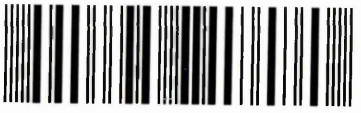

# The effect of anti-D immunoglobulin administration on plasma cytokine profiles

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**Abstract**

Haemolytic disease of the fetus and newborn (HDFN) is, without preventive action, a common cause of fetal morbidity and mortality. The condition is caused by maternal antibody to red cell antigens crossing the placenta and mediating the destruction of fetal red cells. This results in fetal anaemia, jaundice, and in severe cases, death. In western populations the commonest causative antibody is anti-D. For forty years the incidence of alloimmunisation to the RhD antigen, and hence HDFN, has been moderated by the administration of anti-D immunoglobulin to RhD- mothers. The treatment is extremely effective but the mechanism of action remains unresolved.

The aim of this quasi-experimental study is to test the hypothesis that there is a significant difference in maternal plasma cytokine expression before and after anti-D administration, thereby increasing the understanding of the mechanism of action. To recruit a participant cohort, RhD- women called to ant-D prophylaxis clinics at 28 weeks gestation were sent a leaflet describing the study 3-6 weeks before their appointment, and invited to participate. Blood samples were collected from 24 women receiving anti-D at 28 weeks gestation, before, and at 1, and 24 hours after administration. The concentration of plasma cytokines was measured by flow cytometry, and/or by enzyme linked immunosorbant assay (ELISA). Participants were allocated to test and control groups by the RhD group of their babies, determined at delivery. Changes in cytokine concentration greater than 50% (1.5 fold) were detected by ELISA for IL-1ra and TGF-β1. mRNA of white blood cells of these two cytokines was measured by quantitative polymerase chain reaction. When combined, the difference in TGF-β1 responses in RhD+ and RhD- fetus arms were found to be statistically significant ($p=0.047$). When ELISA and PCR results were considered separately, no significant difference was noted in fold changes in test and control groups ($p>0.05$).

TGF-β1 is a powerful immunosuppressant acting on the key cellular elements of the humoral immune response. The results of this study suggest that the mechanism by which anti-D prevents alloimmunisation includes the induction of

TGF-β1 secretion. Monoclonal anti-D selected for trials to replace human products have focussed on antibodies capable of causing clearance of fetal red cells from the maternal circulation, and have to date had poor results. The knowledge that anti-D prophylaxis has additional biological effects which may contribute to immunosuppression should inform the selection of monoclonal antibodies for future trials.

**Contents**

## Appendices

**List of Tables**

## List of Figures

## Abbreviations

| | |
|---|---|
| AE | Acid Elution |
| AHG | Anti-Human Globulin |
| AMIS | Antibody Mediated Immunosuppression |
| APC | Antigen Presenting Cell |
| BCR | B Cell Receptor |
| BCSH | British Committee for Standards in Haematology |
| CD | Cluster of Differentiation |
| cDNA | Complimentary Deoxyribonucleic Acid |
| CT | Cycle Threshold |
| CV | Coefficient of Variation |
| DAT | Direct Antiglobulin Test |
| DC | Dendritic Cell |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| $F(ab)_2$ | Fragment Antigen Binding |
| Fc | Fragment Crystalline |
| FC | Flow Cytometry |
| FcR | Fragment Crystalline Receptor |
| GAPDH | Glyceraldehyde 3 Phosphate Dehydrogenase |
| GC | Germinal Centre |
| GM-CSF | Granulocyte-Macrophage Colony Stimulating Factor |
| HLA | Human Leucocyte Antigen |
| HDFN | Haemolytic Disease of the Fetus and Newborn |
| IAT | Indirect Antiglobulin Test |
| ICAM | Inter-Cellular Adhesion Molecule |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ITAM | Immunoreceptor Tyrosine Activatory Motif |
| ITIM | Immunoreceptor Tyrosine Inhibitory Motif |
| ITP | Immune Thrombocytppaenic Purpura |
| IUT | Intra Uterine Transfusion |
| IV | Intravenous |

| | |
|---|---|
| IVIg | Intravenous Immunoglobulin |
| KBT | Kleihauer Betke Technique |
| LFA | Lymphocyte Function Associated Molecule |
| MCP | Membrane Co-factor Protein |
| MHC | Major Histocompatibility Complex |
| mRNA | Messenger Ribonuceic Acid |
| NHS | National Health Service |
| NICE | National Institute for Health and Clinical Excellence |
| NK | Natural Killer |
| PCR | Polymerase Chain Reaction |
| PGE | Prostaglandin |
| PE | Phyco erythrin |
| qPCR | Quantitative Polymerase Chain reaction |
| RCT | Randomised Controlled Trial |
| REC | Research Ethics Committee |
| RT | Reverse Transcriptase |
| SLE | Systemic Lupus Erythematosus |
| SLO | Secondary Lymphoid Organ |
| TCR | T Cell Receptor |
| TGF | Transforming Growth Factor |
| Th | T Helper |
| TMB | Tetramethylbenzidine |
| TNF | Tumour Necrosis Factor |
| vCJD | Variant Creutzfeld Jacob Disease |

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# Chapter 1 Review of the Literature

## Literature Review Strategy

This review was undertaken using LitSearch software supporting simultaneous interrogation of: PubMed, Medline, Scopus, Biological Sciences, and Intute databases. Key-words used in searches were: anti-D, prophylaxis, immunomodulation, immunosuppression, immunology, Haemolytic Disease of the Fetus and Newborn, pregnancy, cytokine, and Transforming Growth Factor- Beta. This search informed the development and design of this research, and identified that no other published study has investigated the effect of intravenously administered anti-D on cytokine expression measured by ELISA and PCR.

## 1.1 Haemolytic Disease of the Newborn, Pathogenesis, Treatment and Prevention

### 1.1.1 Introduction

Everyday we encounter compelling and diverse evidence that the strategy of mammalian reproduction is effective. An oocyte of maternal origin is fertilised by paternal sperm, and develops in a specialised environment within the maternal uterus, until the developed fetus is sufficiently mature to survive outside the womb (Billington 1992). The manifold physiological and developmental advantages of the strategy are evident from the ecological dominance achieved by mammals since their evolution. Internal rather than external fertilisation has proved a successful strategy shielding the development process from external threats such as predators, toxins, and adverse environmental conditions. The result is that a high proportion of live births is possible and has resulted in greater reproductive success and gene transmission (Mor 2006).

Those benefits can be realised only by virtue of elegant biological mechanisms supporting the development of the fetus, which carries paternal antigens and is, effectively, an allograft, while permitting support of the fetus

by the maternal blood circulation via the placenta. Maternal immune surveillance protects the mother and her baby from bacterial, viral, fungal, or parasitic infection and neoplasms. The relationship between the maternal immune system and the fetus is complex. Under some circumstances fetal red cells become the target for immune destruction and haemolytic disease of the fetus and newborn results. This chapter reviews the biology of the maternal-fetal relationship the immunology of HDFN, and the laboratory and clinical control, treatment and prevention.

### 1.1.2 Normal Fetal Haematology

Erythropoiesis has been demonstrated in the fetal yolk sac at 3 weeks gestation, and the Rh (previously known as Rhesus) peptide detected on fetal red cells from the 3rd week of gestation (Bowman 1994). By 12 weeks, erythropoeisis is proceeding in the liver, and to a lesser degree, the spleen. From 20 weeks, haemopoiesis is transferred to the bone marrow of the long bones and by birth this is the main site (Testa 2001). Where bone marrow production of red cells is insufficient to prevent anaemia due to blood loss or haemolysis, at any stage of life, erythropoiesis may occur in other tissues especially the liver and spleen (Lewis 2001). In the developing fetus and neonate, unlike adults, bone marrow fills every bone cavity available and any requirement for excess erythropoeisis transferred to extramedullary tissues (Pallister 1998). This results in the hepatosplenomegaly characteristic of fetuses and neonates with inadequate bone marrow function.
The control of red cell maturation is poor in extramedullary tissue, in the liver and spleen, resulting in the release of nucleated red cells into the circulation Bowman 1994).

### 1.1.3 The Function of the Placenta

The function of fetal nourishment has been ascribed to the placenta for more than 2,000 years (Huppertz 2007). Until 8-10 weeks gestation, the regulatory and nutritional functions of the developing fetus are provided by the yolk sac. The increasing complexity and scale of the needs of the developing fetus

requires direct maternal support (McNabb 2004) and, from that point the placenta provides all the substrates for fetal growth and allows fetal respiration by gaseous exchange (Smith 2007).

The human placenta facilitates interactions between the maternal and fetal circulation through interdigitating placental villous tree-like structures which extend into the maternal bloodstream (Huppertz 2007).Transport across the placenta is by simple diffusion for oxygen and carbon dioxide. Other species are transported via specific transporter molecules on the villous membrane and large molecules, such as immunoglobulin class G (IgG), by receptor mediated endocytosis (Dunihoo 1990).

### 1.1.4 The Immunology of Pregnancy

The immunological balancing act of pregnancy requires the maternal immune system to tolerate the fetal allograft, while maintaining immune surveillance to defend against infection. This is unique in nature though Medowar, in the early 1950s, recognised the analogy between the fetal allograft and the new science of transplantation (Mor 2006). The focus of maternal tolerance is on the placenta not the fetus; the cells of the placenta are adapted for routine interaction with the maternal immune system without eliciting responses incompatible with viable pregnancy. The array of evasive mechanisms employed by the trophoblast and placental cells is complex and highly developed (Mor 2006).

The placenta fulfils the role of a physical barrier between the mother and fetus but also acts as a mechanism by which the immune privilege status of the fetus is maintained. The barrier is not impregnable to cell migration between fetus and maternal circulations, with cells of maternal origin being detectable in the offspring to adulthood, and fetal cells, derived chiefly from budding of trophoblasts, being detectable in the maternal circulation (Trowsdale 2006).

Fetal evasion of the maternal immune system occurs through a number of routes. The potential for damage to the fetal-placental unit by the maternal

immune system is further reduced by the induction of regulatory T cells (Treg), which are recruited to the materno-fetal interface and down regulate cytotoxic T cells, and NK cell responses. Induction of Treg occurs within 48 hours of fertilisation and is probably stimulated by hormones (Hanson 2009).

There are several mechanisms for the protection of the fetus from the maternal immune system including:

- Trophoblasts do not express the highly polymorphic HLA class I A and B locus, or class II antigens (Hanson 2009). These are replaced by the much less variable HLA-E, F, and G molecules, indeed HLA-G is monotypic (Trowsdale 2008). HLA-C is retained and is a ligand for uterine maternal Natural Killer (NK) cells, the binding of which facilitates placental vascularisation (Trowsdale 2008).
- Expression on trophoblasts of complement downregulatory proteins CD46, CD55 and CD59 (Weetman 1999).
- Shift in cytokine patterns from Th1 to Th2/Th3. Mothers in whom Th1 cytokines predominate are subject to recurrent abortion of pregnancies, and normal, successful pregnancies are considered to require a shift to predominantly Th2 profile (Ragupathy 2000, Levy 2007).

Th1 cytokines, IL-2, IFN-$\gamma$, and TNF-$\beta$ have been shown to be associated with cytotoxic T cell damage to placental cells. Th2 cytokines, IL-4, IL-5, IL-6, IL-10 and IL-13 are strongly associated with successful pregnancy (Ragupathy 2001). The classification of immune responses by Th1/Th2 is simplistic, and it is acknowledged that T cells with other profiles contribute to the support of the fetal allograft. In particular Th3 cells secreting TGF-$\beta$1 an anti-inflammmatory cytokine with a role in mucosal immunity and downregulation of Th1 responses may be important (Ragupathy 2001, Costeas 2004). HDFN is an antibody mediated pathology which may be exacerbated by the strategy of deviating the maternal immune system to Th2/Th3 . Accidental exposure of fetal tissue to the maternal immune system results in such powerful pathologies as HDFN, is indicative of the efficacy of immune evasion (Mor 2006).

### 1.1.5 The Placental Transfer of IgG

The fetal immune system is immature, leaving the neonate vulnerable to extracellular infective agents. Specific immunity transferred from mother to fetus was first reported by Paul Ehrlich in 1892 (cited in Kristofferson 2002). From approximately 12 weeks gestation, maternal IgG is actively transferred across the placenta, conferring passive immunity on the fetus. In humans, maternal IgG is transferred to the fetal circulation by a placenta bound receptor, FcRn which is unlike other IgG receptors both in structure and function, being part of the Major Histocompatibility Complex (MHC) class I protein family and being dependent on dimerisation with β2 microglobulin (Klein 2005). It is widely distributed on endothelial cells and functions include: IgG conservation and salvage, transport in the gut lumen, and into colostrum (Antohe 2001). IgG transport across the placenta is initiated by non-specific pinocytosis, with other plasma proteins including IgM and IgA by syncytiotrophoblasts at the fetomaternal interface (Figure 1.1). IgG is trafficked to vesicles lined with FcRn, the low pH in that environment promotes binding of the IgG to FcRn via specific amino acids at the junction between IgG heavy chains. These are isoleucine 253, histidine 310, and histidine 435 (Firan 2001). Each IgG molecule has two heavy chains and causes dimerisation of the FcRn. Protein not ligated to FcRn bound IgG is directed to lysosomes for enzymatic degradation: IgG is trancytosed (Simister 2003). The vesicles fuse with the basement membrane exposing the FcRn/IgG complex to higher pH and resulting in disassociation and release. (Hadley 2002, Kristofferson 2000). The mechanism by which IgG is transported into the fetal circulation is not known, though the presence of FcRII on fetal vascular endothelium may be important (Simister 2003).

By 24 weeks gestation, the concentration of serum IgG may be up to 1.8g/L in the fetus compared to 12.6g/L in the maternal serum. At this gestation the cytotrophoblast layer, which lacks FcRn and represents a barrier to IgG, transport, becomes discontinuous allowing IgG transfer to the fetus (Simister 2003).

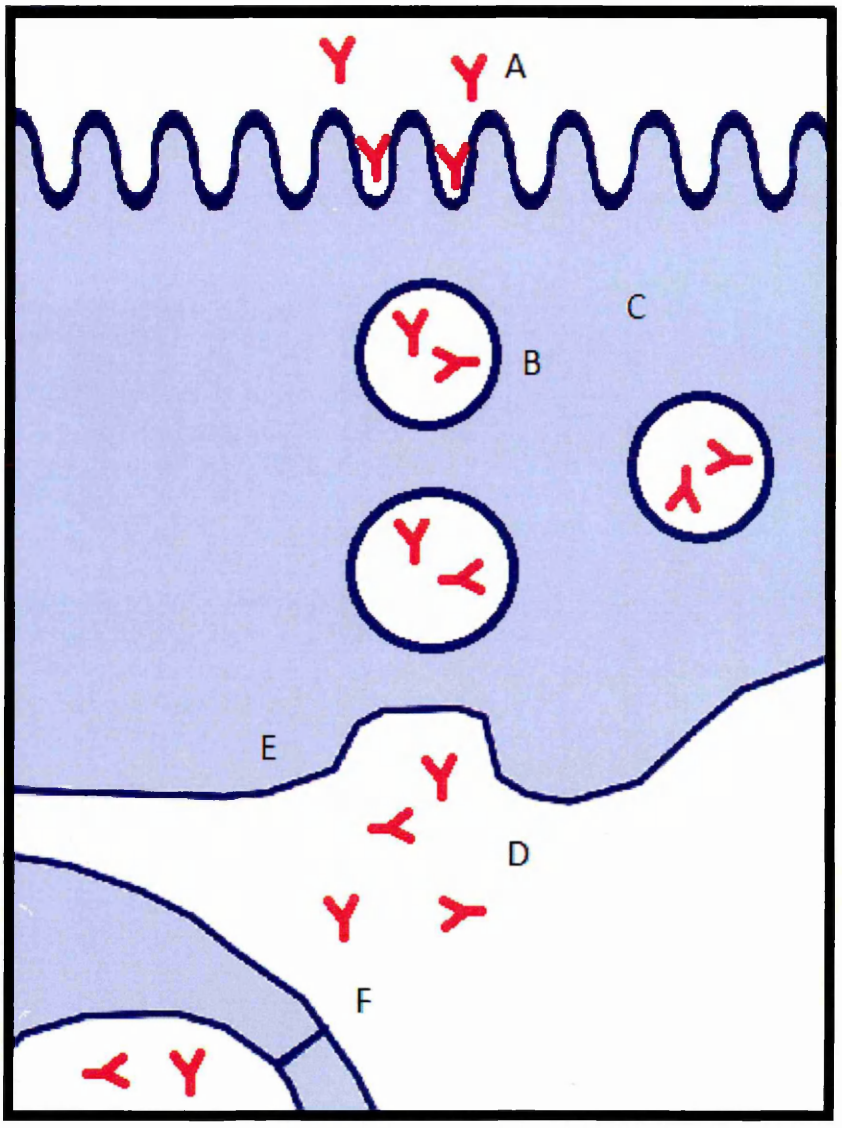



Figure 1.1 Steps in the transport of IgG across the placenta.
Maternal IgG enters the syncytiotrophoblast by endocytosis (A). Vesicles fuse and material is transported in a low pH, IgG binds to FcRn and is rescued from enzymatic degradation (B). Unbound material is degraded (C). At the basal membrane the IgG/FcRn complex dissociates (D). FcRn is recycled (E), and maternal IgG enters the fetal circulation (F) (from Kristoffersen 2000)

From that point, IgG transfer rates increase until, at delivery, overall fetal IgG concentrations, exceed those in the maternal serum (Simister 2003). IgG1 in particular is preferentially transported to the fetal circulation, and IgG2 is transported least, or possibly later. Fetal and maternal IgG3 concentrations are similar at the end of the third trimester, and IgG4 levels very variable (Simister 2003). It may be that the rate of transfer of IgG across the placenta is the rate limiting step in the progression of HDFN (Hughes-Jones 1971). Indeed, HDFN before 24 weeks gestation is rare (Hadley 2002). The transport of pathological maternal autoimmune IgG, specific for fetal tissue antigens, can transfer the maternal condition to the fetus, resulting in heart block and rarely renal dysfunction in the fetuses of women suffering from Systemic Lupus Erythematosus (SLE) (Dahmane 2005) or neonatal Myasthenia Gravis (Telez-Zenteno 2005). Variation in placental transfer of antibody may account for unaffected RhD+ fetuses in pregnancies with high levels of maternal anti-D. This may be associated with placental morphology changes due to fetal anoxia (Lubenko 1994) or antibodies to the paternally derived HLA class II antigens on fetal leucocytes blocking binding sites on FcRn (Dooren 1993). It is when maternal alloantibody is specific for fetal red cells that HDFN occurs.

## 1.2 Aetiology of Haemolytic Disease of the Fetus and Newborn (HDFN)

### 1.2.1 Introduction

HDFN is an unusual disease being discovered, characterised and almost eradicated within a single scientist's career span. An examination of that process illustrates how advances in the understanding of biology in the mid and late 20$^{th}$ century facilitated and informed observation and hypothesis. Unexplained symptoms were translated into understanding of disease pathology, then into treatment and eventually into prevention. The story is punctuated by startling examples of insight and oversight, solutions derived both from the most exquisite technologies and serendipitous application of the mundane. So immediately successful was the prophylaxis, which prevents most HDFN, little attention was paid to the mechanism of its action, a state which perhaps surprisingly persists to the present day.

The first recorded report of HDFN was made in 1609 by Louyse Bourgeois, a French midwife. She described the birth of twins, the first of whom was bloated with hydrops, the other developed severe jaundice and died at a few days of age (Bowman 1994). In view of data from epidemiological studies from the second half of the 20th Century (Clarke 1983, Hussey 1992), many midwives must have encountered these and other symptoms now associated with HDFN. In fact there were many reports of cases of hydropic and jaundiced fetuses in the burgeoning scientific literature of the late 19th and early 20th centuries. Diamond (1932) reviewed a large number of such case reports, which reflected the diverse nature of disease presentation but were characterised by anaemia (congenital anaemia of the newborn), jaundice (icterus gravis neonatorium), and the presence of an abnormally large proportion of erythroid blast cells (erythroblastosis fetalis) in the fetal circulation. The authors recognised the relationship between the conditions by the common clinical features: enlarged fetal liver and spleen (hepatosplenomegaly) resulting from red cell production outside the bone marrow (extramedullary erythropoeisis), fetal oedema and anaemia with jaundice. They also recognised the familial nature of the condition in many cases. Though recognising the common symptoms and suspecting a common cause, the authors favoured the hypothesis that dysfunction of fetal erythropoeisis was the most likely cause of disease.

Interestingly the authors wrote:

> *"the presence of toxins and haemolytic substances derived from the mother have frequently been offered as an explanation for anaemia and icterus"*

Regrettably no reference is offered and they indicate that this might be a separate condition entirely.

The first insight into the pathology of HDFN was provided in 1938 by Ruth Darrow, a pathologist who had herself lost babies to kernicterus. She undertook a comprehensive review of the clinical and pathological presentation of cases classified broadly as icterus gravis neonatorum (Darrow

1938). In this paper Darrow critically considered all the available literature, summarised in a series of observation-based statements, and sequentially eliminated hypotheses that were contradicted by evidence. At the end of the process she was left with a single theory: that, following a leak of fetal red cells into the maternal bloodstream, the immune system had recognised the haemoglobin of the fetal red cells as foreign and had formed an anti-fetal haemoglobin antibody. That fetal haemoglobin differed from adult, and could be antigenic, were both recent discoveries. This remarkable piece of reflection was very close to the truth, only the specificity of the antibody being incorrect. In fact, fetal haemoglobin is formed in small quantities by almost all adults and as a result the alloimmune response described by Darrow is unlikely (Bowman 1994). The causative antibody was discovered one year later following a severe and unexpected transfusion reaction in a group O woman transfused with her husband's group O blood shortly after delivering a stillborn fetus (Levine 1939). At that time the only recognised blood group incompatibility was in the ABO system, and this reaction could not be explained. The patient bled both before and after a subsequent hysterectomy, and was supported with blood transfusions from a further eight professional blood donors selected by the Blood Transfusion Betterment Association, resulting in uneventful and therapeutic transfusions. The crossmatch techniques employed, though unspecified, were described as a "delicate technic". The incompatibility between the patient and her husband's blood was detected, and allowed selection of eight from fifty (16%) compatible professional donors for subsequent transfusion.

This case was the first description of alloimmunisation resulting from pregnancy, though there were previous reports of occurrence subsequent to blood transfusion. The authors hypothesised that the woman had formed an antibody to a red cell antigen expressed on fetal cells, which the child had inherited from its father. In common with alloimmunisation events resulting from blood transfusion, the antibody usually appeared to be transient being undetectable one year after delivery. It is likely that the authors were in fact detecting IgM red cell antibodies in the woman's plasma, incapable of placental passage and hence not the cause of the HDFN, rather a marker for

the co-existing IgG antibody (Issitt 1998). Though transfusion had been used therapeutically for infants born with HDFN, this was the first report of the growing discipline of transfusion science, adding to the understanding of the condition, a link which would strengthen over the subsequent decades in diagnosis, treatment and prevention.

The specificity of the antibody implicated in HDFN was revealed in an experiment in which blood from Rhesus monkeys was used to raise antibodies in guinea pigs. (Landsteiner 1940). When tested against red cells from humans the authors found that red cells from 85% of individuals were agglutinated by the serum (i.e. were Rhesus positive) and 15% remained unagglutinated (i.e., were Rhesus negative). The serum of Levine and Stetson's patient was shown to have a similar pattern of reactivity with panels of human red cells, the woman being confirmed as Rhesus negative and her husband Rhesus positive.

The transient nature of the detectable antibody, in contrast to the recurrence of the disease in subsequent pregnancies, continued to perplex investigators. It was clear that a variant of the detectable (IgM) anti-Rhesus antibodies existed in some bloods detectable only by its ability to prevent agglutination of Rhesus positive red cells by known examples of Rhesus agglutinins. The mechanism for this seemed to be antigen site blocking by an agent specific for the Rhesus antigen but incapable of causing agglutination directly. Within a short period a range of techniques evolved to allow detection of this (IgG) blocking antibody. (Mourant 1983, Tovey 1992), which binds specifically to red cell antigens, without causing direct agglutination. The most significant of the new techniques was the Coombs' test, using anti-human globulin raised in animals to cross-link red cell bound IgG and providing a visible agglutination endpoint (Coombs 1994).

### 1.2.2 The RhD Antigen

The human RhD antigen is expressed on a polypeptide comprised of 417 amino acids expressed only on erythroid tissue. The peptide, has a molecular

mass of 45,000, is strongly hydrophobic and traverses the red cell membrane 12 times (Daniels 2002) (Figure 1.2). The *RH* gene locus on chromosome 1p34-p36 is comprised of the homologous *RHCE* and RHD genes coding for RhD and CcEe peptides and their associated antigens (Van Kim 2006). Genomic rearrangement between the two genes is not uncommon resulting in the creation of *RHD-CE-D* and *RHCE-D-CE* hybrids where the hybrid component may range from a single amino acid to substantial peptide segments. These rearrangements, together with other single amino acid deletions and substitutions, make RhD one of the most polymorphic antigens encountered in red cell immunohaematology (Daniels 2002).

