05	0,10	0,05	0,09	0,05	0,05	0,05	0,08	0,05	0,09	0,08
H	P3,1:100	Blank	N3,1:100	Blank	P5,1:6400	Blank	P3,1:100	Blank	N3,1:100	Blank	P5,1:6400	Blank
Antigen	100 ng per well											
Conjugate	Dilution 1:50						Dilution 1:100					
Super Block blocking buffer												
Each well has infomation of OD value and serum dilution. P = positiv serum, N = negative serum												

Appendix – 13 continued

Table-12g:

Plate 7	1	2	3	4	5	6	7	8	9	10	11	12
A	0,20	0,08	0,19	0,25	0,32	0,15	0,11	0,06	0,13	0,14	0,19	0.12
B	0,08	0,30	0,17	0,3	0,31	0,14	0,06	0,16	0,11	0,18	0,19	0,11
C	0,06	0,13	0,13	0,27	0,32	0,12	0,06	0,08	0,09	0,17	0,19	0.09
D	0,36	0,08	0,21	0,21	0,31	0,07	0,17	0,06	0,15	0,14	0,19	0,07
E	0,16	0,29	0,24	0,12	0,26	0,05	0,08	0,15	0,14	0,10	0,15	0,06
F	0,09	0,16	0,18	0,13	0,18	0,05	0,06	0,08	0,11	0,10	0,13	0,06
G	0,26	0,09	0,17	0,12	0,14	0,05	0,13	0,07	0,11	0,10	0,10	0,07
H	0,13	0,05	0,20	0,05	0,10	0,08	0,07	0,05	0,12	0,06	0,08	0,07
Antigen	50 ng per well											
Conjugate	Dilution 1:50						Dilution 1:100					
Super Block blocking buffer												
Sera and dilutions see plate 6												