Rh is the most complex of the blood group systems described to date with 48 known variant alleles (Wagner 2004). More than 30 D epitopes are recognised (Scott 2002) with more being encountered regularly in pre-transfusion testing (Daniels 2002). There is no *RHd* gene. D negative phenotypes are the result either of an *RHD* deletion or any inactive *RHD* gene. In Caucasians the D- phenotype occurs due to a homozygous deletion of the *RH* gene, which is also widely expressed in non-Caucasian populations. Other inactive genes including the RHDΨ pseudogene expressed in Africans present challenges to accurate genotypic characterisation of D zygosity (Wagner 2004).The RhD antigen is highly immunogenic. Some 80 percent of D- people transfused with an single unit of D+ red cells form anti-D (Issitt 1998), though a more recent study suggests a much lower immunisation rate in the region of 30% (Frohn 2003). Nevertheless this is higher than the immunisation rate for all the other recognised red cell antigens in aggregate, which is unlikely to exceed 5% (Issitt 1998). Most blood group polymorphisms represent single amino acid deletions or substitutions. The immune system of an individual challenged with red cells of a different blood group is presented with relatively small changes in antigen. RhD differs from its nearest homologue, RhCE by 36 amino, acids which are clustered in exofacial portions of the D peptide, representing a much more easily recognised non-self antigen (Figure 1.2, Wagner 2004).

The frequencies of D+ (85%) and D- (15%) in the US populations in the initial report (Landsteiner 1940) and corroborated widely in Caucasian populations since but are not reflected in other populations (e.g. Daniels 2002). The frequency of RhD- is reduced in Asian populations, and is extremely rare in the Far East. In Chinese populations, other red cell antibodies (anti-c, anti-$Di^a$) are more frequent causes of HDFN as the D- phenotype is very rare (Issitt 1998).

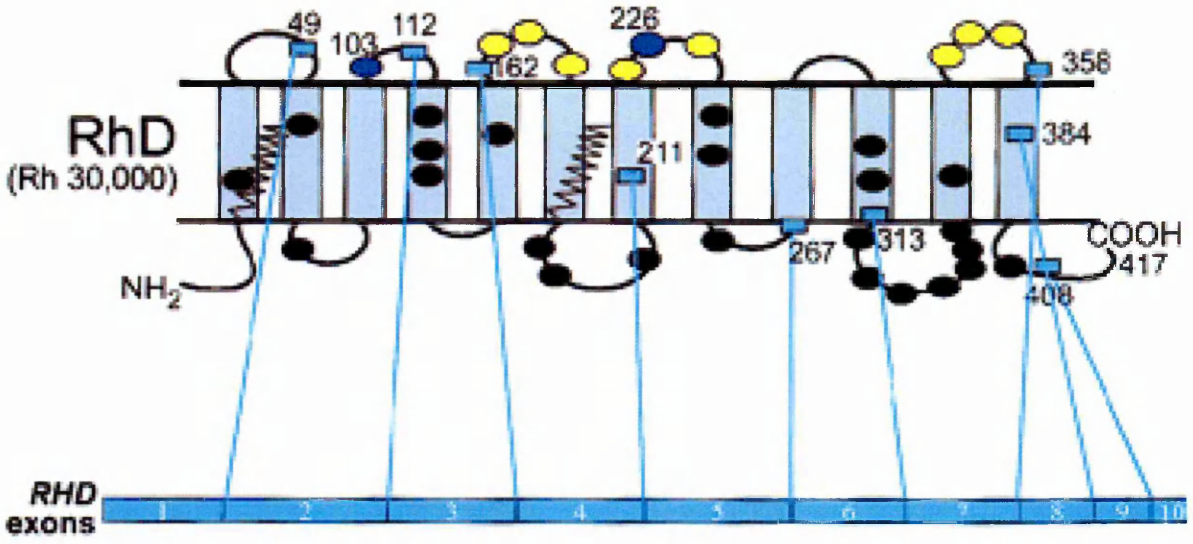



Figure 1.2 The Rh polypeptide showing transmembrane domains.
Black spots identify sites of amino acid substitutions and deletions leading to variant phenotypical expression. Clusters of polymorphisms (yellow spots) on exofacial portion of peptide are particularly immunogenic. and are the sites of polymorphism accounting for partial RhD types, and the difference between the RhD and CE polypeptides (From Avent 2000)

### 1.2.3 The Biology of Alloimmunisation to the RhD Antigen

Maternal anti-D production is almost always stimulated by exposure to fetal D+ red cells. Transfusion stimulated anti-D is now extremely rare in women of childbearing potential as transfusion protocols proscribe the infusion of D+ red cells for such patients except *in extremis* (BCSH guidelines 2004). Instances of stimulation by other means such as shared needles for administration of recreational drugs have been reported (Dimer 1999). For other HDFN causative antibodies, in particular anti-c and anti-K, transfusion is a major cause of alloimmunisation (Koelwijn 2009). In Caucasian populations approximately 10% of pregnancies are D- women carrying one or more D positive fetuses (Kumpel 2002).

Stimulation of anti-D antibody production is much more likely when the fetal red cells are ABO compatible with the mother. Maternal iso-agglutinins are almost universally present in group O , A or B individuals, and cause the rapid destruction of non-compatible fetal red cells either by complement mediated intravascular lysis or extravascular destruction in the liver, where antibody stimulation is much less likely than in IgG mediated splenic destruction (Urbaniak 2000). It has been estimated that group A:O incompatibility confers 90% protection and B:O 55% protection against RhD immunisation (Murray 1965).

The ability of D- individuals to form anti-D in response to D+ red cells is variable (Mollison 1984). Humoral responses to platelet antigens have been demonstrated to be linked to HLA-DR phenotypes, but the evidence that a link exists between HLA types and alloimmunisation to RhD is less clear. Peptides derived from lysis of the RhD protein have been demonstrated to activate T lymphocytes derived from alloimmunised blood donors (Urbaniak 1999). Some groups have attempted to demonstrate a link between HLA type and RhD alloimmunisation (Issitt 1998, Urbaniak 1999, Kumpel 2002) with remarkably varied findings. HLA DRB*1 1501 has been found to be significantly overexpressed in RhD- blood donors who are deliberately alloimmunised with D+ cells (Hall 2005) and 100% of patients alloimmunised

to the $Fy^a$ antigen were found to express HLA DRB1* 04 compared to 19% in the general population (Noizat-Pirenne 2006). The difficulty in obtaining a large cohort of individuals with anti-D, and the extreme polymorphism of HLA make reliable demonstration of a link problematic. In fact the high immunisation rate amongst D- people exposed to D antigen indicated that many HLA types must support the immunisation to D.

Antibody stimulation is more likely in donor alloimmunisation programmes when multiple small doses of antigen are administered rather than single than single large doses (Urbaniak 2000). Seven of eleven volunteers given fortnightly doses of the equivalent of either 0.01mL or 0.1mL of RhD+ whole blood, went on to develop anti-D (Jakobowicz 1972). The frequency of stimulation of anti-D is dose dependent with 15% responding after a single intravenous (IV) injection of 1mL of D+ cells and 70-90% after 250mL (Mollison 1984). Secondary stimulation requires a lower dose; 14/14 volunteers with weak Rh antibodies having the titre boosted by administration of 0.28mL of D+ cells (de Silva 1985). In a programme for immunising volunteers for the production of anti-D immunoglobulin, 26 out of 28 formed anti-D after an initial dose of 200mL of red cells followed by six monthly booster doses of 0.5-1.0mL (Urbaniak 1981). Experimental studies, such as these, present antigen via intravenous injection to non-pregnant, typically male volunteers that may not accurately reflect immunisation rates when Fetomaternal Haemorrhage (FMH) occurs *in utero* and care must be taken in generalising these findings outside the experimental cohort.

That haemorrhage could be the cause of fetal anaemia was known (Weiner 1948), though the bleeding was considered to be from the fetal side of the placenta into the amniotic cavity. It was not until a case of fetal anaemia was rigorously examined that the presence of red cells of fetal origin was demonstrated in the maternal bloodstream (Chown 1954). The imaginative application of the techniques of the burgeoning science of transfusion in this investigation allowed, in addition, the estimation of the size of the feto-maternal haemorrhage in this case as 90-180mL by simple assessment of the proportion of agglutinated cells as viewed by light microscopy and

multiplication by the maternal blood volume. The authors observed that the fetal D+ cells were cleared from the maternal bloodstream shortly after the woman was alloimmunised and formed anti-D antibodies.

The frequency and size of FMH only became apparent with the discovery that fetal haemoglobin was resistant to acid lysis. The proportion of fetal cells in a maternal blood sample and hence the volume of the FMH can be estimated by microscopic examination of maternal blood films from which adult haemoglobin has been eluted by acid, and in which fetal haemoglobin is stained with haematoxylin and eosin (Kleihauer 1957).

## 1.3 The Clinical Effects of HDFN

### 1.3.1 Introduction

Maternally derived IgG may be detected on fetal red cells from as early as the 8$^{th}$ week of gestation and death *in utero* may occur from 18 weeks (Urbaniak 2000, Klein 2005). These are the earliest manifestations of the disease. The spectrum across which HDFN presents is wide indeed. Some cases are only apparent in laboratory tests with no clinical symptoms, other mildly affected infants show anaemic pallor, but deep jaundice, oedema and neurological involvement may occur in the severely affected fetus. Kernicterus occurs in as few as 1/50,000 otherwise well full term babies, and 1-3% of pre-term infants (Ahlfors 2001).

Neonatal jaundice occurs in up to 60% of full term pregnancies as a normal physiological event (Stevenson 2001). Jaundice usually peaks around 3 days after delivery, and becomes undetectable by 5 days. Apparent jaundice, less than 24 hours after delivery of a full term infant requires investigation, as most physiological levels of red cell breakdown in the fetus should be cleared through the maternal liver up to delivery, with no significant increase in fetal bilirubin (Meeks 2004).

Hyperbilirubinaemia occurs in 60-70% of all term infants and virtually all pre-term infants (Stevenson 2001). Fetal red cell breakdown occurs before delivery on a considerable scale associated with the switch from fetal to adult haemoglobin, in preparation for the change from intrauterine to extrauterine life (Meeks 2004). The neonate is faced with a considerable load of bilirubin, as the lifespan of red cells falls from 90 to 70 days, and the delivery process interrupts the flow of unconjugated bilirubin to the maternal circulation (Stevenson 2001).

Bilirubin is formed as a normal breakdown product of haem. It is soluble in lipid but not water. Under normal circumstances, unconjugated bilirubin is conjugated in the liver to albumin forming a complex which is soluble in water and can be excreted. Other causes of neonatal jaundice include re-adsorption of bilirubin through the gut and genetic disorders such as glucose-6 phosphate dehydrogenase deficiency (Meeks 2004).

In some cases, the chief clinical signs of anaemia and hyperbilirubinaemia do not occur due to the ameliorating effects of compensatory mechanisms; marrow hyperactivity replacing lost red cells and the fetal hepatic excretory system exporting red cell breakdown products to the maternal liver. Where haemolysis is severe, the serum bilirubin of the fetus may exceed 350μmol/L. This cannot be rendered non-toxic by albumin conjugation. Where the capacity to conjugate bilirubin to albumin is compromised, or exceeded either by excess red cell destruction (e.g. HDFN) or insufficiency of the neonatal liver then hyperbilirubiunaemia results (Meeks 2004).

Unconjugated bilirubin has an affinity for lipid and may bind to tissues in the brain or other parts of the central nervous system and is particularly toxic to brain tissue, the resulting condition being known as kernicterus (Urbaniak 2000). The risk to the fetus associated with kernicterus is a complex product of factors. Rates of bilirubin production and clearance, serum concentration, and duration of exposure, all exacerbate the condition. Rates of excretion, gestational age and plasma albumin concentration, all reduce the risk to the neonate (Ahlfors 2001). Nevertheless, 70% of infants who develop kernicterus

die between the second and fifth days of life. Of those who survive, a high proportion have suffered permanent cerebral damage resulting in spasticity or, in mild cases, high frequency deafness (Klein 2005).

### 1.3.2 The Epidemiology of HDFN

HDFN affects fetuses and neonates, with the associated loss or impairment of a whole lifetime, and presents in the most distressing ways. The frequency with which the untreated condition affects lives is considerable. In Canada in the early 1940s the perinatal mortality rate was 40 per 1000 births. HDFN accounted for 10% of these deaths (Bowman 2003). In Queen Charlotte's Hospital, London, between 1946 and 1949 fetal deaths due to HDFN occurred at the rate of 3.2 per 1,000 births. Some 0.5% of all women developed Rh antibodies, and 20% of these lost their babies in subsequent pregnancies (Tovey 1990).

Prior to 1945, 50% of babies suffering from HDFN died of hydrops or kernicterus. Improved treatment of the condition reduced mortality in affected cases to 2-3% by the early 1980s (Bowman 2003). Between 1940 and 1970, the proportion of perinatal deaths in cases of HDFN in the UK fell from 50% to 5.9% (Urbaniak 2000). The introduction to the UK in 1969 of routine post-partum anti-D immunoglobulin prophylaxis for RhD- mothers of RhD+ babies reduced Rh alloimmunisation by 70%. The reduced incidence of HDFN in the same period was also associated with a reduction in the birth rate in the 1970s, and that many alloimmunised women, advised by obstetricians, were choosing not to have further pregnancies (Tovey 1978). By the 1990s, the death rate due to HDFN had fallen to around 50 per year (Consensus statement of the Royal College of Obstetricians and Gynaecologists 1997).

It was estimated that by 2002 (NICE guidance 2002) there were approximately 105,000 births to RhD- women annually in the UK. Of these 59% (or 62,000) were RhD+ fetuses, representing 10% of all UK births. In England and Wales at that time, there were an estimated 25-30 fetal or neonatal deaths and 20 spontaneous abortions each year due to HDFN.

Fifteen children were born with major, and a further 30 with minor developmental problems (NICE 2002).Fetal morbidity and mortality due to antibodies other than anti-D (chiefly anti-c and -K) is less common, but there remains no available prophylaxis to prevent alloimmunisation to these antigens. Continued progress in the prevention of anti-D HDFN make these non-anti-D cases an important and increasing proportion of the residual cases (Koelewijn 2009).

## 1.4 The Pathobiology of HDFN

### 1.4.1 Introduction

HDFN is mediated by maternal IgG specific for antigens expressed on fetal red cells. For that antibody to be formed and synthesised in sufficient quantity and quality to cause immune destruction resulting in clinical HDFN, a series of events triggering the maternal humoral alloimmune response must occur. In the first instance, the maternal immune system must be exposed to fetal red cells as a result of Fetomaternal Haemorrhage (FMH). Fetal red cell antigens must be taken up by antigen presenting cells (APC), including B lymphocytes. Interaction and co-stimulation between APC, and T and B lymphocytes leads to activation and differentiation of B-lymphocytes resulting in a powerful alloimmune response leading to the synthesis and release of immunoglobulin, and the development of memory cells capable of staging an even more powerful anamnestic response in the event of repeat challenge with the same antigen ( Hadley 2002, Bowman 2003; Figure 1.3).

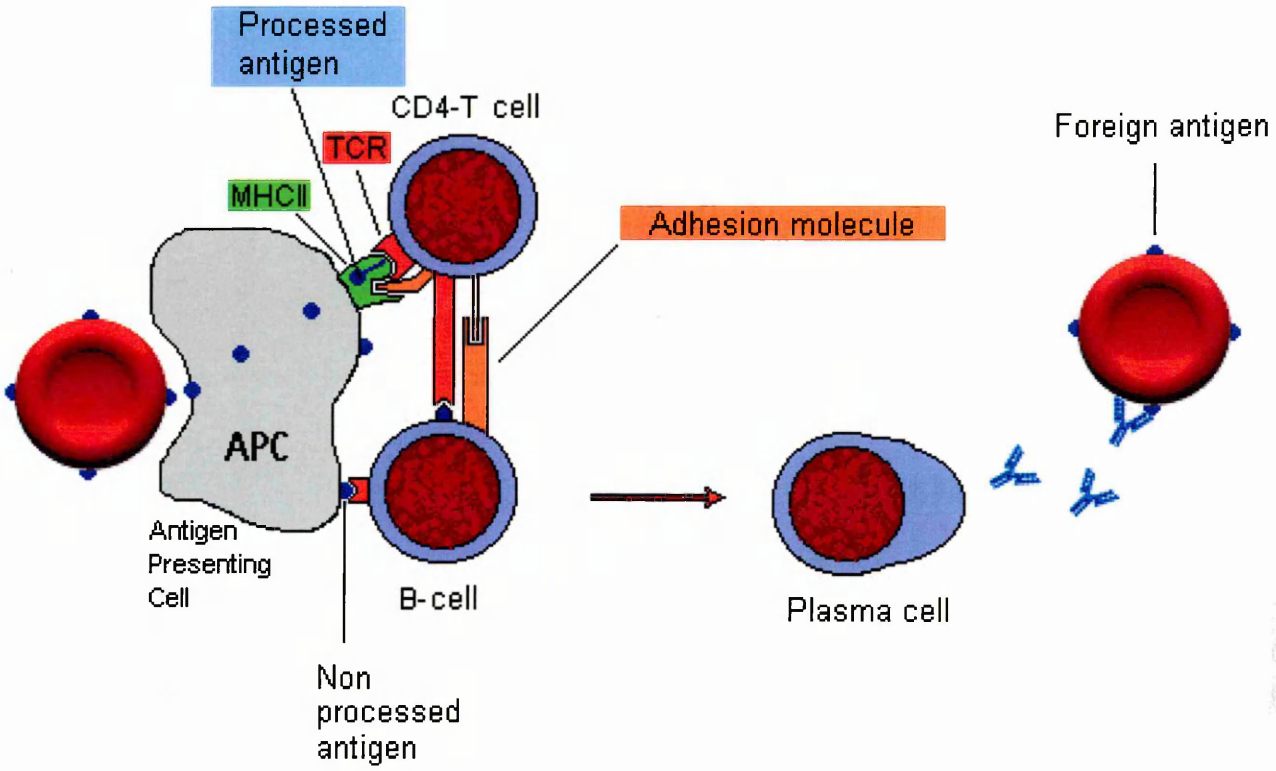



Figure 1.3 The interaction of cells of the immune system leading to red cell alloimmunisation (From Hadley 2000).

Red cells are phagocytosed by antigen presenting cells (APC), and peptides from processed antigen are expressed at the cell surface by MHC II. Interaction between expressed peptide MHC II complex and TCR leads to T cell activation in the presence of costimulatory molecules. B cells bind to red cell antigen expressed at the surface of APC and cross-link with activated T cells. Activated T cells drive B cell activation and expansion by co-ligation and release of cytokines, leading ultimately to the production of plasma cells and the synthesis of antibody specific to the red cell antigen.

### 1.4.2 The Frequency and Pathology of FMH

The dose of antigen delivered into the maternal circulation is dependent on the size of the haemorrhage of fetal cells. FMH is common, with 76% of women having a detectable bleed at some stage of pregnancy, and 45% by the start of the 3rd trimester (Bowman 1994). Only 3% have detectable bleeds in the 1st trimester. The minimum size of FMH required to cause primary or secondary immunisation is unclear and, of the measurable FMH, the proportion that are capable of causing Rh sensitisation is unknown. A dose of 0.1mL of fetal cells has tentatively been suggested, based on red cell doses deliberately administered to blood donors to stimulate anti-D production (see section 1.7.3) (Bowman 1986).

### 1.4.3 How Red Cell Antigens are Encountered by Cells of the Immune System

T cell dependent humoral responses in humans, such as alloimmunisation to red cell antigens, require that antigen specific T and B lymphocytes are exposed to blood borne antigens presented by antigen presenting cells (APC) such as opsonised red cells. Antigen specific T and B cells are rare. To facilitate contact, secondary lymphoid organs (SLO) including lymph nodes and the spleen have evolved complex microarchitecture. These organs are well connected to the lymphatic system and blood supply, respectively. Their structure concentrates antigen, compartmentalises cell types to allow complex interactions triggering differentiation, supported by chemokine gradients maintained on specialised extra-cellular matrix structures required to mount and sustain an effective immune response (Arana 2008, Batista 2009). Unlike lymph nodes, the spleen lacks an afferent lymphatic supply, instead it specialises in dealing with blood borne antigens (Bastista 2009). It is located in the abdominal cavity and is 8-13 cm long and typically weighs 200-300g in adults (Hoffbrand 2001). Under normal conditions, other than pregnancy or other events leading to the exposure to non-self red cells such as transfusion, the red cells processed by the spleen are autologous. The major mechanism for destruction of senescent red cells is phagocytosis in the spleen mediated

by IgG autoantibodies. These bind to red cell antigens which are exposed as the aging membrane loses integrity. Removal from the circulation occurs before reduced plasticity results in impaired function (Hadley 2002). IgG mediated removal of aged red cells is necessary as macrophages lack receptors for red cell antigens, but do express FcγR (Kumpel 2006), though stress induced by high hematocrit and low oxygen levels in the spleen lead to shape changes which promote phagocytosis by macrophages of any abnormal cells (Lewis 2001). Autologous red cells express no foreign antigen and do not cause alloimmunisation. When red cells from another individual enter the circulation, due to FMH or transfusion, then alloimmunisation may occur through the actions of immune cells in the spleen.

The spleen is organised into two zones, the red pulp and the white pulp, reflecting the two chief functions of the organ which are removal of senescent red cells and micro-organisms from the blood, and mounting an immune response (Roitt 2001, Kumpel 2006; Figure 1.4). Compartmentalisation of the spleen allowing controlled contact of the cells types present is key to function (Lokmic 2008). The organ contains 25% of the T lymphocyte pool and 15% of the B lymphocyte pool, representing the largest accumulation of lymphoid tissue in the body (Lewis 2001).

The white pulp is organised into T and B cells zones containing follicles and conduits comprising laminin chains, expressing chemokines, along which DCs and lymphocytes migrate (Lokmic 2008). The T cell zone comprises a network of reticular cells and specialised capillaries which form the periarteriolar lymphoid sheath. This is separated from the red pulp by the marginal zone which is rich in macrophages, dendritic cells and polyreactive B lymphocyte clones (Batista 2009).

Within the B cell zone of the white pulp are aggregates of B lymphocytes, forming follicles which contain DCs. Following exposure to antigen, these cells form germinal centres, which are key to mounting humoral responses to T-lymphocyte dependant antigens, and to the expansion and refinement of

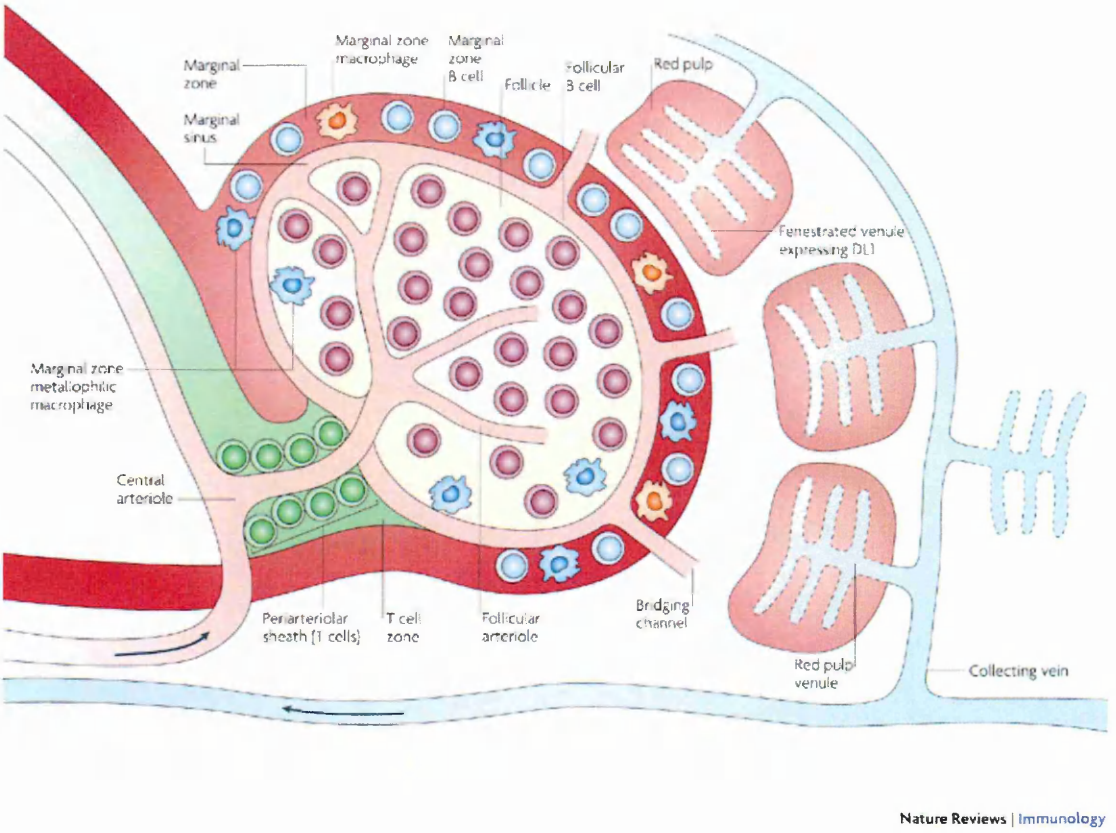



Figure 1.4 Diagrammatic representation of the spleen showing organisation and architecture of B cell follicle including follicular B cells and periarteriolar sheath containing T cells (From Pillai 2009)

Blood borne antigens such as red cells enter the spleen through the central arteriole, then pass through the marginal zone which is populated with macrophages, T and B lymphocytes and dendritic cells. Circulating lymphocytes home to the white pulp and migrate within the spleen along chemokine gradients. Activated lymphocytes and antigen presenting cells migrate to follicles where B cell expansion and maturation occurs, leading to a humoral immune response to blood borne pathogens.

antibody synthesis (Batista 2009). DCs in the white pulp collect and concentrate antigen by interaction with circulating immune complexes through Fc and complement receptors (Gonzalez 2009), though the latter is unlikely to be relevant in processing Rh antigen as Rh antibodies are incapable of fixing complement (Issitt 1998). The white pulp is organised to permit migration of lymphocytes to support interaction between T and B cells.

On entering the spleen, blood cells move through a network of vascular sinusoids in the marginal zone between red and white pulp. Here antigens are first encountered by B cells, macrophages and DC, which remove material from the circulation. Macrophages have excellent phagocytic properties but are poor antigen presenting cells (Roitt 2001). This process as part of normal homeostasis, is non-inflammatory, and results in no tissue damage other than to the opsonised cells (Kumpel 2006). DCs in contrast are less effective as phagocytes, but are armed with an array of receptors which allow detection of pathogens and activation of innate and adaptive immune responses (Bergtold 2005). In processing large particles expressing antigen, such as red cells, it is likely that macrophages and DCs are both involved (Roitt 2001).

### 1.4.4 The Phagocytosis of IgG Opsonised Red Cells

Phagocytosis of IgG opsonised particles, including red cells coated either with auto- or specific IgG alloantibody, is effected through Fcγ receptors (FcγR). FcγR are immunoglobulin-like molecules, which are widely expressed on the effector cells of the immune system including monocytes, macrophages, and dendritic cells (Janeway 2001).

There are four major groups of Fcγ receptors, FcγRI-III and FcRn. The structure of the groups is variable, and functions include control of IgG catabolism (FcγRI), mediation of phagocytosis (FcγRIIA), endocytosis leading to antigen presentation (FcγRIIB-2, and FcγRIII), and regulation of cellular responsiveness (FcγRIIB). Most cell types express FcγR both activatory and inhibitory types. The exceptions are NK cells which express only activatory

FcγRI, and B cells which express only inhibitory FcγIIRB-1, which in this form has low affinity for its ligand and is a poor mediator of phagocytosis (Heyman 2003). In particular FcγR are key to the humoral immune response through participation in immune complex maturation of DCs, the presence of both activatory and inhibitory FcγR of DC is key to maintaining activation thresholds for these cells (Nimmerjahn 2008).

In particular, FcγIIR which is the most widely expressed of the group has a strong inhibitory effect on DC and B lymphocytes when cross linked by multiple, bound IgG molecules, FcγRI is a high affinity receptor for IgG1 and IgG3 in humans, binding with an affinity 100-1000 times that of the other receptors (Nimmerjahn 2008)

When FcγRI and III are cross linked by multiple binding of Fc domains, other FcγR are activated to capture and enclose the particle in an internal phagosome. Fusing of the phagosome with one or more lysosomes leads to enzyme lysis of protein at low pH (Janeway 2001), making available antigen derived peptide for loading on to MHC I and II for presentation to T cells (Bergtold 2005). Large particles such as red cells may not be internalised in their entirety, but fragments of membrane may be degraded (Petz 2004).

When bound and cross-linked by their ligand, phosphorylation of intracellular domains results in cell signalling (Roitt 2001). FcγR can initiate both activating and inhibitory signals and are key elements of the balance, in healthy individuals between pro-and anti-inflammatory conditions.

### 1.4.5 The Role of T Lymphocytes in the Humoral Response

When DCs present antigen derived peptide at the cell surface on MHCII they recruit naïve CD4 T cells which can bind to the peptide complex through TCR (Fazilleau 2007). The accompanying information about the immunological threat, imparted through co-stimulation, causes those T cells to differentiate into specific subsets with characteristic functions. There is some evidence that

the affinity of the bond between MHCII/peptide and TCR positively influences the subsequent degree of T cell clonal expansion (Malissen 2009). Classically these were described as Th1, and Th2 sets based on signature cytokine profiles, but this paradigm is now considered simplistic, and several other subsets have been characterised, including Th3, Tho, Th17 and Treg. Each of these is reported to have characteristic surface markers and cytokine profiles, Th3 cells are a major source of TGF-β, and have distinct functions (Malissen 2009). Follicular T helper cells (TFH) have been described which derive from naïve T cells expressing TCR with particularly high affinity for antigen. Functionally they are characterised by expression of IL-21 which drives B cell differentiation, and by expression of CXCR5, a chemokine receptor which directs lymphocytes in the direction of germinal centres (Malissen 2009).

With the presented peptide localised to the binding groove on MHCII on the APC, the T cell receptor (TCR) on T helper cells can bind to the peptide MHC complex. This interaction is facilitated by co-stimulatory and cell adhesion molecules including Lymphocyte Functional Antigen 3 (LFA-3) binding to CD2, Intercellular Adhesion Molecule-1 (ICAM-1) binding to LFA-1, and CD80/86 binding to CD28. Without this co-stimulation providing a second signal T cell activation cannot occur. Th activation is facilitated by interleukins (IL) -1, -6, -12, and -15 and tumour necrosis factor-α (TNF-α) and proliferation is driven by IL-2 (Roitt 2001, Janeway 2001, Kumpel 2000).

### 1.4.6 Lymphocyte Homing in the Spleen

Circulating lymphocytes entering the spleen home to the white pulp; T lymphocytes to the periarteriolar lymphatic sheath, and B lymphocytes to the adjacent lymphoid nodules (Pillai 2009). Naïve B cells enter the spleen from the circulation to optimise potential to encounter specific antigen. Most B cells migrate to follicles dependent on expression of the chemokine receptor CXCR5 and the expression of its ligand CXCL13 on the extracellular matrix of follicular tissue (Okada 2006, Pillai 2009).

Follicular B cells are highly mobile, migrating within the follicle at up to 6μm per minute, presenting opportunities to survey antigen presented on follicular DCs (Okada 2006). As part of those movements within the follicle, B cells migrate toward the T cell zone dependent on moderate expression of CCR7, its ligand CCL21 is expressed in T cell zones (Okada 2006). This movement facilitates interaction between B and T cells. Naïve B cells migrate through follicles for 12-18 hours, and may migrate to other follicles before leaving the spleen. It is estimated that more than 99% of cells encounter APC, before leaving the spleen (Schwickert 2007).

If follicular B cells fail to encounter specific antigen, they return to the circulation. In the event of B cells encountering specific antigen they migrate within hours to the border of the B cell and T cells zones, having rapidly upregulated CCR7 expression upon engagement, and can remain there for several days (Okada 2005).

Photon microscopy studies have demonstrated the role of antigen engaged B lymphocytes in binding to specific, activated T cells at the T-B border, and leading the migration of both cells to the germinal centre (Okada 2005).

### 1.4.7 Antigen Uptake by B Lymphocytes

The production of a significant humoral response to thymus dependent protein antigens, such as Rh on red cells, requires the action of T and B lymphocytes. (Janeway 2001). In B cells, the B cell surface antigen receptor (BCR) has a function analogous to the TCR in T cells. The B cell receptor is immunoglobulin, IgM and IgD being expressed on mature B cells. When cross-linked by antigen, surface immunoglobulin effects intracellular signalling via immunoreceptor tyrosine-based activation motifs (ITAMS) expressed on two associated heterodimeric proteins Igα and Igβ (Lydyard 2005, Arana 2008). Other molecules key to cell function are expressed early in the cell's development including MHC class II to permit antigen presentation, and co-

receptors including complement receptors (CD2), FcRγII-B1 (McHeyzer Williams 2006).

The co-ligation of BCR and antigen along with these and other B cell surface molecules with ligands on T cells (e.g. CD40 and CD40L) is required to avoid clonal anergy, and to permit intracellular signalling resulting in B cell proliferation (Lydyard 2005).

Through the BCR, B cells are capable of recognising soluble or membrane bound antigen, the latter when associated with professional APCs is both a more frequent and more important route for lymphocyte activation due to co-stimulation (Bergtold 2005, Batista 2009). The presentation of antigen to follicular DCs is undertaken by marginal zone B cells, which migrate between marginal zone and follicle by alternate expression of CXC chemokine receptor 1 (CXCR1) and sphyngosine 1-phosphate receptor (S1PR). This is achieved through receptors other than BCR, and the high level of complement receptors on marginal B cells may be important for presentation of immune complexes including complement (Batista 2009), though this is unlikely to occur in Rh alloimmunisation as Rh antibodies do not cause complement fixation.

### 1.4.8 The Activation of B Lymphocytes

Activated T helper cells interact with antigen primed B lymphocytes via HLA class II/peptide complex (Figure 1.2). Additional co-stimulatory and adhesion molecules on T and B cells are required for activation (CD28 binding to CD80/86; CD40 ligand to CD40; LFA-1 to ICAM-1 or ICAM-3; and CD2 to LFA-3). B-cell activation and division are stimulated by IL-4 and IL-6. In particular the interaction between CD40 and CD40 ligand, upregulates IL-4 receptors on B-lymphocytes and the release of IL-4 from Th2 cells drives B-lymphocytes, into the cell cycle resulting in proliferation and expansion (Janeway 2001). On contact with DCs, B cells undergo a conformational change, spreading along the antigen presenting surface to form a synapse

which supports the BCR interaction with antigen and the required co-stimulation to drive B cell expansion (Arana 2005). Under further influence from Th derived cytokines, B-lymphocytes switch the class of antibody production, the combined effects of IL- 4, -5, -6, -13 and IFN-γ resulting in the production of IgG (Roitt 2001). In particular IgG production is down regulated by TGF-β1, both by direct inhibition of B cell proliferation, and by immunoglobulin class switching to IgA (Li 2006).

Unregulated B cell expansion carries the risk of tissue damage due to proliferation of autoreactive B cells, and among regulatory mechanisms the action of the inhibitory FcγIIB-1 expressed on B cells offers a checkpoint to prevent development of autoreactive antibodies (Nimmerjahn 2008). Reduced expression or absence of FcγRIIB-1 results in exacerbation of antibody mediated autoimmune conditions. Inhibitory signals are generated by IgG ligated FcγIIRB-1 through immunoreceptor-tyrosine-based inhibitory motifs (ITIMs), FcγIIRB-1 cannot bind monomeric IgG and requires immune complex, allowing multiple crosslinking of FcγIIRB-1 with BCR (Heyman 2003). This results in inhibitory signalling through ITIMs, which can down-regulate activatory signals from activatory tyrosine based motifs (ITAMS) linked to BCR. This leads to B cell downregulation and ultimately to apoptosis (Kumpel 2002).

Once activated, B cells can recruit specific CD4+ T helper cells by processing and presenting antigen through their own MHC class II molecules (Batista 2009). Activated B cells can differentiate along two lines. Firstly extrafollicular plasmablasts which are essential for rapid, protective antibody production, and secondly plasma cells in germinal centres can produce a long lasting antibody response that can, after affinity maturation, result in high titre, high affinity antibody production (Batista 2009). The development of B cells is summarised in Figure 1.5.

In the event of a second challenge with the same antigen, an enlargement of existing germinal centres is induced along with formation of new ones. B-

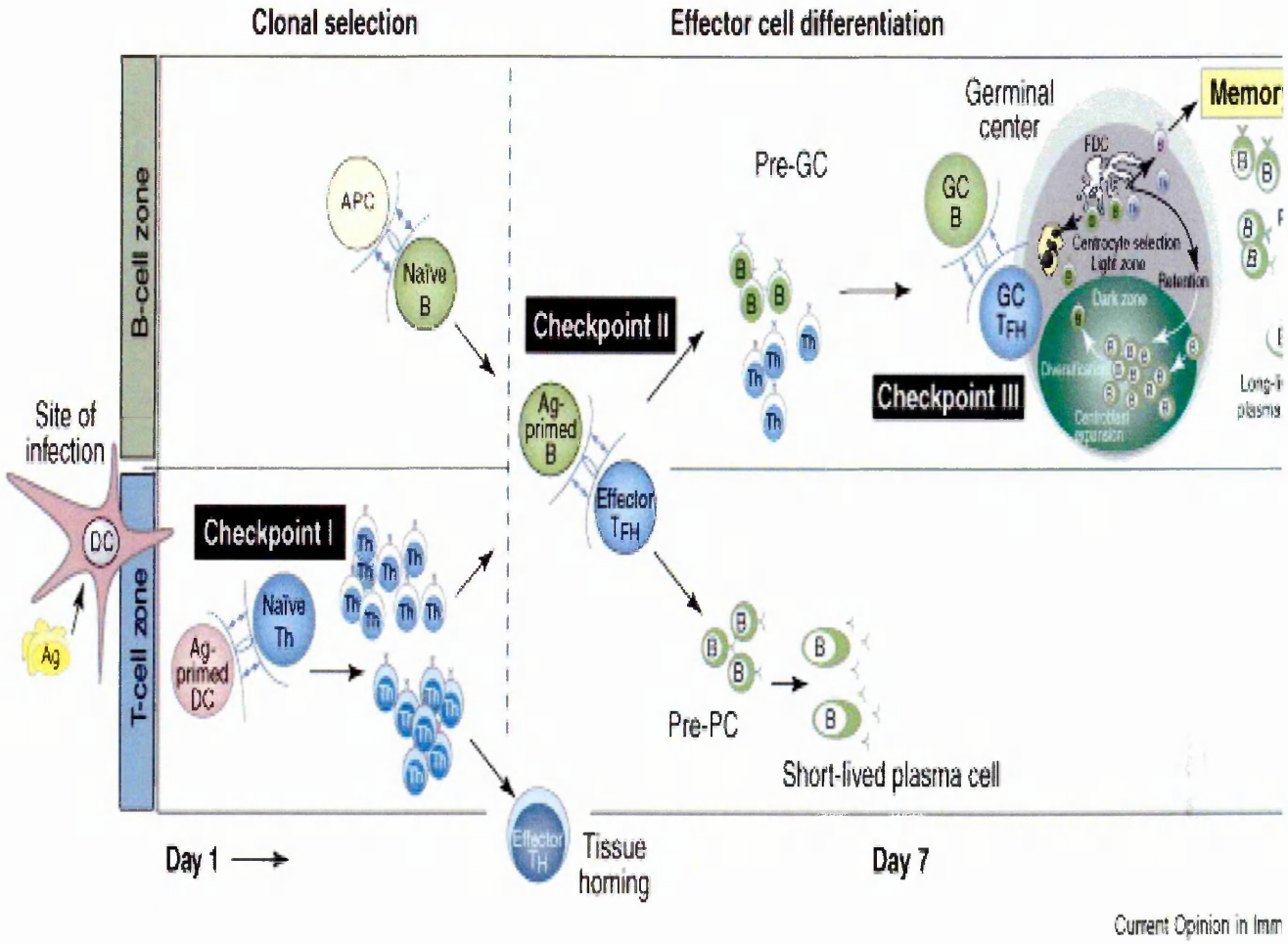



Figure 1.5 B cell expansion and differentiation into Plasma cells and memory cells, (From McHeyzer Williams 2006).

After exposure to antigen DCs migrate to the T cell zone of the spleen, and engage with naive T cells (checkpoint I). B cells specific for the antigen interact with activated T cells (checkpoint II) and develop either into short lived plasma cells, or following further maturation with T cell help (checkpoint III) into long lived plasma cells.

lymphocytes, primed with newly presented antigen migrate, and divide in response to stimuli from dendritic cells and Th cell derived cytokines. Through a competitive process in which B-lymphocytes become centrocytes and undergo apoptosis (unless continuing to be bound to antigen) which is reduced in concentration. Clonal selection ensures that only antibody producing cells with high affinity for the antigen survive, mature and expand to secrete antibody in significant quantities (Roitt 2001, Fazilleau 2007). By this competitive mechanism of refinement, antibody response to low doses of antigen results in high affinity antibodies. High antigen doses generate much lower affinity antibodies. As FMH are typically less than 4mL of fetal packed cells (Mollison 1972), affinity of anti-D antibodies is generally high, leading to powerful mediation of immune destruction. There is little evidence to indicate that anti-D prophylaxis can prevent or reduce secondary immune responses (Mollison 1984). Follicular dendritic cells store antigen and can present it long after the initial challenge. This is key to the process of affinity maturation associated with T cell dependent antibody responses (Batista 2009).

### 1.4.9 IgG Biology

There are five classes of immunoglobulin, IgM, IgG, IgA, IgE, and IgD. Of these IgG is the most abundant in human plasma, diffusing rapidly through tissues to neutralise bacterial toxins and enhance phagocytosis of microorganisms (Roitt 2001). IgG is further differentiated in structure and function by four subclasses IgG1-4, which are characterised by variation in the heavy chains. IgG classes 1 and 3 are: bound more effectively by Fc receptors on macrophages, more readily transported across the placenta (Roitt 2001) and more pro-inflammatory in humans than IgG2 and IgG4. The heavy chains of IgG are glycosylated with galactose residues, which stabilise the tertiary structure of the protein. This is required to permit binding with FcR. The degree of glycosylation varies between individuals and increases during pregnancy (Nimmerjahn 2008).

The evidence that IgG isotype is strongly linked to the severity of HDFN and is therefore a potential predictor of HDFN severity has been the subject of much research (Hadley 2002). The anti-D antibody in pregnant women was shown predominantly to be a mixture of IgG1 and IgG3, in a study of 98 pregnancies of women with alloimmune anti-D. A third of these cases contain only IgG1, and of 3 pregnancies in which only IgG3 was detected, none were affected by HDFN (Pollack 1990). The ratio between fetal and maternal IgG1 concentration, but not IgG2, 3, or 4, was found to be much higher in pregnancies affected by HDFN than those unaffected, suggesting that IgG transport is a key factor in determining disease severity (Lubenko 1994). In 40 affected pregnancies, the concentration of IgG1 detected by ELISA in eluates prepared from fetal cells was found to be higher than in maternal plasma (Lambin 2002). *In vitro* studies have indicated that IgG3 is a more effective mediator of red cell lysis, prompting speculation that the longer hinge region is more effective in bridging the gap between negatively charged red cells and effector cells, than IgG classes with shorter reach (Hadley 2002). Though this conflicts with the limited evidence that IgG3 in isolation is a poor effector of HDFN (Pollack 1990).

### 1.4.10 Red Cell Destruction in the Fetus

Very few studies have examined the mechanisms of fetal red cell destruction. The mechanisms observed in adults and *in vitro* studies are generally accepted as relevant to the fetal environment (Hadley 2002).
Once maternal anti-D has been transported across the placenta it binds via the Fab domains to fetal D antigen. Opsonised red cells are recognised by macrophages bearing receptors which bind to the IgG molecules mediating phagocytosis and red cell destruction (Hadley 2002). The presence of competent macrophages has been demonstrated in the fetal spleen as early as 13 weeks gestation (Billington 1992). The severity of HDFN due to antibodies to antigens not restricted to red cells, is reduced by antibody binding to antigen expressed on other tissues including placenta and endothelium (Kumpel 2002).

## 1.5 Laboratory Testing to Support Prevention and Prediction of HDFN

### 1.5.1 The Role of the Laboratory in Predicting HDFN

Historically HDFN has been a disease which progresses, unseen to the clinician, until it presents at birth, too late for remedial action. As a result, a generation of clinicians and scientists have sought predictive examinations or laboratory tests that might indicate pregnancies in alloimmunised women in which the risk of intervention is exceeded by the risk of the disease. Fetal examination, either by clinical observation or by laboratory tests is inherently hazardous. Observation of the mother, and tests on maternal peripheral blood much less so, and that has been the basis of clinical and laboratory practice (Abdel Fattah 2002).

In fact, laboratory testing has been more successful in support of prevention through prophylaxis than prediction of disease severity. The human immune system is exquisitely complex and the multifactorial nature of the pathogenesis of HDFN has not adequately been captured, either by individual or by panels of laboratory tests, to predict the clinical course of HDFN with confidence. (Figure 1.6). In fact the evolution of informative laboratory assays has happened in parallel with the development of clinical techniques to allow diagnosis and treatment of HDFN.

The pathological mechanisms of HDFN are similar in many respects to that of the immune response to transfused red blood cells. As a result, the laboratory tests associated with HDFN are performed in transfusion laboratories. Safe blood transfusion requires matching donor red cells with the ABO and Rh groups of the recipient. Transfusion scientists employ simple reliable and cost-effective techniques for determining patient blood groups using, in almost all cases: automated test systems interfaced with information technology that allow safe interpretation, storage and dissemination of test data to support transfusion practice (BCSH 2006).

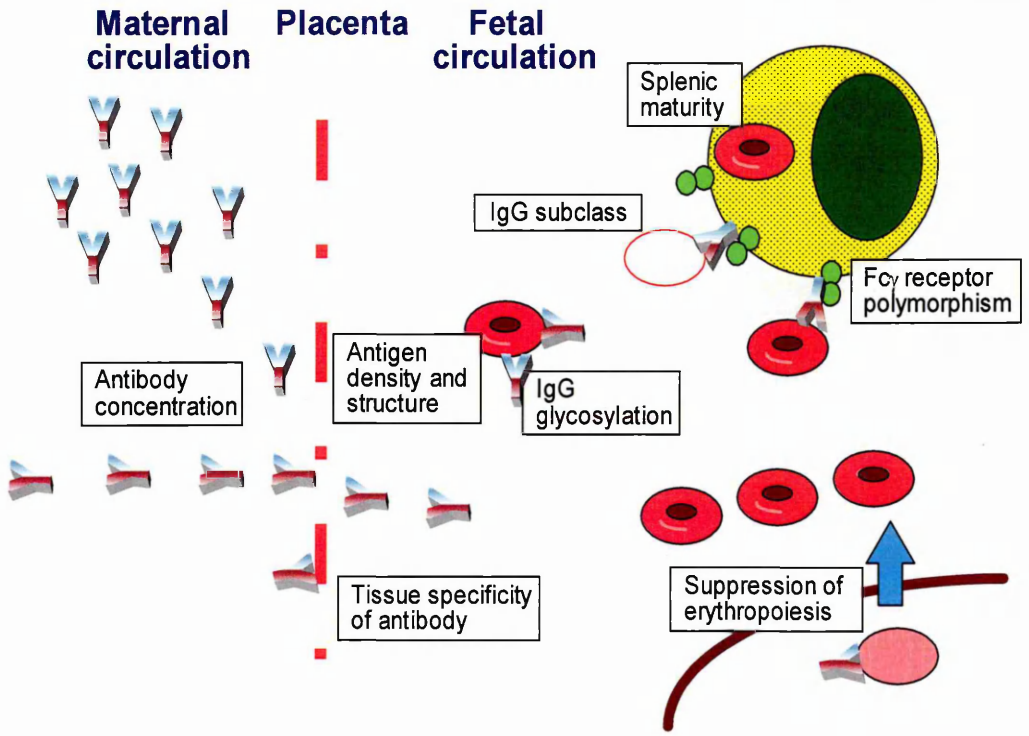



Figure 1.6 Illustration of the range of factors which influence the occurrence and severity of HDFN in candidate cases, critical elements include:

- Antibody concentration, isotype and degree of glycosylation
- Efficiency of transport across the placenta
- Antigen expression on fetal red cells and other tissues
- Efficiency of fetal immune system

(Image from Hadley 2002)

In the UK, all women should have ABO and RhD group determined twice in pregnancy, at the time that they book with a midwife at 10-14 weeks gestation and at the beginning of the third trimester (BCSH 2006). The determination of ABO group is of little significance for HDFN, but may be useful in the event of requirement for maternal blood transfusion at delivery. The RhD group is used to determine the need for, and plan the administration of routine anti-D prophylaxis if required, at 28 weeks and at delivery, and to allow timely treatment (BCSH 2006a).

Antibody screening techniques for pre-transfusion testing are suitable for use in detection of antibodies which may cause HDFN. Again automated tests are in use in most laboratories. Red cells used for screening are obtained from two or three blood donors, selected for strong expression of antigens to clinically significant red cell antibodies. Tests are performed by the Indirect Antiglobulin Technique (IAT) standardised to detect IgG antibodies to red cells that are active at 37°C, which might mediate red cell destruction either in reducing the survival of transfused red cells or destruction of fetal red cells in HDFN (BCSH 2006). Not all antibody specificities are associated with immune destruction of red cells, due either to the characteristics of antigen (likely to detach from red cell surface or low antigen density) or to those of the antibody (cold-reacting antibodies with low thermal amplitude) (Daniels 2002a).

An alternative approach for laboratory investigation of HDFN is the detection of IgG bound to fetal red cells by Direct Antiglobulin Test (DAT). Anti-human IgG (AHG) is added to a suspension of patient's cells in saline. If IgG is bound then the AHG cross-links the red cells causing visible agglutination (Poole 2002). Though indicative of the presence of IgG on fetal red cells, a positive DAT is not diagnostic for clinical HDFN. The pathogenesis of HDFN is dependent on many factors including IgG subclass, extent of IgG glycosylation, and the efficiency of the fetal reticulo-endothelial system. As a result, in some cases, fetal red cells coated with maternal IgG may survive normally with no evidence of red cell destruction (Stevenson 2001, Klein 2005).

It is recognised that many examples of antibodies to red cell antigens, even of significant specificities, do not cause accelerated destruction of transfused antigen positive red cells. No simple, reliable and timely tests are available to differentiate between benign and lytic antibodies (Hadley 2002). When an antibody significant for transfusion purposes is identified, biomedical scientists select blood lacking the corresponding antigen for transfusion. The differentiation between lytic and benign antibody is non-critical as risk can be managed simply. In the majority of cases this is straightforward and results in little extra work, expense or delay. In contrast, in the case of antibodies in pregnant women the actions a practitioner might undertake are much more hazardous for mother and baby. Invasive techniques such as amniocentesis or fetal blood sampling, to determine the severity of disease have been practiced with significant process-related loss of fetuses. Consequently, it has been the focus of many immunohaematologists over more than last forty years to develop tests which would accurately and reliably allow the prediction of HDFN severity from maternal peripheral blood samples, in order to allow clinicians to reduce the requirement for invasive procedures, to minimise risk and maximise predictive benefit (Hadley 2002).

### 1.5.2 The Assessment of Antibody Concentration

Red cell agglutination assays have been used with serially diluted patient serum or plasma to measure the concentration of antibodies. The IAT has been used for this purpose since the 1950s (Bowman 1994). The "strength" of the antibody is reported as the reciprocal of the last dilution which causes agglutination of test cells. Titration, however, is inherently imprecise with variation associated with test conditions, operator and endpoint interpretation (AuBuchon 2008), though rigorous application of more modern serological techniques, including column agglutination technologies, may improve performance.

More reproducible, objective and effective in testing large numbers of samples is autoanalyser quantification. The technique also uses diluted patient plasma against red cells treated with a proteolytic enzyme to promote agglutination in

a continuous flow system. The mixture is passed through a settling coil, to separate agglutinated from non-agglutinated cells. These are lysed and passed through a colorimeter that measures the concentration of released haemoglobin, which is inversely proportional to that of the antibody. Antibody concentration in test samples is calculated using a calibration curve against an international standard (National Institute for Biological Standards and Control , South Mimms, UK) gives a result in International Units per mL (IU/mL) (Hadley 2002).

As well as technical deficiencies, IAT titration has a poor record as an indicator of disease severity. In the USA, where the technique is still widely practiced for anti-D, a critical titre of 16 is generally accepted as an indicator that intervention is required. Some authors point to data indicating that 20% of anti-D cases with titres below 16 require exchange transfusion suggesting that titration is an unreliable predictor of HDFN (van Dijk 1995). Autoanalyser quantification has a marginally better record with clinical HDFN recorded in very few pregnancies with anti-D concentrations of less than 4IU/mL, though many pregnancies with anti-D concentration greater than 4IU/mL are unaffected by HDFN. The limitation of agglutination assays is that they measure the effectiveness of the Fab region of the antibody to bind to red cells antigen whereas it is the Fc domain which effect immune destruction through Fc$\gamma$R mediated phagocytosis by splenic macrophages.

A range of cellular assays has been developed with a view to offering a better *in vitro* model of the *in vivo* process and hence better reliability as a predictor of disease. In fact these tests, involving the measurement of the interaction of effector cells with red cells sensitised with maternal antibody, have all to some degree succeeded at being better predictors of disease severity than titration or autoanalyser quantification (Hadley 1993, Urbaniak 1985). However the specialised nature of these tests, the length of time they take to complete and the requirement for referral to a distant centre, means the timelines of results is compromised. More importantly the ability to detect even relatively mild HDFN by ultrasound has made much of this work redundant. The role of the

laboratory is now to flag moderate levels of risk to obstetricians as a trigger for non-invasive investigation (Hadley 2002).

## 1.6 Clinical Assessment and Treatment of HDFN

### 1.6.1 Pre-natal Assessment of HDFN

In much the same way that the assessment of severity of HDFN has changed in the laboratory as new technologies have promoted better modelling, the approach to clinical assessment has evolved. In fact, the ability to base clinical judgements on non-invasive clinical assessments has greatly reduced emphasis on laboratory based diagnosis and has improved focus on deferral of potentially dangerous invasive techniques.

The most accurate way to assess fetal anaemia remains the measurement of haemoglobin concentration by direct fetal blood sampling by cordocentesis. The risks are high, both of fetal loss (estimated at 1%) and boosting the maternal antibody by induced FMH exacerbating HDFN (Abdel Fattah 2002). Some clinicians aim to time the first intervention in the current pregnancy at around 10 weeks before that in a previous affected pregnancy (Abdel Fattah 2002). This reflects the incremental exacerbation of HDFN in successive pregnancies, and the lack of firm evidence on which practice is based.

Ultrasonography is a widely applied technology for assessing pregnancy. The symptoms of hydrops as an indicator of fetal anaemia, ascites, pleural effusions and oedema, are visible to the ultrasonographer and are indicators of profound anaemia, requiring urgent intra-uterine transfusion. Attempts to find an earlier indicator of fetal anaemia have been less successful (Abdel Fattah 2002, Divakaran 2001). Hepatosplenomegaly resulting from extramedullary erythropoeisis measured by ultasonography is a useful indicator of anaemia, but optimally after 30 weeks gestation. Similarly, abnormal fetal heart rate patterns are indicative of severe fetal anaemia late in pregnancy (Abdel Fattah 2002).

Recently applied techniques, particularly Doppler velocimetry, allow relatively accurate estimation of fetal haemoglobin concentration. Measurement of fetal blood flow in the median cerebral or splenic arteries is possible and is indicative of fetal haemoglobin concentration due to changes associated with the haemodynamic compensation in the fetal circulation to manage anaemia (Scheier 2004, Bahado-Singh 2000). Though Doppler screening is less accurate than cordocentesis, sufficient sensitivity can be achieved with an acceptable false positive rate to allow the procedure to be used as a screen. In a study of 55 scans of 26 fetuses, a threshold measurement of 1.5 times the median peak systolic velocity gave 100% detection of affected fetuses, with a 20.6% false positive rate (Badaho Singh 2000). The associated reduction in risk of procedural loss of the fetus, and further alloimmunisation associated with reduced numbers of cordocentesis procedures makes this a considerable improvement to antenatal surveillance (Divakaran 2001).

### 1.6.2 Post natal Assessment of HDFN

Even after delivery, diagnosis of mild HDFN is problematic. Neonatal haemoglobin concentrations fall over the first 2 months of life (physiological anaemia of the newborn) in unaffected infants. This is associated with reduced erythropoeisis and in particular low level of erythropoietin, as the baby matures from the low oxygen environment of the uterus and to breathing oxygen-rich air. Serum bilirubin rises over the first 2-3 days after birth occurs in almost all infants (Klein 2005).

### 1.6.3 The Treatment of HDFN

By the time the effects and mechanism of HDFN were being clarified and characterised in the late 1930s and early 1940s, blood transfusion was emerging as a useful, if incompletely understood therapy. Its use to correct anaemia in babies presenting with HDFN was unsuccessful in many cases, being ineffective against kernicterus (Bowman 1994). A more successful strategy for transfusion of the affected neonate was exchange transfusion in which fetal RhD+ red cells were drawn from the fetal circulation and replaced

with RhD- donor red cells, both maintaining haemoglobin levels and removing the source of the bilirubin. By this means some clinicians were able to reduce the fetal mortality rate associated with HDFN from 50% to 25% (Bowman 1994).

For women with high concentrations of alloantibody, particularly early in pregnancy, exchange plasmapheresis has been used. The process involves regular removal of large volumes of maternal plasma and replacement variously with albumin, normal immunoglobulin, or whole plasma. It is a disruptive and sometimes uncomfortable treatment for the mother, often requiring multiple large exchanges per week (Bowman 1994, Gottval 1995). It is common for antibody production to increase such that levels cannot be diluted below pathological levels: such a "bounce-back" effect can be noted as early as 48 hours after plasma exchange. (Knott 1987, Moise 2002). IgG synthesis is controlled by feedback mechanisms (Bouchard 1995), it is possible that the sudden removal of large quantities disturbs such mechanisms resulting in a significant increase in IgG synthesis and release. The effectiveness of the process is difficult to assess from the available literature, as other therapies including IUT and IVIg, have been applied as an adjunct to plasmapheresis in some cases (Moise 2002). Since ultrasound guided intravenous fetal transfusion has become widespread, plasmapheresis is rarely used to treat HDFN, though some authors believe the treatment may delay or make unnecessary the use of more invasive techniques (Knott 1987). Exchange plasmapheresis continues to be a treatment option for other fetal pathologies associated with maternal antibodies including prevention of fetal heart valve damage from maternal anti-Rho associated with SLE (van der Leij 1994).

### 1.6.4 Induced Premature Delivery

When presented with a pregnancy in which the fetus is likely to become hydropic, clinicians are faced with balancing the considerable risk of early delivery against the risk associated with the disease process (Bowman 1994). The point at which the risk of immaturity is exceeded by the risk of hydrops

was established as 32-34 weeks (Chown 1954), though a more modern view of the same question may reduce that gestational age in view of improvements in neonatal care. By 1961 early induced delivery had reduced the mortality rate of babies suffering from HDFN from 25% to 16% (Bowman 1994).Though the risk was understood, there was no means of discerning high from low risk pregnancies. The presence of anti-D in the maternal plasma was no indicator of fetal RhD type and many RhD- pregnancies were exposed to the risks associated with early delivery. Spectrophotometric analysis of amniotic fluid at 450nm allowed accurate estimates to be made of bilirubin concentration, hence levels of haemolysis and anaemia (Liley 1961). Assessment of risk of frank HDFN by amniocentesis allowed clinicians to choose the pregnancies for which early delivery would be therapeutic. In Manitoba between 1961 and 1964 the perinatal mortality rate from HDFN fell further to 13% (Bowman 1994).

The risk to the fetus of early delivery decreases with fetal maturity. In the 1960s premature fetuses were unlikely to survive before 32 weeks gestational age but 8% of affected fetuses become hydropic before that gestational age (Bowman 1994). The prognosis in these cases was poor until the first *in utero* transfusions (IUT) were performed. Guided by radiography, blood was transfused into the peritoneal cavity, to be adsorbed via the right lymphatic duct into the venous circulation, with 10-12% of cells transfused in this way being adsorbed per day. Absence of fetal breathing movements and moderate to severe ascites both inhibit the process. Though offering a therapy where previously there was none, the risks of intraperitoneal transfusion *in utero* are considerable. The procedure has a 7% traumatic death rate and 30% spontaneous labour rate and has largely been discarded as a treatment option (Bowman 1994).

For transfusion outside the uterus, intraperitoneal transfusion had long been replaced by venous infusion. The potential benefits of venous transfusion *in utero* were apparent: rapid increase in fetal haemoglobin levels and the potential to be therapeutic for moribund ascitic fetuses lacking breathing movements (Bowman 1994). Early attempts to transfuse directly into fetal

veins had little success, apart from a very few highly skilled practitioners who were able to overcome the turbidity in the amniotic fluid to visualise accurate placing of a needle in the umbilical vein by fetoscopy (Tovey 1992). The process became widely available with the advent of ultrasound guidance. Fetal blood sampling and intra-uterine transfusion become part of the same process (Urbaniak 2000). The procedure is superior to intraperitoneal transfusion in survival rates, and risks to both mother and fetus. Fetal survival rates after IUT are 84%, 70% of hydropic fetuses survive, though if the first IUT is administered before hydrops occurs the survival rate is 94% (Moise 2002) By contrast intraperitoneal transfusion resulted in traumatic death in 7% of procedures, and spontaneous labour in 30% (Bowman 1998).

The risk of fetal loss due to IUT is moderated by ultrasound guidance; further maternal alloimmunisation remains a risk and may exacerbating HDFN, as well as making selection of blood for further IUT problematic. Of 91 women whose fetuses received IUTs, 24 (26%) formed alloantibodies resulting from exposure to donor or fetal red cells exacerbating risk of HDFN in current and subsequent pregnancies (Vietor 1994).

## 1.7 Anti-D Prophylaxis

### 1.7.1 The History of Anti-D Prophylaxis

That the presence of passive antibody prevents active immunisation to the corresponding antigen had been demonstrated as early as 1900 with passively administered anti-ox antibodies preventing a humoral response to ox red blood cells injected into rabbits (Von Dungern 1900 cited in Urbaniak 2000). It was sixty years later, and twenty years after the discovery that RhD was the chief causative agent of HDFN, that antibody mediated immune suppression (AMIS) was attempted experimentally in association with HDFN (Clarke 1963). In that study, RhD positive red cells, previously coated with anti-D in *vitro,* were injected on four or five occasions into each of 16 volunteers in the test group. None of the volunteers developed anti-D

antibodies. When 10 of the group were subsequently injected with uncoated D+ red cells, 5 produced anti-D antibodies (Stern 1961).

At the same time a similar experiment was prompted by the observation of partial protection from RhD immunisation by ABO incompatibility (Finn 1961). Using high titre anti-D antibodies injected into volunteers shortly after 5mL of $Cr^{51}$ labelled RhD+ red cells, rapid clearance of 50% of the injected cells was noted, demonstrating the potential for protection and the antibody dose-dependent nature of red cell clearance. This striking experiment prompted studies in the prevention of Rh alloimmunisation in Liverpool (Clarke 1963) and New York (Freda 1963). The Liverpool group expanded on Finn's work exploring the importance of the anti-D dose administered, showed that "complete" (IgM) anti-D enhances rather than suppresses immunisation and, importantly, recognised the greater safety, consistency and convenience of using the IgG plasma fraction rather than whole plasma.

The New York group delayed their experiment until they had a concentrated anti-D immunoglobulin prepared by the Ortho Research Foundation (Freda 1963). This was safer than the whole plasma, used initially by the Liverpool group, being sterile and lacking some immunogenic plasma proteins and minimising the risk of enhanced alloimmunisation. In addition, the concentrate provided a standard product, which could be administered in small volumes; these were pre-requisites if prophylaxis on a population scale was to be realistic. Initial experiments, on nine inmates at Sing-Sing Prison, consisted of administration of anti-D immunoglobulin twenty-four hours before five, monthly doses of 2mL of whole RhD+ blood. Six months after the last administration, a period chosen to allow decay of the exogenous anti-D to below detectable levels, none of the four individuals in the protected arm, compared to four of the five in the unprotected arm, had been alloimmunised. Realising that in clinical situations anti-D would be administered after the potential sensitising event; the authors undertook a further study in which anti-D was administered 72 hours after a single dose of 10mL whole RhD+ blood. The 72 time period between sensitising dose, and anti-D administration was dictated by prison visiting times. Of the 13 "unprotected" controls 6 were immunised, none of the

"protected" volunteers formed anti-D. This experiment was so successful in demonstrating the effectiveness of administration anti-D after the potentially sensitising episode that the 72 hour period remains accepted as the maximum safe elapsed time some 45 years later (BCSH 2006a). Evidence to support or challenge this regime would be difficult to generate but would be valuable in revisiting our perception of best practice.
Use of the newly described technique for detecting and accurately measuring feto-maternal haemorrhage (Kleihauer 1957) provided evidence that transplacental haemorrhage is most common at delivery (MRC Combined study report 1974). That, and the concern that exogenous IgG anti-D might cross the placenta and destroy fetal RhD+ cells, prompted workers to attempt antibody mediated immunosuppression (AMIS) shortly after delivery.

The first clinical trials were conducted soon afterwards (MRC Combined study report 1974). The first study was designed to provide information as rapidly as possible on the efficacy of AMIS with the limited amount of anti-D immunoglobulin available. In this multi-centre trial the authors attempted to randomise treatment and non-treatment alternately to primiparous women in whom there was demonstrable feto-maternal haemorrhage at delivery. In a refreshing display of openness the authors describe the difficulties in randomisation associated with obtaining test results for weekend deliveries, and the intermittent availability of anti-D immunoglobulin. This resulted in the default allocation of some participants from the treatment group to the control group. Nevertheless, in tests performed 6 months after delivery, of the 78 participants in the control group 19 (24%) became alloimmunised forming anti-D. Of the 78 participants in the test group none were confirmed to be immunised, though 3 had inconclusive antibody screens (MRC Combined study report 1974).

### 1.7.2 The Introduction of Anti-D Prophylaxis

In 1966 a working party established by the Medical Research Council (MRC) began a further controlled trial to demonstrate the effectiveness of anti-D. Within a year of the commencement there was sufficient evidence of the

effectiveness of anti-D prophylaxis. As the UK supply of anti-D allowed treatment of only 12% of at risk primiparous women, the trial was converted to establishing the minimum protective dose (MRC combined study report 1974). Approximately 200 women in each of 4 dose groups (200 μg, 100μg, 50μg and 20μg) received anti-D immunoglobulin at delivery were followed to the end of a second RhD+ pregnancy. Each participant was screened for alloimmunisation. Dose group immunisation rates between 1.5% (200μg) and 2.9% (20μg), approximately one tenth of that in untreated pregnancies. The authors concluded that a dose of 100μg (500 IU) of anti-D was optimal.

A further study included experimental elements designed to determine the alloimmunisation rate in unprotected volunteers, whether alloimmunisation was dose dependent, and how the characteristics of the anti-D immunoglobulin preparation affected function (Pollack 1971). In this experiment, volunteers (200 men 2 post menopausal women, aged 20-65 years, mean age 37 years) were protected with a standard dose of anti-D approximating to 180IU were separated into 6 groups, each with an untreated control, and challenged with RhD+ whole blood doses ranging from 11.6-37.5mL (Pollack 1971).The results confirmed that antibody response was dose dependent, as was AMIS with volunteers receiving the largest dose red cells being the most frequently alloimmunised (untreated group), and least protected (treated group). Interestingly the authors speculate in their discussion that a mechanism other than simple antigen destruction was in operation.

Routine post partum anti-D prophylaxis was introduced in the United Kingdom through the early 1970s for RhD- women bearing RhD+ babies, with a significant reduction in the morbidity and mortality rates from HDFN (Tovey 1978). The incidence of Rh alloimmunisation was reduced from 17% to approximately 1% in less than a decade (Urbaniak 1998, Tovey 1978).

Many authors comment on the continued residual alloimmunisation rates. Failure to prevent alloimmunisation includes cases in which anti-D was not given after abortion, representing a third of all new cases, or failure to determine and report the D group of the baby (Tovey 1978, Clarke 1983, Urbaniak 1985). Guidance was subsequently issued that anti-D should be offered after all abortions, spontaneous or therapeutic and other specified potentially sensitising episodes during pregnancy. These include: invasive prenatal diagnostic techniques such as amniocentesis, ante partum haemorrhage, and external cephalic version to correct breech presentation. Standard doses of 250IU for pregnancies before 20 weeks gestation, and 500IU thereafter were specified based on maximum bleed size associated with fetal blood volume (BCSH guidelines 2006a). A small proportion of FMH are larger than 4mL and hence require larger doses of anti-D to be protective. Failure to recognise these events, and act accordingly, accounts for some cases of alloimmunisation. The rate of decline of clinical HDFN slowed through the 1970s. Some recurring cases related to women who had been immunised before the introduction of post-natal prophylaxis (Tovey 1978), others to continued failure of prophylaxis.

The residual alloimmunisation rate continued to concern clinicians. The consensus conference of the Royal Colleges of the Physicians of Edinburgh and of Obstetrics and Gynaecology in1997 made a statement addressing the problem and potential solutions. In that statement the successes of post natal prophylaxis are celebrated, though the lack of awareness of guidance among patients and some clinicians, and the failure systematically to follow established protocols, was highlighted. Importantly, despite an acknowledged lack of evidence of the scientific or economical benefit, the expert opinion was strongly in favour of adoption of routine antenatal prophylaxis as a means of further reducing the alloimmunisation rate among RhD- women (Royal College of Obstetricians and Gynaecologists Consensus statement 1997).

Routine postnatal prophylaxis was introduced in the UK in the early 1970s Thirty years later, the childbearing span of a generation of women, routine antenatal prophylaxis was recommended by NICE-the National Institute for

Clinical Excellence (NICE 2002). The NICE assessments committee undertook a meta-analysis of 8 studies in which women were given 500IU of anti-D at 28 and 32 weeks (4 studies), 1500IU at 28 weeks only (3 studies) or 1500IU at 28 and 34 weeks (1 study). The studies included 5 non-randomised trials, and 5 trials which used historical control data with the attendant risk of overestimating the effect of anti-D prophylaxis because of the beneficial effect of improved clinical care. The committee concluded from sensitisation rates that the regimes were equally effective and recommended both for routine practice. A subsequent review in 2008 used the same data augmented with a single randomised controlled trial (RCT) that was designed to investigate the benefit of IV rather than IM administration of a single 1500IU dose. Based on this review the committee recommended consideration of cost effectiveness in choosing between the approaches. The committee also commented that their decision was based on financial evidence in the absence of clinical data, and that an RCT should be undertaken to investigate the relative merits of anti-D administration protocols. Modelling of the effect on mortality in the UK based on these analyses, indicate that deaths from HDFN might be reduced from approximately 27 per year to approximately 10 (NICE 2008).

### 1.7.3 The Use of Monoclonal Anti-D for Prevention of Alloimmunisation

Though the supply of anti-D is adequate in the UK and North America, some countries have ceased production of anti-D immunoglobulin on the grounds of cost (Bowman 2003, Kumpel 2007). The alternative is a monoclonal or recombinant anti-D preparation. The potential benefits to patient safety and securing adequate supplies are significant. The first anti-D immunoglobulin preparations used plasma from alloimmunised women as source material; indeed, plasma from women undergoing apheresis procedures in which their own plasma was exchanged with that of blood donors was used in some centres. To this extent the anti-D prophylaxis programme has been a victim of its own success. Fewer women are alloimmunised and, as the existing donors retired, they were replaced by volunteer blood donors who were deliberately alloimmunised with cells from accredited RhD+ donors (Urbaniak 1981). Ethical concerns about this process (Tovey 1990), lead to bans in

some European countries (Beliard 2006) but these concerns were overtaken in the UK by the presence of variant Creutzfeld-Jacob Disease (vCJD) in the donor population and the associated risk of transmission via transfusion. As a result all UK plasma for fractionation, including the production of anti-D immunoglobulin is sourced from the USA. The discovery of vCJD in American cattle further threatens the ongoing supply of anti-D (Hall 2005). Though anti-D is regarded as a safe product, there remains a finite risk associated with, as yet, unknown pathogens, from the infusion of any product of human origin, as demonstrated by Hepatitis C infections associated with anti-D immunoglobulin in Eire, before donor screening was available (Power 1995).

In view of these benefits and not inconsiderable commercial interest there have been many *in vitro* and clinical trials of monoclonal and recombinant anti-D products. Studies include the injection of autologous $^{51}Cr$ labelled red cells coated with anti-D to male RhD+ volunteers. In these trials monoclonal antibodies have been shown to clear red cells, but more slowly than polyclonal anti-D preparations. Similarly monoclonal anti-D administered to D- volunteers cleared $^{51}Cr$ labelled D+ cells, unless administered at saturating doses clearance was slow. In this study 0 of 24 participants were alloimmunised (Beliard 2006). A further study recruited 95 male D- volunteers who were injected with $^{51}Cr$ labelled D+ red cells 24 hours before a mixture of IgG1 and IgG3 monoclonal antibodies (BRAD-5 and BRAD-3). One participant was alloimmunised to the D antigen at 12 weeks. To confirm that the participants could mount a response to D+ cells the remainder were given further D+ cells 24 and 36 weeks later. One participant mounted a strong response to the second challenge by 28 weeks, suggesting that the monoclonal anti-D had not prevented a primary response. Only 24% of the cohort was alloimmunised after two unprotected challenges with D+ red cells. This low figure was taken by the authors to indicate the possibility of long term protection afforded by the anti-D treatments (Kumpel 2002).Both alloimmunised participants had cleared the D+ cells within 3 days of administration (Kumpel 2007).

An assumption made by workers undertaking trials is that the ability to mediate rapid red cell clearance is the key property of monoclonal anti-D in effecting AMIS (Kumpel 2002, 2007, Beliard 2006). The important characteristic of antibodies useful in AMIS is the immunosuppressive effect. Overall the results of these trials, examining protection from immunisation rather than clearance, have been disappointing, with many preparations failing to clear red cells *in vivo*, or doing so slowly, or variably. Some antibodies appear to have enhanced alloimmunisation; none to date have been as effective as human polyclonal preparations (Kumpel 2007). Monoclonal and recombinant antibodies differ from polyclonal IgG in the degree and quality of glycosylation, and sialylation. Potentially adverse effects on the interaction with FcR on effector cells and on mediation of inflammation as an enhancement of the humoral response have been attributed to these characteristics (Kumpel 2007, Kaveri 2008).

Anti-D in association with HDFN prophylaxis is the most widely applied example of the therapeutic administration of exogenous immunoglobulin Beliard 2006). Other specific therapies include anti-tetanus or anti-rabies products which may be administered prophylactically or in response to specific exposures to infective agents and are effective in preventing disease onset (Kobayashi 1996).

### 1.7.4 IgG in Therapeutic Immunomodulation

Intravenous Immunoglobulin (IVIg) has long been used to treat primary or secondary immune deficiencies including ataxia telangectasia, Wiskott Aldrich Syndrome and DiGeorge Syndrome (Mazer 2005). Since the early 1980s, IVIg has, in addition, been used to treat other conditions associated with allo- or auto-immune reactivity or systemic inflammatory states. These include Immune Thrombocytopaenic Purpura, Guillaine-Barré syndrome, Myasthenia Gravis and Kawasaki syndrome (Ephrem 2005). The effectiveness of these treatments is supported by clinical data, but there is some clinical and experimental evidence to suggest that IVIg might be therapeutic in other diseases of the immune system (Ephrem 2005). The range of functions of

circulating normal immunoglobulin is wide and diverse and that is reflected in the range of mechanisms by which IVIg has been shown, or is considered to exert its therapeutic effects. These effects are non-mutually exclusive and are considered to be synergistic (Tha-In 2008).

IVIg is prepared from pools of plasma from thousands to tens of thousands of blood donors, and contains immunoglobulin expressing a wide array of the variable and constant regions and of immunoglobulin isotypes expressed on a population scale (Mazer 2005). Preparations consist mainly of IgG, with smaller amounts of IgM and IgA, reflecting the characteristics and levels of circulating immunoglobulin in the donor population (Tha-In 2008). In autoimmune cases IVIg may be administered in doses up to 2.0g/kg leading to serum levels of 5-25 mg/mL (Dussault 2008) consistent with the normal concentration of 8-16mg/mL (Roitt 2001). In antibody deficiency cases, lower doses of 400-600 mg/kg are used (Mazer 2005). A high proportion of the antibody molecules are of self-reactive specificities associated with the regulation of self and non-self recognition, destruction of senescent cells, and maintenance of the idiotype-anti-idiotype network (Ephrem 2005).

The mechanisms of action of IVIg are both unclear and controversial. Many of the disease states which can be treated exist in an inflammatory immune environment. Licensed use includes Kawasaki Disease, chronic inflammatory demyelinating polyneuropathy, and Guillain-Barre syndrome, though "off-label" applications are increasing resulting in short supply, and increasing product price (Ascherman 2009).

There is evidence from clinical and *in vitro* studies that IgG has an anti-inflammatory effect through:

- modulation of FcR expression
- T and B cell activation,
- anti-idiotype antibodies, and IgG dimers,
- the induction of anti-inflammatory cytokines
- the inhibition of IFN-$\gamma$

(Ephrem 2005, Clynes 2007) .Though the complexity and number of putative mechanisms is challenging, it does reflect the diversity of function of circulating, native immunoglobulins. The presence of soluble HLA class I and II molecules and CD40 in IVIg preparations may interfere with antigen recognition by T and B lymphocytes and subsequent co-stimulation. Immunomodulation of B lymphocytes by IVIg has been shown to be via phosphorylation of intracellular signalling kinases (Dussault 2007). *In vitro* studies have demonstrated down regulation of expression of co-stimulatory molecules on DCs by exogenous IgG resulting in reduced secretion of IL-12 (Ephrem 2005).

There is some evidence that the anti-inflammatory effect of IgG is attributable to a fraction of IgG which expresses a terminal sialic acid residue on asparginine 297 linked glycan. This has been demonstrated experimentally both with IgG recovered from plasma pools made from blood donations, and with a recombinant IgG1 fraction. The immunomodulatory effect appears to occur through up-regulated expression of the inhibitory FcR$\gamma$IIB, or may reflect the presence of a novel receptor for sialic residues on IgG (Nimmerjahn 2008a).

In a study using a mouse model, IVIg was demonstrated to have multiple immunosuppressive effects. Mice immunised with ovalbumin and treated with IVIg were shown to generate fewer splenic antigen specific T cells, as measured by cytokine release than mice in an untreated control group. The authors also demonstrated lower titres of antibodies to ovalbumin in the treatment groups, and speculate that this may be the result of reduced T-cell activation, Using *ex vivo* APC and T cells, the author demonstrated almost complete inhibition of T cell response to APC previously treated with IVIg suggesting that the mode of action is mediated through APC rather than directly on T cells (Aubin 2010).

The potential for improving the cost, risk and effectiveness of IgG therapies if recombinant product can successfully applied clinically is significant (Kaveri 2008).

### 1.7.5 Use of High dose IVIg in Alloimmunised Pregnancies

An alternative application for IVIg is in improving fetal cytopaenias, by administration to alloimmunised women who are at high risk of having affected pregnancies due to HDFN, or neonatal alloimmune thrombocytopaenia (Urbaniak 1999, Gottstein 2003). The practice is considered less risky than alternative treatments such as early delivery, IUT, or exchange transfusion at delivery, particularly as administered by those unfamiliar with the practice. Some workers (Gottval 1995) report good results, with concentrations of maternal alloimmune anti-D apparently being controlled despite repeated cordocentesis procedures, and better conservation of fetal haemoglobin levels than in an untreated control group. The authors report similar ratios of maternal to fetal serum anti-D levels, and conclude that placental transfer of IgG is unaffected by the therapy and the means of action is fetal Fc blockage by the exogenous IVIg. Other authors report reduced requirement for exchange transfusion, with the associated extended hospital treatment (Gottstein 2003). There is some *in vitro* experimental evidence that IVIg preparations can inhibit placental transfer of anti-D (Urbaniak 1999a). Some workers have administered IVIG directly to babies soon after delivery, with similar beneficial effects on reducing the need for exchange transfusion (Gottstein 2003, Miqdad 2004).

Though offering a limited insight into possible mechanisms of AMIS when high doses of antibody are used, these studies are small scale, with mixed results. Improvements in the safety and efficiency of ultrasound guided IUT (Abdel Fattah 2002) mean that the use of IVIg in treating HDFN has not been widely adopted.

### 1.7.6 The Use of Intravenous Anti-D to Treat Immune Thrombocytopaenic Purpura

The role of IV anti-D in the treatment of immune thrombocytopaenic purpura is similarly celebrated as an effective therapy, but authors continue to debate the means of action. The treatment has been in use since 1983, and theories on the mechanism of action include: FcR blockade, anti-idiotype regulation, and modulation of cytokine networks (Semple 2008). Fc blockade is supported by the evidence that anti-D therapy is only effective in RhD+ patients. IgG anti-D bound in multiple copies to D+ red cells might conceivably block and/or cross-link FcR on the patient's phagocytic cells, thereby preventing destruction of platelets.

In a study investigating the effect on serum cytokines of anti-D treatment of ITP, 7 patients were given 50μg/kg of anti-D when their platelet counts were below $30x10^9$/L. Blood samples were collected before treatment, and 3 hours and 8 days afterwards. Significant rises in the concentrations of both pro- and anti-inflammatory cytokines (IL-1ra, IL-6, GM-CSF, MCP-1α, and TNF-α) was noted at 3 hours, with return to baseline at 8 days after treatment. The authors suggest that interaction between IgG opsonised red cells and FcγR induce a cytokine storm which downregulates the function of the reticulo-endothelial system (Semple 2002).

In another study (Cooper 2004), 27 ITP patients were administered 50-75μg/kg of anti-D in response to low platelet counts; samples were taken before treatment, and 2 hours, 24 hours, and 7 days after treatment. In 24 of this cohort, polymorphisms in FcγIIR (HH/HR) and FcγIIIR (VF/VV) were determined. Increases in the concentrations of the 4 analytes measured were noted at 2 hours (IL-6, IL-10, MCP-1, TNF-α). Higher responses were recorded in individuals expressing HH FcγIIR than HR, and higher platelet increments in individuals expressing VF FcγIIIR than VV. In a cohort receiving IVIg (1g/kg), only IL-10 showed an increase at 2 hours. The authors

concluded that IVIg and anti-D relieve ITP by different mechanisms and that anti-D bound to D+ red cells interacts with the patients Fc receptors.

The pathology of transfusion reaction is analogous to AMIS by anti-D in D+ patients, and some of the clinical features of transfusion reactions, particularly fever and rigors have been associated with the release of cytokines and are common to both groups of patients. Pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IL-8 were detected in monocytes challenged with D+ red cells in an *in vitro* model (Davenport 1993). The same cytokines were detected at elevated concentrations in a patient undergoing a delayed haemolytic transfusion reaction due to anti-D (von Zabern 1998).

### 1.7.7 The Mechanism of Action of Anti-D: Antibody Mediated Immunosupression.

That anti-D prophylaxis is effective in suppressing Rh alloimmunisation is clear, though in common with other immunoglobulin therapies, the mechanism underpinning this suppression is not. The parallel chronologies of anti-D prophylaxis, the advance of the science of immunology, and the tightening control on studies involving human participants have contributed to our lack of understanding of the mechanism of antibody mediated immunosuppression (AMIS). Two measures of the efficacy of anti-D prophylaxis have been widely applied: the ability to clear fetal D+ red cells rapidly from the maternal circulation, and the prevention of maternal alloimmunisation. For many practitioners and some scientists, the link is perceived to be directly causal, though there is little direct evidence to support the hypothesis.

#### 1.7.7.1 The Clearance of RhD+ Red Cells

It is suggested that anti-D antibody binding to RhD antigen on fetal red cells causes clearance of cells expressing alloantigens, before they can be recognised by the immune system (Brinc 2009). In fact anti-D coated fetal red cells are cleared to the maternal spleen, a secondary lymphoid organ which is

the chief site supporting humoral immune responses to blood borne antigens (Batista 2009, Kumpel 2002). Trafficking of alloantigen positive cells to the spleen is likely to enhance rather than suppress the humoral response in the absence of other other suppressive mechanisms. This theory of antigen deviation away from main immunogenic sites is consistent with the protection offered by fetomaternal ABO incompatibility. In this case maternal ABO IgM isoantibodies effect destruction of fetal cells either by complement mediated lysis in the intravascular compartment or by phagocytosis in the liver where there are relatively few APC (Kumpel 2002). Other evidence from animal models suggest that AMIS correlates more closely with the quantity of IgG bound to target cells, and to its ability to interact with FcR rather than the rate of cell clearance (Heyman 1984).

Small FMHs appear to need clearance within 5 days to avoid alloimmunisation (Mollison 1984). There is some evidence that in larger bleeds, or sensitising episodes associated with inadvertent transfusion of RhD+ blood to RhD- patients, clearance facilitated by large doses of anti-D immunoglobulin can take much longer without alloimmunisation occurring (Lubenko 1999, Mollison 1984). These case reports are based on small cohorts of patients and, experimental confirmation of these findings would be problematic.

### 1.7.7.2 The Suppression of the Humoral Response by Antigen Blocking

This theory demands that exogenous anti-D binding to fetal RhD+ red cells does so in sufficient quantity to prevent interaction with B lymphocytes. It is clear that AMIS is dose dependent, with too small a dose being ineffective (Pollack 1971, Mollison 1984). There are estimated to be 10-40,000 RhD antigen site per red cell (Daniels 2002). As little as 20μg of anti-D can effect AMIS against 1mL of D+ red cells containing $10^{10}$ red cells and $10^{14}$ D antigens. Under these conditions it has been calculated that fewer than 5% of D sites are blocked (Klein 2005). In the context of the small volumes of red cells required to elicit an alloimmune response to the D antigen, it is hard to

see how routine doses of anti-D immunoglobulin could block sufficient antigen sites to prevent an immune response by the mechanism of steric hindrance. Further, in animal models, the use of Fab and $Fab_2$ fragments fails to suppress alloimmunisation, whereas whole IgG with Fc region intact does (Urbaniak 2000). It has been demonstrated that the effect is particle, rather than epitope specific by experiments in which alloimmunisation to the RhD antigen was suppressed in volunteers injected with D+K+ red cells and potent anti-K. The K antigen is co-expressed with RhD on red cells but much less densely with 2-5,000 sites per cell (Woodrow 1975).

### 1.7.7.3 Down-Regulation of B lymphocytes

At least one author (Kumpel 2002) favours the theory that immunosuppression is the result of down regulation of B cells. The proposed mode of action is co-ligation of multiple RhD antigens on red cells with the BCR, and the Fc portion of red cell bound anti-D with the FcγRIIb IgG receptor which is also expressed on B lymphocytes. Interaction between the BCR and antigen is stimulatory through tyrosine based activation motifs in the cytoplasmic domains of proteins associated with the BCR. When phosphorylated, this motif activates a signalling pathway which results in B cell activation, proliferation and, ultimately, production of antigen specific antibody. Where co-ligation occurs, tyrosine based inhibitory motifs activate an inhibitory signal resulting in down-regulation of the B lymphocyte response and possibly apoptosis (Kumpel 2002). The argument is supported by some *in vitro* studies, and by the evidence that immunosuppression is effected by whole IgG but not $F(ab')_2$ portions (Brinc 2009) reinforcing the role of the Fc portion of IgG. In contrast experiments using $Fc\gamma RIIB^{-/-}$ mice have demonstrated that the inhibitory FcγRIIB is not required for AMIS to be effective, suggesting that more than one mechanism in effective (Heyman 2003, Brinc 2009). IgG3 has been shown to be an effective AMIS agent, but does not bind to FcγRII (Heyman 2003). Though FcγRII B may have a role in immunosuppression, there is good evidence that this is not the sole mechanism.

Experiments using mice injected with sheep red blood cells as a model of AMIS showed that B cells from mice protected with anti-sheep antibodies were much less effective than those from untreated mice in presenting sheep red blood cells to T cells *in vitro*, indicating inhibition of B cell priming *in vivo*. The same authors report no significant effect on T cell priming in the same model. Caution must be exercised in extrapolating the results obtained in murine *in vitro* or *ex vivo* studies into a human clinical setting (Brinc 2008).

Alternative hypotheses, involving the inhibition of B lymphocytes by IgG opsonisation of red cells, include prevention of B-cell spreading and synapse formation with APC, and that bound IgG prevents MHC II presentation of antigen, internalised by B lymphocytes (Brinc 2009).

This collection of hypotheses to explain a clinically demonstrable effect has strong analogies with the ongoing debate on the mechanism by which IVIG is effective in modulating a number of inflammatory autoimmune diseases, and the mechanism by which IV anti-D improves platelet counts in patients with Immune Thrombocytopaenic Purpura (ITP). Perhaps as a result of this, some researchers have focussed on the effect that anti-D administration in pregnancy has on maternal cytokine profiles (Branch 2006). The authors, noting the documented effect of IV anti-D on cytokine profiles when used to treat ITP, undertook a small study on a cohort of pregnant women at the time that anti-D prophylaxis was administered.

In this study, ten women between 28 and 32 weeks gestation were recruited who were known either to be carrying a D+ fetus or to have a D+ partner. Samples of whole blood were collected before and 48 hours after intramuscular administration of 1500IU of anti-D antibody (WinRho, Cangene Corp. Winnipeg, Canada). The levels of 17 different inflammatory mediators in the participant's plasma were measured by Enzyme Linked Immunosorbent Assay (ELISA), of which 10 were below the detectable level in all samples tested and 4 others showed no change. Of the three remaining analytes IL-1ra showed a moderate increase after treatment in 7 of the 10 women, though this was not statistically significant, and 2 analytes ($PGE_2$, and TGF-

β1) showed significant increases with 5/10 women giving 1.5 or greater fold increase in PGE-2, and 7/10 volunteers having 1.3 or greater fold increase in TGF-β1. Of these, one woman had a >5 fold increase.

The authors suggest a causal link between administration of exogenous anti-D and changed anti-inflammatory cytokine profiles, and in particular highlight the recognised role of TGF-β1 in modulating humoral immune responses. The study of Branch et al (2006) had a small cohort of participants, with no control group. The time series involved just two points before and after treatment, and there was no attempt to correlate the presence or absence of fetal D+ cells in the maternal circulation with any observed effect. Nonetheless the findings point to the potential to further investigate a causal link between anti-D administration and changed cytokine profiles, which may offer an insight into the mechanism by which AMIS operates.

### 1.7.8 Cytokines and Control of the Immune System

Cytokines are small soluble proteins which can be produced by almost any of the nucleated cells in the body and may act on in an autocrine or paracrine manner (Mantovani 2000, Janeway 2001). Cellular interaction of the immune system is regulated by cross-talk, mediated by a system of cytokines, which are released in response to different stimuli by a range of cell types, and can be inhibitory or synergistic (Theze 1999) and the effect on cell growth, differentiation, function and death are complex and variable (Mantovani 2000). Functions of cytokines involve driving T cell responses and coordinating the immune response between the cell mediated, and humoral arms. Individual cytokines may have a range of functions, and some effects may be achieved by alternative cytokines, offering flexibility and versatility in the immune response by redundancy of cytokine function. For example, B cell antibody production is driven by IL-2 but even in the absence of IL-2, IL-4 and-10 can stimulate immunoglobulin synthesis (Theze 1999). Changes in cytokine expression both locally at the site of implantation, and more generally in the maternal immune response, are key to establishment and maintenance of successful pregnancy (see section 1.1.4). That cytokine profiles change in

response to exogenous IgG is well documented (Sewell 1999, Semple 2008), and the interaction between anti-D and red cell antigens has also been reported to result in a powerful cytokine response (Zabern 1998). Of particular interest is the evidence that TGF-β1 expression is altered by the administration of exogenous anti-D antibody (Branch 2006).

TGF-β is a 25kDA homodimer which is highly conserved among mammalian species (Lawrence 2001), the evolution of the TGF-β system can be traced to the split of arthropods from vertebrates more than 1 billion years ago (Li 2006). TGF-β1 is secreted by Type 1 regulatory T cells and Th3 lymphocytes, which are induced from primed CD4+ lymphocytes and possibly naïve lymphocytes, when activated by appropriate antigen (Taylor 2006).

Three forms of TGF-β are expressed in humans: TGF-β1, TGF-β2 and TGF-β3, of these, TGF-β1 is predominant in the immune system, though the *in vitro* functions of all three forms are similar. TGF-β is secreted by almost all nucleated cells as high molecular weight, latent complexes (Lawrence 2001), with latency associated protein, and in some cases latent TGF-β binding protein (Li 2006). Secretion as a latent complex is an effective mechanism to control TGF-β biology, averting damaging pathologies associated with uncontrolled active TGF-β. In addition, the release of latent complex increases the circulating half life of TGF-β from two to ninety minutes (Lawrence 2001). Activation can be achieved *in vitro* by heating, or low pH (Li 2006). *In vivo* this occurs by the action of cell surface proteases, such as plasmin, cathepsin, and metalloproteinases, that cleave the active form of TGF-β from the associated protein (Lawrence 2001).

TGF-β is a potent and versatile mediator of immunosuppression, instigating and maintaining immune tolerance, with diverse effects on hemopoeitic cells (Li 2006, Wahl 2006). The effects are dose, cell type, and context dependent and in many cases are modulated by other cytokines (McKarns 2003). In addition, TGF-β has an important role in the repair of tissue damage, released by platelets at the site of injury or infection acting as a chemoattractant for monocytes, lymphocytes and fibroblasts, and stimulating the deposition of

extracellular matrix (McKarns 2003, Wahl 2004). The effect of TGF-β1 is modulated by the presence of inflammatory cytokines including (IFN-γ), and signalling derived from co-stimulatory molecules such as CD28 and CD40, which results in control over immune response to self or non-harmful antigens whilst permitting immune surveillance or, and response to pathogens. TGF-β1 has a profound effect on T lymphocytes including proliferation: differentiation and survival, especially anti-proliferatitve effects on naïve T cells. The effects are dependent also on co-stimulation, the state of differentiation of the target cells, and the presence of inflammatory cytokines, so that the final outcome of the action of TGF-β1 may very according to the immunological environment (Li 2006). Antigen presentation by macrophages is inhibited by TGF-β1 by reduction of IFN-γ induced MHC class II expression (Wahl 2006). In addition TGF-β1 inhibits the expression of co-stimulatory molecule CD40 and IL-12p40, which together reduce the antigen presenting effectiveness of macrophages (Li 2006).The effect of TGF-β1 on B cells is analogous to that on T cells, with powerful inhibitory effect on proliferation, activation and antibody class switching to isotypes other than IgA and IgG2 (Li 2006, Taylor 2006).

The most important receptor for TGF-β is TGFβRII. Signalling occurs through associated activin receptor-like kinases (ALK-5 and ALK-1), the cytoplasmic domains of which directly phosphorylate Smad signalling proteins which in turn form an enzyme cascade leading to regulation of transcription of target genes (Li 2006). The effect of TGF-β on T cell proliferation is generally suppressive , through the down regulation of IL-2, though TGF-β induces the differentiation of naïve T cells to Foxp3+ T reg cells, which themselves produce TGF-β on exposure to epitopes of α-fetoprotein (Alisa 2008). Th3 cells express TGF-β and drive oral tolerance, and class switching of immunoglobulin synthesis to IgA (Li 2006). B cell proliferation and differentiation are inhibited by TGF-β, and maturation of differentiated DCs is similarly regulated (Li 2006).

IgG and TGF-β can form a complex, which has a powerful suppressive effect on B cell proliferation (Bouchard 1995). TGF-β1 IgG complexes have been reported with an immunosuppressive effect estimated as 500 fold that of unbound cytokine, and it has been proposed that the conformational change required for binding is effective in activating TGF-β (Lawrence 2001).

Our understanding of the mechanism of anti-D AMIS is limited, further evidence of the effect of the treatment on cytokine profiles, and how that might mediate immunosuppression will improve our knowledge. In this study the authors intend to generate new knowledge in a quasi-experimental inquiry including a control group, an extended time series, and to seek evidence of a link between detectable FMH and cytokine response following the administration of anti-D.

### 1.8. Aims, Hypotheses and Objectives

This study aims to investigate the effect of anti-D prophylaxis on the expression of cytokines in the maternal circulation, and that any alteration is dependent on the presence of fetal RhD+ red cells.

Two pairs of hypotheses are presented:

**Hypothesis 1**

$H_o$ There is no significant difference in cytokine expression in maternal circulation before and after anti-D prophylaxis

and

$H_1$ There is a significant difference in cytokine expression in maternal circulation before and after anti-D prophylaxis

**Hypothesis 2**

$H_o$ There is no significant difference in cytokine expression in maternal circulation after anti-D prophylaxis in the presence or absence of D+ cells and

$H_1$ There is a significant difference in cytokine expression in maternal circulation after anti-D prophylaxis in the presence or absence of D+ cells.

Thus the objectives of this study were to:

1) Recruit up to 30 women to participate
2) Determine the role of fetal RhD group by establishing the group, and measuring the presence of fetal D+ cells in the maternal circulation
3) Measure cytokines before and after anti-D prophylaxis.

# Chapter 2 Materials and Methods

## 2.1 Introduction

Experimental inquiry, generally in the form of randomised controlled trials, is regarded as the best approach by which to establish causal relationships in healthcare research. In true experiments, the researcher identifies a variable within a system then manipulates it and measures the effect on other variables. The internal validity of the inquiry is maintained by the use of control groups in which the treatment is withheld. Participants are allocated randomly to either test or control group to avoid accidental or deliberate bias in the samples, which might produce results that are misleading and cannot be generalised to the population from which the sample is drawn (Field 2003).

In some circumstances, including those pertaining to the current study, true experiments in which the investigator is in full control of the variables cannot, for logistical and ethical reasons, be undertaken. NICE guidance is clear that all D- women must be offered prophylaxis at 28 weeks gestation, and although a small number of women choose not to accept treatment, forming a cohort from these would be logistically challenging, and ethically questionable. There are alternatives to in vivo human studies in many cases. Animal models and in vitro experimental models might be applied to gain the advantages of internal validity associated with experimental inquiry, without the ethical problems associated with withholding proven prophylaxis to patients. (Mollison 1984).

The animal models used in AMIS experimentation have limitations however, in that significant differences between animal and human immunology prevent generalisation of results. Mice, for example have no blood groups, and challenge with non-mouse erythrocytes leads to rapid complement mediated destruction, unlike the slow lymphocyte mediated response in human Rh alloimmunisation (Kumpel 2006). The immunology of human pregnancy is exquisitely complex (Weetman 1999) and no adequate *in vitro* model has been created, which allows true experimental research on the immunology of

pregnancy, without serious doubts as to how the results apply to the in vivo condition.

For these reasons a quasi-experimental methodology was selected to allow investigation of the in vivo effects of anti-D administration in human participants, rather than accept the confounding variables associated with in vitro or animal models, in this complex immunological environment.
In this model, the researcher relinquishes control over a number of variables, including the timing of the experimental manipulation and the random assortment of patients into test or control groups. Quasi-experimental investigation offers advantages over true experimental research in supporting investigation when participants cannot be allocated randomly to treatment, or non-treatment groups by researchers such as the current study. The use of *in vivo* models facilitated by quasi-experiment reduces the risk of results being adversely affected by the artifice of laboratory investigation. The approach does bring limitations to the study, being less effective than true experimental research in establishing causal relationships between treatment and effect, as non-random assignment of participants to groups risks the associated allocation of confounding variables, beyond the control of the researcher. (Field 2003).

## 2.2 Study design and Ethical Approval

### 2.2.1 Ethical approval

This study required the collection and testing of peripheral blood samples from pregnant women being treated in the NHS, beyond that required in standard care protocols. As a result Research Ethics Committee approval was sought from York Local Research Ethics Committee and granted. (REC reference number 08/H131/22, copy of approval in Appendix I). NHS R&D approval was granted by Leeds Teaching Hospitals (Appendix II).

### 2.2.2 Selection of Women for Test and Control Groups of the Study

The purpose of this study was to investigate the mechanisms underlying antibody mediated immune suppression caused by anti-D prophylaxis. This effect occurs only when D- mothers carry D+ fetuses. Approximately 40% of D- mothers carry D- fetuses, with no risk of alloimmunisation or any possible specific immunomodulation of the maternal response to fetal red cells, as they are D-. Use of a placebo group of D+ pregnant women to which saline was administered was considered, and ethical approval obtained. This group would have comprised women attending antenatal clinic at 28 weeks gestation for glucose tolerance tests. It is acknowledged that inclusion of a placebo control would have offered evidence of non-specific alteration of cytokine expression, induced, for example by phlebotomy, but as that was not the primary research question, and any non-specific reaction would be detectable in the control arm, a placebo group was not included. The use of women bearing D- fetuses was considered a better model of the immune process, being more similar to the test arm, and as the D group status of participants' babies was not known and could not be predicted at the time of recruitment, offered better randomisation. Accordingly patients were allocated to test and control groups of the study based on fetal blood group, described from this point as "D+ fetus group" and D- fetus group". The fetal blood group is determined routinely at delivery by standard automated blood grouping technologies when D- women deliver, to support appropriate anti-D prophylaxis, treatment is not being offered to the mothers of D- babies (BCSH 2006). The information required for this allocation was obtained from the records of Leeds Teaching Hospitals after completion of recruitment and testing phases of the study.

### 2.2.3 Calculation of sample size

The raw data from Branch's 2006 work on cytokine profiles were obtained to inform the current study (Donald Branch personal communication). The data (n=10) were demonstrated to be normally distributed using the Anderson-Darling test., and detected fold changes were found to have a standard

deviation of 1.43. A power calculation to determine sample size required to detect a 1.5 fold difference in cytokine concentration in pre- and post-treatment samples with a power of 90% and a significance level of 5%, using Minitab software (Minitab Ltd, Coventry, UK). the minimum sample size required was found to be 15. It was anticipated that 40% of recruited participants would have D- fetuses and therefore would not be included in this test group. Accordingly a minimum cohort size was set at 24, in the expectation that 15 participants would be recruited to the D+ fetus group and 9 to the D- fetus group. Numbers of participants recruited to each group are reported in section 3.1.

### 2.2.4 Criteria for Inclusion in the Study

Pregnant RhD- women are routinely invited to anti-D clinics at St James' University Hospital, Leeds, at gestation between 28 and 32 weeks. All attendees at 24 clinics were invited to participate in the study, and no other selection criteria including age, ethnicity, parity, or health were considered. It is acknowledged that this strategy may result in the introduction of confounding variables. It is not intended that the generalisation is extended to individuals who are outside the core selection criteria of pregnant women, and 28 weeks gestation receiving anti-D prophylaxis. The value of the study would be enhanced if the results were applicable to all individuals meeting those criteria. It must be recognised that other variables in other patient groups, in particular route of administration, dose size or source of anti-D might limit the generalisation of the results of this study (Field 2003).

### 2.2.5 Timing of Samples

The short duration of the time series of this study (approximately 24 hours) offers some advantages in avoiding some confounding variables associated with longer time series. Participants once recruited and informed of the nature of the study are unlikely in the short time of the investigation to be exposed to factors, either associated with their own circumstances, publicly available information, or the study itself, which might cause them to withdraw from the

study. Longer time series are much more likely to be subject to these problems (Field 2003).

The administration of routine anti-D prophylaxis by the intravenous route, was unusual at the time of the current study, although the practice is increasing, following NICE guidance update (NICE 2008), indicating the need for cost effective prophylaxis. Conventionally anti-D is administered through the intramuscular route. The advantages of IV administration are largely logistical and financial, but in this study IV administration ensures that the kinetics of anti-D dispersal through the maternal circulation are more predictable, along with the timing of any cytokine burst, which may be a response to IgG administration is likely to be both rapid, and short lived (Semple 2002, Malinowska 2001).

### 2.2.6 Testing Strategy

Testing the hypotheses which define this study requires estimation of cytokine release in test and control groups, the detection of low levels of fetal D+ cells in the circulation of participants, and correlation of the data gathered.
For cytokine estimation, a strategy was selected which involved screening plasma for a panel of cytokines by flow cytometry (FC). This method was selected as rapid, sensitive and cost effective for investigating a number of analytes. The second stage of the strategy was to use FC data, or that from previous studies (Branch 2006) to identify specific cytokines for further analysis by ELISA. These data were then used to select analytes for investigation by real time quantitative PCR (q PCR) on samples of peripheral blood.

### 2.2.7 Selection of Analytes

A range of analytes was selected based on literature reports indicating modulation by anti-D AMIS IL-1ra, TGF-β1, IL-12p70 (Branch 2006), modulation by IVIg TNF-α, IL-10, IL-6 (Cooper 2004), IFN-γ (Clynes 2007),

as well as IL-1β, and IL-17 as key pro-inflammatory cytokines, and IL-4 for its role in Th2 type immunity (Fazilleau 2007).

### 2.2.8 Recruitment of Participants to the Study

Lists of women invited to anti-D administration clinics at St James' University Hospital in Leeds, were obtained from the clinic administrators 3-6 weeks prior to each appointment. From the details on the list, patient addresses were obtained using the NHS patient tracing system. The study information leaflet (Appendix III) and Participant information sheet (appendix IV) was sent by mail to each potential participant, at least one week before the scheduled clinic visit. The recruitment leaflet was designed to inform potential participants of the study in a concise format, giving an indication of the aims of the research, and what would be required of participants. The participant information sheet offered greater detail on the nature of the study, bothe documents were included in the ethics committee application. Contact details for the chief investigator were included. This gave potential participants 3-6 weeks to consider whether to join the study or seek additional information.

One hundred and three potential participants were sent leaflets and information sheets, of whom seven contacted the chief investigator before their clinic appointment indicating willingness to participate, all of whom did so. The remaining seventeen participants agreed to join the study at the clinic. Of the women who agreed to participate in the study, all gave a full set of samples, all of which were of sufficient volume to test.

At the clinic, the study was explained by the chief investigator to each potential participant in the presence of a midwife, with an invitation to join the study. A copy of the leaflet and the participant information sheet was offered. In discussion with potential participants it was stressed that participation was voluntary, participants could withdraw at any stage, and that that the decision whether to join the study would not affect their care. Women agreeing to participate completed 2 copies of the consent form (Appendix IV), one copy

being retained by the chief investigator in the research site file, the second being stored in the patient held notes.

A full antenatal check was performed by the midwife on each woman comprising: blood pressure, urine analysis, abdominal palpation, and a check on the baby's heartbeat. Venesection was performed by midwives using a 21 gauge ¾ inch butterfly needle, one 6mL EDTA sample was collected by the vacutainer system for routine antenatal blood group and antibody screen, and a further 6mL EDTA and 4mL into RNA preservative (Tempus tube, Applied Biosciences Ltd) sample collected, for the current study. Immediately after collection the Tempus tube was shaken for 10-15 seconds (Applied Biosystems product insert) to promote cell lysis and stabilisation of free RNA, then stored at room temperature pending transfer to storage at -20°C within 2 hours of collection.

1500IU (300 μg) of Rhophyllac anti-D immunoglobulin, in a volume of 2mL was then administered to the patient via the same butterfly needle. Rhophyllac is a human polyclonal anti-D product, fractionated from plasma donated by volunteers known to have high titre IgG anti-D antibodies, and is marketed by CSL Behring (Marburg, Germany). Rhophyllac is presented in single use pre-filled 2mL glass syringes. Each syringe nominally containing 1500IU (300μg) anti-D immunoglobulin and in addition a maximum of 30μg/mL of other human plasma proteins, of which 10 μg/mL is human albumin as a stabiliser, 95% of the remainder being IgG (Rhophyllac package insert, CSL Behring). The product is licensed in the UK for intravenous or intramuscular injection, and the recommended dose for routine antenatal prophylaxis is a single dose between 28 and 30 weeks. Alternative prophylaxis regimes are recommended following potentially sensitizing episodes. Rarely, allergic (<1 in 100) or anaphylactic (<1 in 1000) reactions occur, anti-D recipients are observed in clinic for 10-20 minutes before release from hospital (Rhophyllac package insert).

Women remained in clinic for 20 minutes after anti-D administration, then travelled to the Blood Transfusion Centre at Seacroft, Leeds some 5 miles

away, either by their own transport or taxi arranged by the chief investigator. Blood samples were collected into Tempus and EDTA tubes as before, nominally 1 hour after Rhophyllac injection (mean 57 minutes range 35-105 minutes). The final samples were collected on the following day at the Blood Transfusion Centre (mean time 21 hours 45 minutes, range 17 hours 15 minutes to 26 hours 5 minutes, after anti-D administration).

EDTA samples collected at St James' University Hospital, were transferred to a laboratory facility on site and centrifuged for 5 minutes at 3000g (Sorvall Legend centrifuge) to remove cellular components from the plasma. Four to six, 0.5mL aliquots of plasma were transferred to labelled 1 mL cryotubes (Nunc Ltd) using 1mL Pastettes (Alphalabs, Eastleigh, UK). The plasma aliquots were transferred to a freezer at -65°C within 1 hour of collection, and subsequently to -80° C at the Blood Transfusion Centre for long term storage. The same protocol was used for samples collected at the Blood Transfusion Centre, but plasma was transferred directly to -80°C storage. The collection period comprised 24 clinics held between 6$^{th}$ January and 7$^{th}$ April 2009, with a cohort totalling 24 participants.

## 2.3 Method Estimation Of The Size Of Fetomaternal Haemorrhage By Flow Cytometry

### 2.3.1 Introduction

Kleihauer Betke Technique (KBT) is widely used in Western Europe both as a screening and semi-quantitative test. The technique has been well described elsewhere (BCSH 2009) and is rapid, inexpensive and requires no specialised equipment, but is laborious, difficult to automate and subjective in its interpretation. The technique is prone to false positive results associated with staining of adult cells in patients with haemoglobinopathies or hereditary persistence of fetal haemoglobin. KBT is recognised as having intrinsically high intra- and inter-assay variance, associated with the subjectivity in

interpretation and the relatively small sample examined; typically 50,000 cells are observed (BCSH 2009).

Flow cytometry (FC) using monoclonal anti-D directly conjugated to fluorochromes offers a robust, objective assay for fetal D+ red cells and is generally recommended as the technique of choice for confirmation of positive screen results obtained by Kleihauer and for accurate determination of the size of FMH larger than 4mL (BCSH 2009). Though widely accepted as the reference technique, FC has a reputation for being less effective at detecting small FMH than KBT .

### 2.3.2 Principles of Flow Cytometry

Flow cytometry is a versatile technique which allows assessment of the characteristics of particles by causing a single stream of particles to pass through the beam of a laser. Collection of light scattered by the particles allows characterisation of size shape and granularity. The presence of intracellular and extracellular markers can be detected by fluorescent dyes conjugated to specific antibodies. Light emitted by the fluorophores is detected and measured by specific detectors (Figure 2.1).

### 2.3.3 Flow Cytometry for the Estimation of Fetomaternal Haemorrhage

As well as robust laboratory technique, the reliable and reproducible detection and estimation of levels of rare events, such as D+ cells in the circulation of a D- mother after FMH, is dependent upon the number of events analysed. Lower coefficients of variance (CV) and narrower confidence intervals can be obtained by increasing sample size (Hoy 1994). This study incorporated an investigation of the characteristics of FMH estimation by FC in our laboratory, the specific objectives being to determine the lower level of sensitivity of flow cytometry in our laboratory in detecting FMH. In this optimisation study artificial mixtures of adult RhD- cells and fetal RhD+ cells were prepared in a range of concentrations, nominally at and below the 4mL bleed size, above which an anti-D greater than the standard 500IU dose would be required.

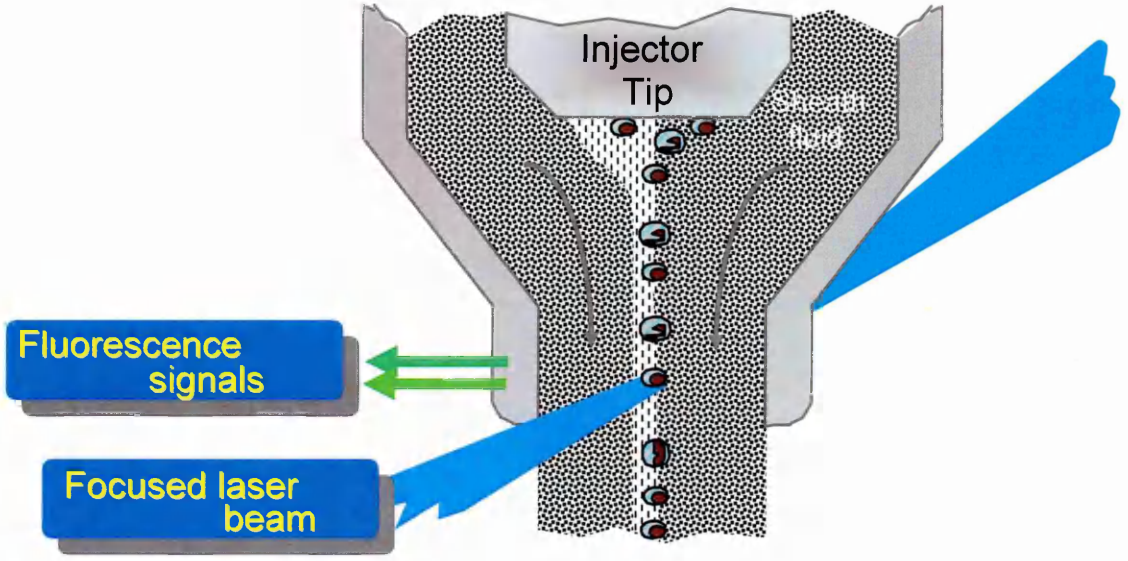
These were tested by the standard flow cytometric method (BCSH 2009). Data was collected in a format to allow comparison of performance across as range of data set sizes: 25,000, 50,000, 100,000, 250,000, 500,000, and 1,000,000 event collections for cells incubated both with FITC-conjugated BRAD-3 and AEVZ5.3. Both reagents are recombinant IgG antibodies, BRAD-3 has been characterised as reacting with all D+ and most partial D phenotypes, AEVZ is specific for Varicella zoster, and is not does not react specifically with red cells, and is used as an isotype matched control (Lloyd-Evans 1999).

Validation was performed using group O ccddee donor and group O CcDee fetal red cells less than 48 hours after collection. These were used to prepare test mixtures of D+ and D- at the following concentrations: 0.2%, 0.1%, 0.05%, 0.025% and 0.01% (approximately simulating bleed sizes of 4mL, 2mL, 1mL, 0.5mL and 0.025mL) as well as D- cells as a negative control. Cells were stored in CellStab (DiaMed Ltd) at 4 °C for up to 4 weeks, in accordance with the manufacturer's recommendations.

### 2.3.4 Method: FMH Detection by Flow Cytometry

EDTA samples from study participants were tested within 48 hours of collection. All samples were thoroughly mixed on an orbital mixer for at least 10 minutes, 100μL aliquots were removed and the red cells were washed twice in PBS pH 7.0+/- 0.2 (Inverclyde Biologicals, Bellshill, Scotland). Centrifugation for these steps was at 1000g for 2 minutes in a centrifuge with a full swing-out rotor, and aspiration was undertaken carefully to avoid removal of fetal cells, which are less dense than those of adults (BCSH guidelines 2009). Red cell suspensions of approximately 3% were prepared by adding 1mL of PBS to the resuspended cell pellet.

A




B

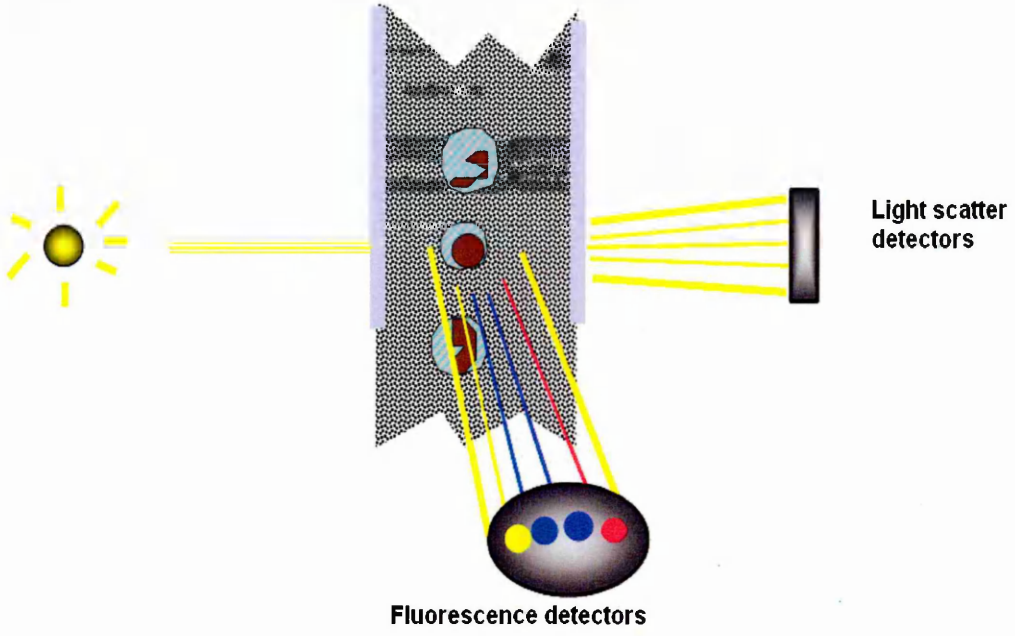



Figure 2.1 Diagram of flow cytometer flow cell. Showing single stream of particles created by fluidics of flow cell (A). Laser light incident on particles is scattered forward or to the side allowing characterisation of particles by size, shape and granularity all of which affect light scatter. The light emitted by any adherent fluorophore labelled antibodies is measured by fluorescence detectors (B)

BRAD-3 anti-D and inert AEVZ5.3 control FITC conjugates (both IBGRL, Bristol, UK) were prepared by diluting 1 in 10 in PBS. Six, 50 µL aliquots of diluted FITC BRAD-3 and AEVZ were dispensed into 75x12mm polyethylene tubes and to each was added 50 µL of mixed cell preparation, using clean pipette tips for each aliquot. Tubes were covered and incubated for 30 minutes in the dark in a 37°C water bath. After incubation, each cell suspension was washed once in 3mL of PBS with centrifugation at 1000g for 2 minutes in a centrifuge with a swing-out rotor and the pellet re-suspended in 1mL of PBS. Analysis was performed on a Beckmann Coulter Epics XL flow cytometer at 498 nm. Red cells were gated on forward and side scatter and data regions for positive and negative events were set equidistant between peaks obtained for a standard 1% control (Figure 2.2).

Each dilution was analysed 5-7 times, and results captured in Windows Excel for analysis. The number of events in corresponding AEVZ 5.3 controls was subtracted from corresponding counts obtained using FITC BRAD-3 to remove background fluorescence, and the estimated size of simulated bleed calculated using the standard formula, in which 1800mL is assumed to be the standard circulating maternal red cell volume, and fetal red cells are assumed to be 22% larger than adult red cells (Mollinson 1972).
Formula for calculation of the volume of D+ fetal red cell in the maternal circulation

$$\frac{\%\ \text{fetal cells}}{100} \times 1800 \times \frac{122}{100}$$

p values for the difference between means of each cell dilution with the D- data, 95% confidence intervals, and %CV for each dilution were calculated using Microsoft Excel software.

Samples from study participants were run by the same protocol, except that data acquisition was managed to collect a total of $1x10^6$ events in 2 aliquots.

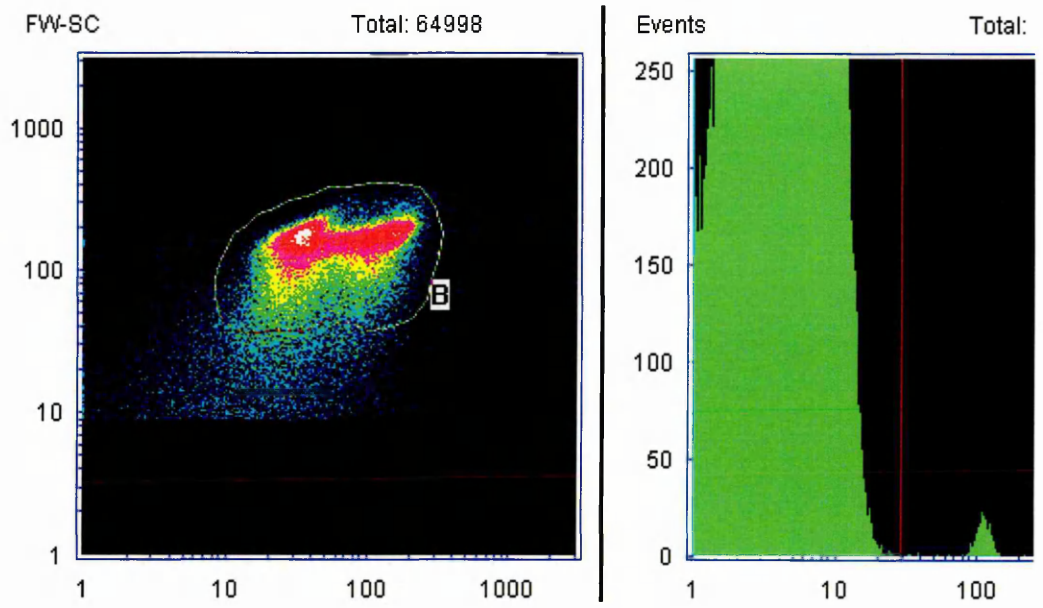
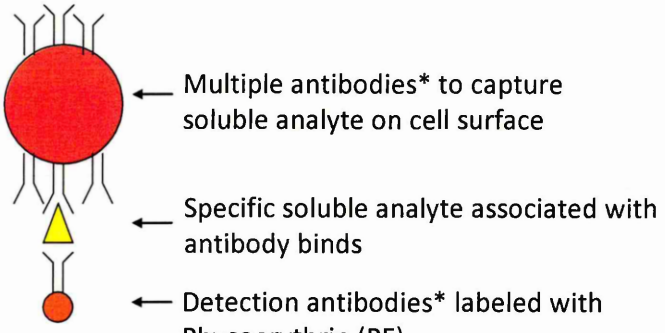



Figure 2.2 , Scatter graph (left) showing gate (B) around red cell population drawn on forward (y axis) and side scatter (x axis), and histogram (right) showing fluorescence (x-axis) and events gathered (y-axis) and the position of data regions, defined by vertical red line. Unstained (D- cell) events are in the population on the left, stained (D+ cells) to the right.

## 2.4 Measurement of Plasma Cytokine Concentration by Flow Cytometry

### 2.4.1 Introduction

Cytokine detection by flow cytometry is a technique which uses the capacity of flow cytometry to measure multiple analytes by means of specific monoclonal antibodies immobilised on beads, which can be recognised by pre-determined fluorescent characteristics when exposed to laser light at 635nm. Samples containing the analyte are mixed with the beads and bound material is detected in a sandwich assay by adding fluorochrome labelled detector antibody (Figure 2.3). Measurement of fluorescence from the detector reagent when exposed to light at 532nm, associated with each bead type, allows rapid and cost effective analysis of multiple analytes from small samples of test material (Becton Dickinson, product insert). Quantification is achieved by comparison with reactions obtained with recombinant cytokine standards (Figure 2.4) The technique offers similar sensitivity to standard ELISA, though the range of analytes covered by available, validated flow cytometry assays is not as extensive, in particular TGF-β, a key analyte for this study was unavailable as a bead assay.




* Antibody matched pairs

Figure 2.3 Multiplex bead assay. Analyte capture, and reporter mechanisms in multiplex cytokine flow cytometry assay, showing fluorochrome labelled bead with bound antibody (red circle), analyte (yellow triangle) and reporter antibody labelled with fluorochrome (orange circle)

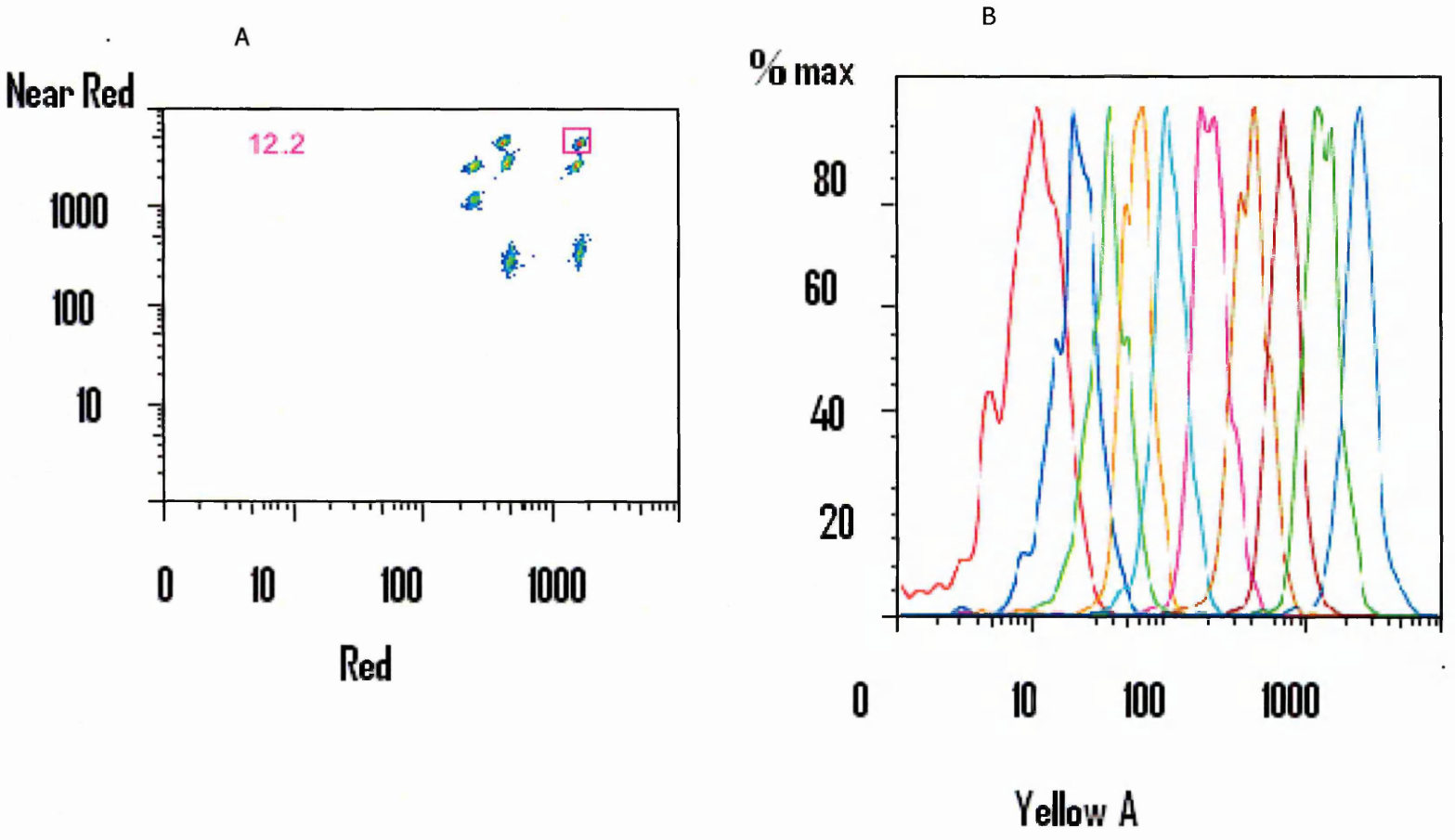



Figure 2.4 Cytokine Flow cytometry output. The dot plot (A) demonstrating how capture beads are characterised by Red- (x axis) and Near-red (y axis) fluorescence. Data are captured by gating (pink box). The calibration chart for IL-1β (B) shows fluorescent intensity (x axis, yellow A) for 10 standard dilutions.

### 2.4.2 Method for Cytokine Detection by Flow Cytometry

Cytokines IFN-γ, IL-1β, IL-4, IL-10, IL-6, IL-12p70, IL-17A, TNF-α were assayed by flow cytometry Using Becton Dickinson reagent kits. All reagents and consumables were obtained from Becton Dickinson, unless stated otherwise. Kits included lyophilised cytokine preparations for use as standards. See Figure 2.5 for summary of method. Standard samples were prepared by pooling the lyophilised standards for all the analytes in a conical 15mL plastic tube labelled "Top Standard", and reconstituted by the addition of 4mL of assay diluent. The tube was mixed gently and left on ice in the dark for 30 minutes. Dilutions for the standard curve were prepared by adding 500μL of assay diluent to each of 9, 12x75mm plastic tubes labelled 1:2, 1:4, 1:8;, 1:16: 1:32 1 :64:1:128, and 1:256, the last tube labelled "true zero" contained only dilution buffer. A 500μL aliquot of the top standard was pipetted into the tube labelled 1:2, the contents were well mixed and doubling dilutions performed through to the 1:256 standard.

The capture beads sufficient for 90 tests (10 standards+72 participant samples+working volume) were prepared, pooled and placed in a labelled tube on ice in the dark until required. The volume of PE detection reagent required was calculated as 1 μL per analyte per test and dispensed into a labelled tube. To this was added 45μL of each detector, labelled PE detection reagent, and stored on ice in the dark until required.

Tests were prepared in polypropylene tubes (ELKAY laboratories Ltd, Basingstoke, UK) by adding 25μL of bead mixture to each test and standard tube. 25μL of standard or freshly thawed test plasma was added to each labelled tube. Tests were incubated for 1 hour in the dark at ambient temperature to allow binding of analyte to antibody immobilised on capture beads.

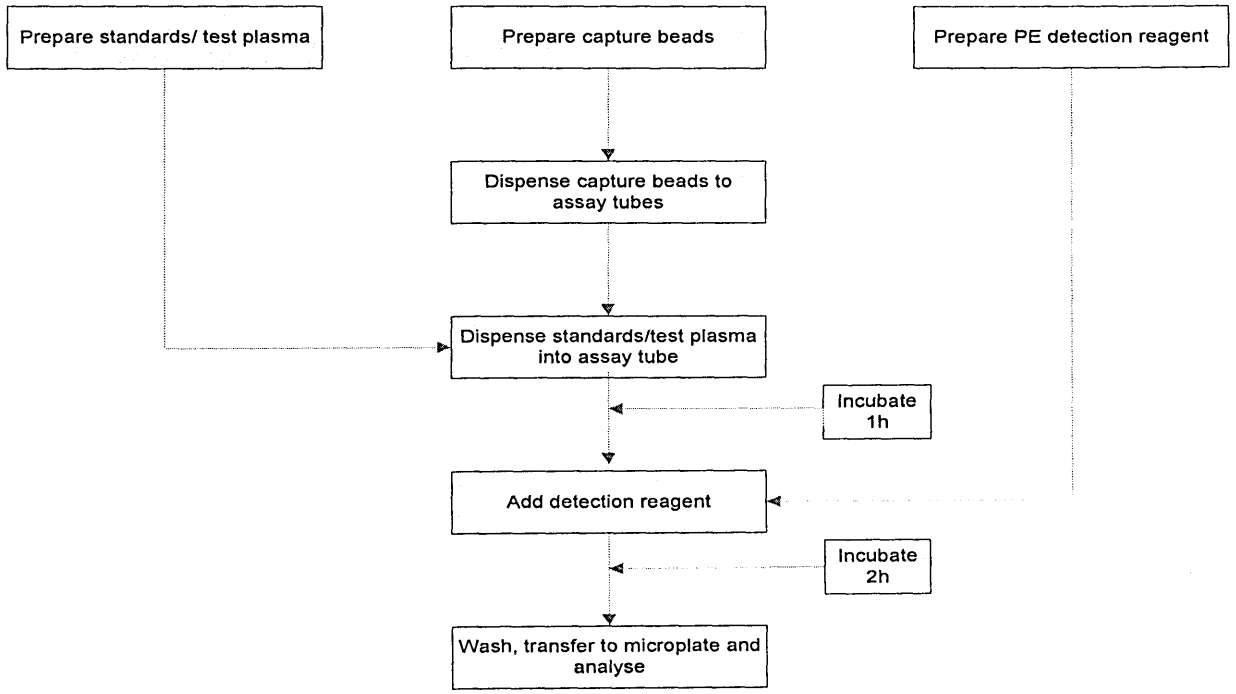



Figure 2.5 Workflow diagram for cytokine detection by flow cytometry method.

After 1 hour, samples were washed once in 300μL of wash buffer centrifuged for 5 minutes at 200g (Sorvall Legend Centrifuge) and 25μL of detection reagent was added to each tube followed by mixing by gentle pipetting. Samples were incubated for a further 2 hours in the dark at room temperature to allow PE conjugated antibody to bind to analyte bound to capture beads. After incubation samples were washed once as before and the supernatant was aspirated to leave approximately 50μL in the tube, and a further 200μL of wash buffer added. Samples were transferred using a pipette to a 96 well Falcon microplate with gentle mixing. Samples were analysed on a Facs Array flow cytometer (BD Biosciences, Oxford , UK), and data gathered using Facs Array system software. Analysis and reporting was through FCAP array analysis software (SoftFlow Inc, Minnesota, USA).

Beads were identified by type within the multiplex analysis, within gates drawn from red and near infra-red fluorescence characteristics associated with each bead, and detected at 635nm filter. Analyte was detected from red fluorescence from PE conjugated detector reagent at 532nm.

## 2.5 Measurement of Plasma Cytokine Concentration by ELISA

### 2.5.1 Introduction

ELISA is a well established technique widely used in immunology and virology, which can be used to detect soluble antigen or antibody. A ligand for the analyte which may be antibody or antigen is fixed to the solid phase. The test material is added and any analyte present binds to the solid phase. Free material is washed away and peroxidase conjugated secondary antibody added followed by washing and then addition of substrate, tetramethylbenzidine chromogen (TMB) which, on exposure to the peroxidase conjugated antibody, causes a measurable colour change proportional to the amount of bound analyte. A pure, standard preparation of the analyte is required to allow the results of the assay to be

expressed as a concentration of analyte, by preparation of a calibration curve (Janeway 2001).

A variation of the technique, used in this study to quantify TGF-β, IL-1 and IL-1ra, is the sandwich or antigen capture ELISA. The principle of which is that a monoclonal antibody specific for the analyte is bound to the wells of the test microplate (Figure 2.6). The target molecule added to wells in standards or test samples binds to the immobilised monoclonal antibody. After the initial incubation, wells are washed to remove unbound material and a conjugate of enzyme and analyte-specific monoclonal antibody added. An incubation phase allows the development of the sandwich of analyte between plate bound and enzyme conjugated monoclonal antibodies. A further wash stage removes free conjugated antibody. Enzyme substrate is added resulting in a colour change proportionate to the amount of analyte bound in the initial incubation. After a further incubation the colour reaction is stopped by the addition of 2N sulphuric acid. Absorbance is measured at 450nm with a correction for optical variation of the plate made at 570nm, which is subtracted from the 450nm reading. Values obtained from the standards were used to prepare a standard curve, the slope of which was calculated and used to calculate analyte concentrations in test samples using Microsoft Excel software.

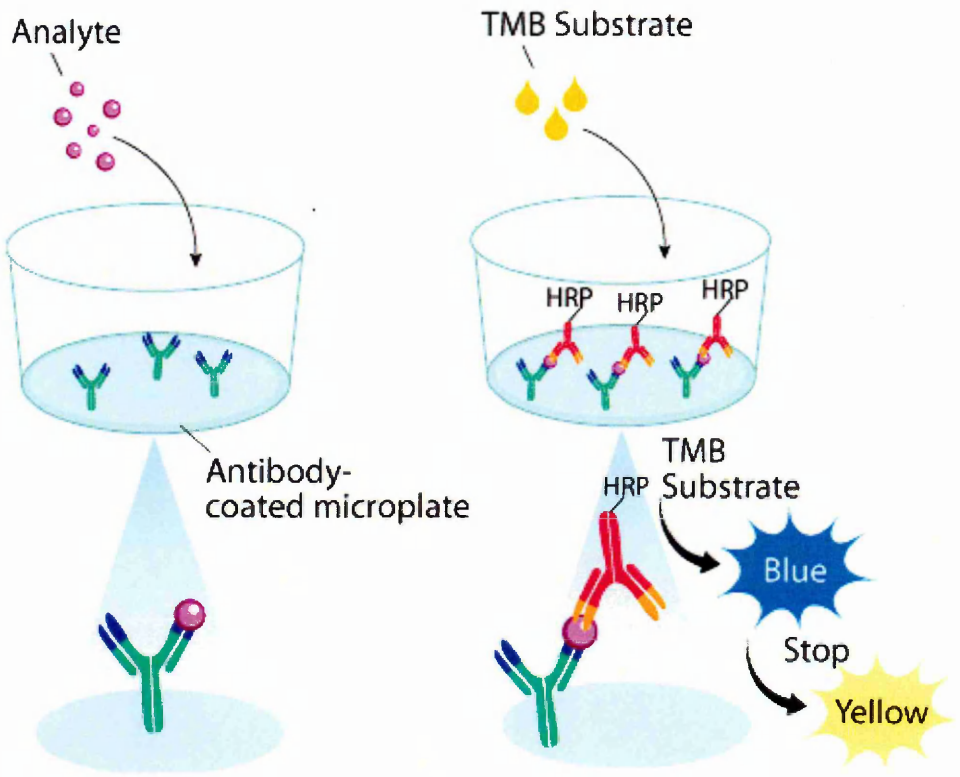



Figure 2.6 Illustration of sandwich ELISA, showing monoclonal antibody immobilised on solid phase of microplate capturing analyte (left). The reaction is detected by addition of horse radish peroxidase labelled antibody binding to captured analyte, then causing measurable blue to yellow colour change on the addition of TMB substrate (right)

Diagram reproduced with permission from R+D systems website http://www.rndsystems.com/product_detail_objectname_elisa_assay_product.aspx

### 2.5.2 Method for Measuring the Concentration of IL-1 β by ELISA

All kits were supplied by R+D systems Ltd. Tests and assays were performed in accordance with the manufacturer's instructions (R+D Systems Ltd, Europe). Briefly, all reagents were brought to room temperature. IL-1β standard was prepared by adding 5mL of calibration diluent (product RD6C) to the supplied lyophilised standard to prepare a 250pg/mL standard. Reconstitution was over 30 minutes with occasional gentle mixing. Dilutions were prepared by adding 500μL of calibrator diluent to each of six 1mL polypropylene tubes (Eppendorf, Hamburg Germany), then adding 500μL of reconstituted standard to the first tube. Doubling dilutions were prepared using a clean pipette tip for each step, tubes were mixed thoroughly by vortex to prepare standards of: 250, 125, 62.5, 31.2, 15.6, 7.8, and 3.9 pg/mL. The zero control was the calibrator diluent.

Plates were removed from foil pouches, and 50μL of assay diluent (RD1-83) added to each well. Using a clean pipette tip for each, 200μL of standard, diluent control or test plasma was added to duplicate wells. Plates were sealed with adhesive strip and incubated for 2 hours at room temperature. Wash buffer was prepared, by adding 20mL of supplied concentrate to 480mL of deionised water. Plates were aspirated by inversion and flicking the contents of wells into a sink, wash buffer was added to fill the wells, using a wash bottle, a total of 3 washes were performed. After each wash, the buffer was removed as above and the plate vigorously blotted on a paper towel.

200μL of anti-IL-1β secondary antibody conjugated to horseradish peroxidase was added to each well. Plates were sealed and incubated for a further 2 hours. Substrate solution was prepared by mixing equal volumes of stabilized hydrogen peroxide and TMB chromogen immediately before use.
After incubation, plates were washed three times as before, blotted and 200μL of substrate solution added to all wells. Plates were incubated uncovered at room temperature in the dark for 20 minutes. The colour reaction was stopped by the

addition of 50μL of 2N sulphuric acid and a colour change from blue to yellow was noted. Tests were read on a Wallac plate reader using Wallac Software reading at 450nm. Readings at 570nm were made and subtracted from 450nm readings, to correct for any optical imperfections in the plate.

### 2.5.3 Method for Measurement of IL-1ra Concentration by ELISA

All reagents were brought to room temperature. IL-1 ra standard was prepared by adding 1.0mL of deionised water to the supplied lyophilised standard to prepare a 20,000 pg/mL standard. Reconstitution was over 30 minutes, with occasional gentle mixing. Dilutions were prepared by adding 900μL of calibrator diluent (RD5-33) to the first of 7 polypropylene tubes, and 500μL to the remaining 6 tubes. One hundred μL of reconstituted standard were added to the first tube. Doubling dilutions were prepared using a clean pipette tip for each step, and vortexing dilutions to facilitate thorough mixing, to prepare standards of 2,000, 1,000, 500, 250, 125, 62.5, and 31.2 pg/mL. The zero control was the calibrator diluent.

Plates were removed from foil pouches, and 100μL of Assay Diluent (RD1S) added to each well. Using a clean pipette tip for each dispense 100μL, of standard, diluent control or test plasma was added to duplicate wells. Plates were sealed with adhesive strip and incubated for 2 hours at room temperature. Wash buffer was prepared, by adding 20mL of supplied concentrate to 480mL of deionised water. Plates were washed as above for the I-1β ELISA assay.

200μL of anti-IL-1ra secondary antibody conjugated to horse radish peroxidase was added to each well and plates were sealed and incubated for a further 2 hours. Tests were completed and read as in 2.5.2.

### 2.5.4 Method for Measurement of TGF-β1 Concentration by ELISA

TGF-β1 is secreted as a latent complex with another protein, which must be dissociated before the active form can be measured (Lawrence 2001). In this assay latent secreted TGF-β1 was activated to the immunoreactive form by acidification. 40μL of each test plasma sample was placed in a polypropylene tube and 20μL of 1N HCl added. Acidified samples were incubated on the bench at room temperature for 10 minutes, then neutralised with 20μL of 1.2N NaOH/0.5M Hepes (Sigma Ltd, Poole, UK) to pH 7.2-7.6.

All reagents were brought to room temperature. TGF-β1 standard was prepared by adding 2.0mL of calibrator diluent RD5-53 to the supplied lyophilised standard to prepare a 2,000 pg/mL standard. Reconstitution was over 5 minutes with gentle agitation. Dilutions were prepared by adding 400μL of calibrator diluent (RD5-53) to each of 6 polypropylene tubes. 400μL of reconstituted standard were added to the first tube. Doubling dilutions were prepared using a clean pipette tip for each step, and vortexing dilutions to facilitate thorough mixing, to prepare standards of: 2,000, 1,000, 500, 250, 125, 62.5, and 31.2 pg/mL. The zero control was calibrator diluent.

Plasma samples from participants were further diluted by adding 10μL of activated plasma to 190μL Calibrator Diluent in a polypropylene tube to make a final dilution of 1 in 40). Plates were removed from foil pouches, and 50μL of assay diluent (RD1-73) to each well. Using a clean pipette tip for each dispense, 50μL of standard, diluent, control or diluted test plasma was added to duplicate wells. Tests were completed and read as in 2.5.2.

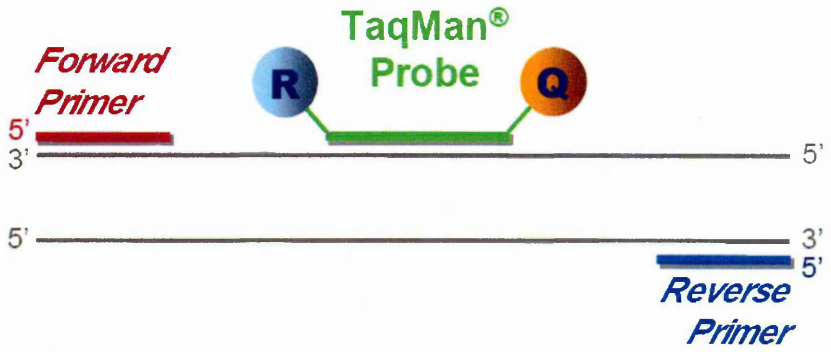
## 2.6 Method for Reverse Transcriptase Quantitative Real Time PCR for Measurement of IL-1ra and TGF-β1 mRNA

### 2.6.1 Introduction

Real time quantitative PCR allows measurement of mRNA, using synthetic oligonuceotide primers, and is a well established technique for quantifying the expression of specifically transcribed genes in cells and tissues. By synthesising cDNA from RNA using reverse transcriptase (RT) it is possible to assess those genes which are expressed by RT PCR. The more recent development of real time PCR facilitated by the development of probes, which emit fluorescence only at the extension phase of the PCR cycle, allows assessment of the level of expression of genes (Overburgh 2003).

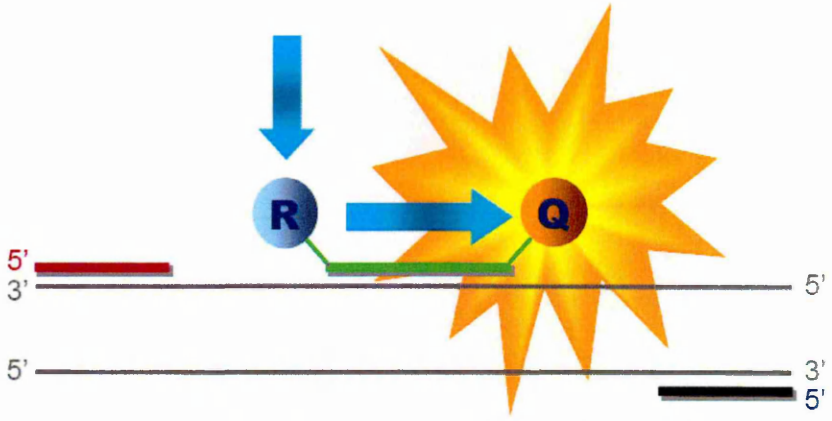
In the current study TaqMan technology was used (Applied Biosystems, Foster City, California, USA). TaqMan probes are designed to bind to cDNA in the target sequence between forward and reverse primers and carry both a reporter fluorophore and a quencher, and do not fluoresce when intact. As Taq polymerase effects extension of the new DNA chain its exonuclease activity degrades the Taq probe, releasing the reporter from the quencher, resulting in measurable fluorescence (Figure 2.7). The measured fluorescence is proportional to the quantity of specific cDNA for that gene in the sample, and can be expressed numerically as the number of PCR cycles required to raise the level of fluorescence above background or cycle threshold (CT value, Figure 2.8). Relative gene expression is best measured by comparison with an endogenous control gene, which is selected for widespread and consistent expression between tissues (Bustin 2005). The difference in CT values between endogenous control gene and test gene is expressed as ΔCT, and changes in ΔCT across a time series, as in the present study are described as ΔΔCT.

## 5' Nuclease Assay Using TaqMan® Probes




- PCR specificity (primer)
- Hybridization specificity (probe)

## 5' Nuclease Assay Using TaqMan® Probes




- Fluorescence Resonance Energy Transfer (FRET) from high energy (Reporter) to low energy (Quencher) dye
- No reporter signal with intact probe

Figure 2.7 Illustration of TaqMan chemistry. The primer bonds to specific DNA sequence with reporter and quencher in close proximity (top). Taqman 5' exonuclease activity releases quencher resulting in fluorescent signal (bottom). Reproduced with permission of Applied Biosystems Ltd

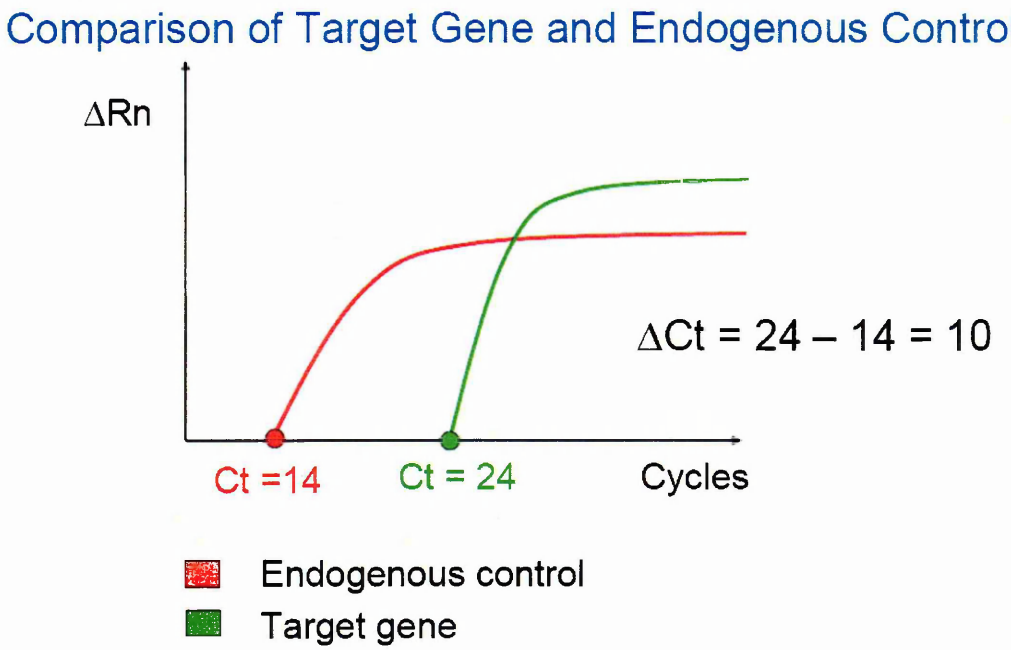



Figure 2.8 Illustration of amplification curves for analyte and endogenous control. Showing CT vale on the x-axis, and fluorescence on the y-axis, the point at which fluorescence exceeds background, and ΔCT value representing the difference in CT values between the test gene and the endogenous control

Reproduced with permission from AB systems

### 2.6.2 RNA Extraction

Blood samples collected in Tempus tubes (Applied Biosystems Ltd) were stored at -20°C until extraction (period 6-10 months). The supplier's product advice indicates that mRNA is stable indefinitely when stored in this way. Extraction and purification was performed using Tempus Spin RNA extraction kits (Applied Biosystems Ltd). All reagents and consumables used were supplied with the kit, unless otherwise stated.

Samples were thawed at room temperature and decanted into clean, labelled 50mL tubes (Falcon, BD UK Ltd). Phosphate Buffered Saline (PBS) was added to bring the volume to 12mL (typically 3mL of PBS was added). Each tube was mixed by vortex for 30 seconds then centrifuged at 3000g for 30 minutes at 4°C (Sorvall 6000D). After centrifugation, the supernatant was gently poured off and the RNA pellet re-suspended by the addition of 400μL of RNA purification suspension solution, with a brief vortex mix. Re-suspended RNA samples were stored on ice between process steps from this point.

RNA purification filters were labelled and pre-wetted with 100μL of RNA purification wash solution 1, taking care to dispense the solution to the centre of the filter, the re-suspended RNA was added to filters, which were placed inside RNase free, 2mL elution tubes. Tubes were centrifuged at 16,000g for 30 seconds at 4°C (Hereus Fresco 17). All subsequent centrifugation steps used this protocol, unless stated otherwise. After centrifugation, filters were removed; liquid waste discarded from the bottom of the elution tube, and filters replaced. 500μL of purification wash solution 1 was added to each filter before centrifugation and waste then discarded as before. Two washes were performed using Purification Wash Solution 2, then the filters were air dried by performing one further centrifugation step without addition of wash solution. Filters were transferred to clean, labelled elution tubes, and elution of RNA from the filter performed, by adding 100μL of nucleic acid elution purification solution, then incubating the tube

at 70°C in a heating block for 2 minutes. Tubes were centrifuged and the eluate harvested and returned to the filter. Tubes were centrifuged again, this time at 16,000g for 2 minutes at 4°C. Filters were removed and discarded. The purified RNA was transferred to clean, labelled PCR grade 0.5mL Eppendorf tubes (Sigma, Poole, UK) in four 20μL aliquots, which were immediately transferred to -80°C for storage, with a small additional aliquot reserved on ice for assessment.

The quantity of RNA extracted from each sample was assessed using Nanodrop ND-1000 spectrophotometer by measuring the percentage absorbance of light at 280nm, which is proportional to the concentration of analyte. The ratio of absorbance by the sample at 260 and 280 nm indicates sample purity, a ratio of greater than 2 representing acceptably pure RNA. (nanodrop manual www.nanodrop.com).

### 2.6.3 cDNA Synthesis Method

cDNA synthesis was performed using Superscript II Reverse Transcriptase (RT) (Applied Biosystems Ltd) as part of a mastermix solution prepared on ice as described in Table 2.1.

| Reagent | Supplier | Product code | X1 (μL) |
|---|---|---|---|
| DEPC treated water | Invitrogen | 750024 | 2 |
| 5x 1st strand buffer | Invitrogen | 18065-014 | 4 |
| Dithiothreitol 0.1M | Invitrogen | | 2 |
| RNase out | Invitrogen | 1077-019 | 0.5 |
| Random primers | Invitrogen | 48190-011 | 0.5 |
| dNTP | Bioline | Bio-39025 | 0.5 |
| Superscript II RT | Invitrogen | 18065-014 | 0.5 |

Table 2.1 Reverse transcriptase mastermix reagent volumes for 1 preparation (x1). Mastermix for multiple preparations prepared to the same formula.

Mastermix was prepared without reverse transcriptase and a 9.5μL aliquot removed for the No RT control. Reverse transcriptase was added to the mastermix, which was mixed gently by pipette aspiration and transferred to labelled, RNase free Eppendorf tubes in 10μL aliquots. RNA samples were removed from -80°C storage thawed on ice and 20μL added to 10μL aliquots of mastermix. Negative control tubes were prepared as in Table 2.2

| Control | Mastermix no RT | Mastermix +RT | DEPC water | Pooled random RNA* |
|---|---|---|---|---|
| No RT | 9.5μL | | | 10.5μL |
| Negative | | 10μL | 10μL | |

Table 2.2 qPCR Negative control reagent volumes. *Prepared from a pool of 4 randomly selected participant RNA samples from the study.

All tubes were capped, spun gently to ensure mixing of reagents, then incubated for 2 hours in a heating block (Biometra trio thermablock) at 42°C, and synthesised cDNA transferred to -20°C for storage.

### 2.6.4 Method for the measurement of IL-1ra and TGF-β1 mRNA by q-PCR

PCR primer mix was prepared on ice in RNase free 1.5mL Eppendorf containers as detailed in Table 2.3

| Reagent | Supplier | Product code | Per well(μL) |
|---|---|---|---|
| DEPC treated water | InVitrogen | 750024 | 3.5 |
| TAQMAN mastermix | Applied Biosystems | 4366072 | 5 |
| Gene Specific Primer | Applied Biosystems | See table 2.4 | 0.5 |

Table 2.3 PCR primer mix

| TaqMan probe | Supplier | Product |
|---|---|---|
| GAPDH | Applied Biosystems | Hs99999905-m1 |
| Cyclophilin | Applied Biosystems | Hs99999904-m1 |
| B actin | Applied Biosystems | Hs99999903-m1 |
| IL-1ra | Applied Biosystems | Hs00610318-m1 |
| TGF-β | Applied Biosystems | Hs00893626-m1 |

Table 2. 4 Primers used in study for assessing test RNA and as endogenous controls

PCR primer mix was transferred to labelled 0.5mL RNase free Eppendorf tubes in 18μL aliquots. cDNA samples were retrieved from storage, thawed on ice, then 2μL aliquots added to primer mix. Controls were included from each batch of cDNA synthesis performed. All tubes were centrifuged briefly to ensure reagents were mixed.

Dilution templates were obtained for the endogenous control and each target gene by preparing dilutions of pooled cDNA from the study at neat, 1/10, 1/100, and 1/1000 to demonstrate the efficiency of the primers. Tests were transferred to Microamp Optical 96 well plates (Applied Biosystems) in duplicate in 9μL aliquots. Plates were sealed with adhesive sealers (Applied Biosystems) and centrifuged briefly (Sorvall 6000D) to ensure plate contents were mixed and located at the bottom of the wells. PCR and analysis were performed using the StepOne Plus instrument (Applied Biosystems Ltd).

### 2.6.5 Quantification of IL-1ra and TGF-β1 mRNA from qPCR Results

CT values were captured from StepOnePlus software (Applied Biosystems Ltd), and the mean of values of replicates of endogenous control target gene tests calculated. The differences between the CT for the endogenous control gene and target genes for the pre-test sample from each participant were established and

expressed as ΔCT. The difference between this value and the ΔCT of the one hour and twenty four hour post- test samples was calculated and expressed as ΔΔCT. Fold changes were calculated using the formula.

$$\text{Fold Change} = 2^{-\Delta\Delta CT}$$

## 2.7 Statistical Analysis

### 2.7.1 FMH Estimation

Estimated FMH, coefficient of variation (CV), and standard deviation were all calculated using Microsoft Excel software. The significance of difference between means of multiple validation mixtures and samples from D+ and D- fetus groups was calculated by ANOVA, using Microsoft Excel software.

### 2.7.2 ELISA and qPCR

Fold changes from raw data were calculated using Microsoft Excel software. Normality of fold change data was determined using Kolmogorov-Smirnoff, and significance of means between D+ and D- fetus groups with independent T test.. SPSS software was used for these calculations (SPSS Inc, Chicago, Illinois).

### 2.7.3 Combined Results

The proportion of participants undergoing fold change >1.5 detected either by ELISA or qPCR was calculated. The significance of the difference between proportions in D+ and D- fetus groups was calculated using $Chi^2$.

# 3 Results

## 3.1 Fetal Blood Groups

The blood groups of babies born to women participating in the study were obtained after all laboratory tests were completed, by interrogating the Patient Information System of Leeds Teaching Hospitals. All 24 fetal groups were available, having been determined after delivery and are listed in Table 3.1.

17/24 (70.8%) neonates were RhD+, and these participants were allocated to the D+ fetus group of the study; 7/24 (29.2%) neonates were RhD- and these participants were allocated to the D- fetus group. Typically 40% of RhD- women in the UK carry RhD- fetuses (NICE 2002). In this study the proportion of RhD- fetuses is lower than might be expected than that expected using data extracted from population-scale studies. This finding can be explained by the relatively small size of the sample in the current study..

## 3.2 FMH by Flow Cytometry

### 3.2.1 Flow Cytometry Optimization Results

Using the standard protocol (BCSH 2009), acquiring 500,000 events per test, the smallest simulated bleed which could be detected with a significant difference between the counts in the D- and test samples was 0.05% (approximately 1mL, Table 3.2e ) Broadly, protocols acquiring fewer events permitted similar levels of discrimination, though coefficients of variation were higher and the 95% confidence intervals were wider, suggesting that the accuracy of the test is lower than when more events are acquired (Tables 3.2 a-f). By considering all the accumulated data, collecting $10^6$ events the sensitivity of the test was increased such that a simulated bleed of 0.025% (approximately 0.5mL) could be detected

| Participant number | Maternal ABO and RhD group | Fetal ABO and RhD Group |
|---|---|---|
| 1 | A- | AB- |
| 2 | O- | O+ |
| 3 | O- | O+ |
| 4 | A- | O+ |
| 5 | O- | O+ |
| 6 | A- | A+ |
| 7 | A- | O+ |
| 8 | O- | O- |
| 9 | O- | O+ |
| 10 | A- | A+ |
| 11 | A- | B- |
| 12 | O- | A+ |
| 13 | AB- | A- |
| 14 | O- | A- |
| 15 | A- | A- |
| 16 | A- | O+ |
| 17 | O- | A+ |
| 18 | O- | A+ |
| 19 | A- | A+ |
| 20 | O- | O+ |
| 21 | A- | O+ |
| 22 | A- | A- |
| 23 | A- | O+ |
| 24 | A- | A+ |

Table 3.1 Maternal and fetal blood groups of the participants in the study. Participants invited to the study based on previously determined RhD- group. Approximately 40% of RhD- mothers in the UK carry RhD- babies, in this cohort 29.1% of the babies were RhD-.

with a significant difference between the number of events in positive and negative populations (Table 3.2f).
Using the 500,000 event protocol with a simulated bleed of 0.2% (approximately 4mL) the confidence interval at the 95% level in this test was 0.72 (lower and upper limits 3.08-3.80) (Table 3.2e) with a percentage coefficient of variation (CV) of 8.13. Increasing the events acquired to $10^6$ reduced both the confidence interval at the 95% level to 0.578 (lower and upper limits 3.19-3.77) and the % CV to 6.46 (Table 3.2f). Protocols acquiring fewer events had wider 95% confidence intervals, using 50,000 events for the simulated 0.2% bleed this was 1.156 (lower and upper limits 2.498-3.654), %CV 14.1 (Tables 3.2 a-d ).

Based on this assessment a protocol acquiring $1x10^6$ events was selected for use in this study.

### 3.2.2 Results of FMH Investigation on Pre-Treatment Samples

FMH estimations were performed on pre-treatment samples from all 24 participants using the $1x10^6$ event protocol based on the data obtained in 3.1. (Appendix V). In each group, some tests gave marginally higher event-counts with the control than with anti-D, hence indicated in negative bleed values; this is commonly encountered in clinical samples. In these cases the FMH values were set to zero for subsequent calculations.

Considering the data by D+ and D- fetus groups of the study, the range of detected apparent FMH, the mean FMH and standard deviation were similar in each group (Table 3.3). Applying the independent T test to determine the significance of difference in the mean FMH between means of D+ and D- fetus groups, indicate that the differences are not significant (p=0.779).

FMH results in the D- fetus group should have approximated to zero. Even if FMH had occurred, there should have been no binding of FITC-anti-D. Positive

values up to 0.98 mL (mean 0.31mL, SD 0.37, n=7)) in this group may be the result of non-specific binding of FITC anti-D to red cells or other particles in the samples. Similar mean and range values for the D+ fetus group (mean 0.37mL range 0-1.4mL, SD 0.41) were obtained and indicated that no large FMH (>4mL) had occurred among the cohort before the study samples were collected.
The SD obtained in the participant's samples is much higher than that in the validation exercise performed, again suggesting that this protocol is insufficiently robust to detect or measure FMH at these levels, which are far below those required for routine testing performed on clinical samples (BCSH 2009).

### 3.3 Cytokine Measurement by Flow Cytometry

Eight cytokines were tested in all 72 samples using a multiplex assay (Figure 3.1). Of the samples tested, fluorescence above background was detected only sporadically, and was above the bottom standard in only 4 tests, which were all for IL-17a, each in one sample from separate patients, No pattern could be inferred from these results (appendix VI).

Test sensitivity was adequate to detect level of circulating analyte, consistent with those reported by other authors who measured elevated cytokine concentrations by ELISA in disease states or in response to treatment (Table 3.3) (Makhseed 2000, Jonsson 2006, Branch 2006).

Tables 3.2a-c Results of FMH optimisation. Nominal FMH of spiked samples, mean measured FMH and associated standard deviation (SD) ANOVA p values for difference between means of negative control and each D+/D- mixture, and 95% confidence interval (CI) expressed for each nominal FMH size (4.0-0.2mL) using each flow cytometry protocol (25,000-100,000 events)

Table 3.2a Results of fold change measurement of prepared mixtures of D+ and D- cells mimicking FMH samples acquiring 25,000 events

| Nominal FMH (mL) | Measured FMH (mL) | CV (%) | SD | p | 95% CI |
|---|---|---|---|---|---|
| 4.0 | 3.145 | 11.85 | 0.295 | <0.005 | 0.962 |
| 2.0 | 2.039 | 7.53 | 0.137 | <0.005 | 0.446 |
| 1.0 | 0.847 | 52.40 | 0.432 | <0.005 | 1.41 |
| 0.5 | 0.069 | 81.30 | 0.225 | 0.64 | 0.734 |
| 0.2 | -0.173 | 178.24 | 0.294 | 0.556 | 0.739 |

Table 3.2b Results of fold change measurement of prepared mixtures of D+ and D- cells mimicking FMH samples acquiring 50,000 event data

| Nominal FMH (mL) | Measured FMH (mL) | CV (%) | SD | P | 95% CI |
|---|---|---|---|---|---|
| 4.0 | 3.076 | 17.57 | 0.354 | <0.005 | 1.156 |
| 2.0 | 1.901 | 23.41 | 0.314 | <0.005 | 3.024 |
| 1.0 | 0.186 | 35.75 | 0.408 | <0.005 | 1.331 |
| 0.5 | -0.095 | 91.29 | 0.288 | 0.39 | 0.94 |
| 0.2 | -0.106 | 181.69 | 0.5 | 0.856 | 1.257 |

Table 3.2c Results of fold change measurement of prepared mixtures of D+ and D- cells mimicking FMH samples acquiring 100,000 events

| Nominal FMH (mL) | Measured FMH (mL) | CV (%) | SD | P | 95% CI |
|---|---|---|---|---|---|
| 4.0 | 3.365 | 8.71 | 0.226 | <0.005 | 0.738 |
| 2.0 | 1.564 | 16.92 | 0.209 | <0.005 | 0.681 |
| 1.0 | 1.024 | 29.02 | 0.231 | <0.005 | 0.752 |
| 0.5 | 0.259 | 78.08 | 0.175 | 0.047 | 0.57 |
| 0.2 | -0.109 | 298.93 | 0.194 | 0.467 | 0.488 |

Tables 3.2d-f Results of FMH optimisation. Nominal FMH of spiked samples, mean measured FMH and associated standard deviation (SD) ANOVA p values for difference between means of negative control and each D+/D- mixture, and 95% confidence interval (CI), expressed for each nominal FMH size (4.0-0.2mL) using each flow cytometry protocol (250,000-$10^6$ events)

Table 3.2d Results of fold change measurement of prepared mixtures of D+ and D- cells mimicking FMH samples acquiring 250,000 events

| Nominal FMH (mL) | Measured FMH (mL) | CV (%) | SD | p | 95% CI |
|---|---|---|---|---|---|
| 4.0 | 3.164 | 11.64 | 0.374 | <0.005 | 1.22 |
| 2.0 | 2.065 | 4.05 | 0.221 | <0.005 | 0.722 |
| 1.0 | 0.601 | 24.60 | 0.433 | <0.005 | 1.413 |
| 0.5 | 0.573 | 126.68 | 0.515 | 0.063 | 1.41 |
| 0.2 | -0.228 | 129.04 | 0.794 | 0.649 | 1.997 |

Table 3.2e Results of fold change measurement of prepared mixtures of D+ and D- cells mimicking FMH samples acquiring 500,000 events

| Nominal FMH (mL) | Measured FMH (mL) | CV (%) | SD | p | 95%CI |
|---|---|---|---|---|---|
| 4.0 | 3.446 | 8.13 | 0.222 | <0.005 | 0.723 |
| 2.0 | 1.9 | 16.01 | 0.24 | ,0.005 | 0.782 |
| 1.0 | 0.96 | 21.23 | 0.175 | <0.005 | 1.242 |
| 0.5 | 0.207 | 66.35 | 0.154 | 0.065 | 0.499 |
| 0.2 | 0.082 | 136.6 | 0.18 | 0.419 | 0.3224 |

Table 3.2f Results of fold change measurement of prepared mixtures of D+ and D- cells mimicking FMH samples acquiring $10^6$ events.

| Nominal FMH (mL) | Measured FMH (mL) | CV(%) | SD | p | 95% CI |
|---|---|---|---|---|---|
| 4.0 | 3.479 | 6.46 | 0.177 | <0.005 | 0.578 |
| 2.0 | 1.883 | 10.14 | 0.156 | <0.005 | 0.51 |
| 1.0 | 0.925 | 16.80 | 0.136 | <0.005 | 0.442 |
| 0.5 | 0.283 | 74.00 | 0.171 | 0.031 | 0.565 |
| 0.2 | 0.055 | 100.96 | 0.16 | 0.389 | 0.403 |

| Analyte | Concentration in lowest detected standard sample (pg/mL) |
|---|---|
| IFN-γ | 18.16 |
| IL-1β | 10.35 |
| IL-4 | 9.58 |
| IL-10 | 9.62 |
| IL-6 | 9.63 |
| IL-12p70 | 8.54 |
| IL-17A | 16.38 |
| TNF-α | 10.3 |

Table 3.3 Lower level of detectability of analytes tested by cytokine flow cytometry

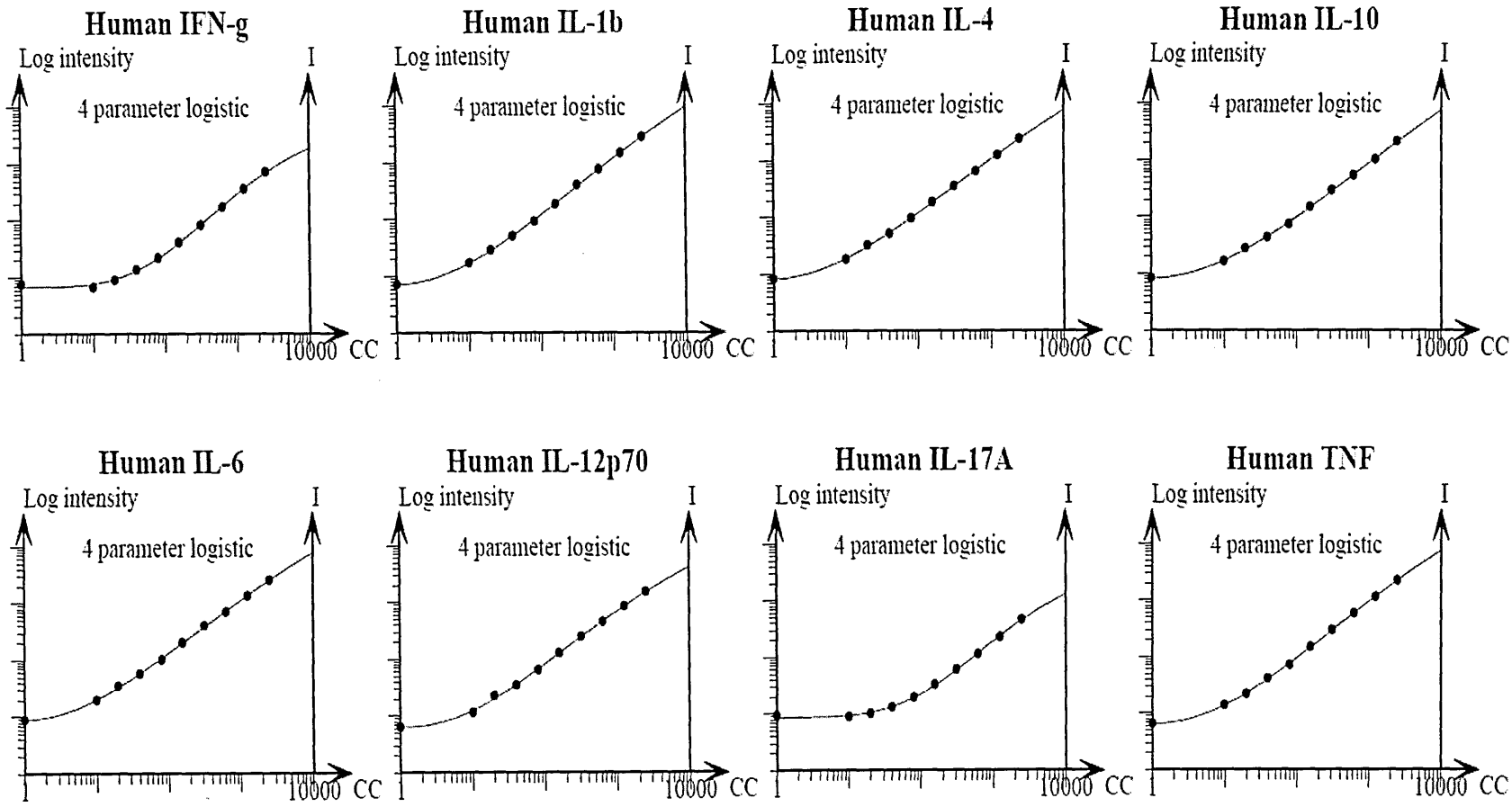



Figure 3.1 Cytokine flow cytometry standard curves for IFN-γ, IL-1β, IL-4, IL-10, IL-6, IL-12p70, IL-17A, TNF-α, showing dilution of standard on x-axis and fluorescent intensity on y-axis

## 3.4 The Measurement of IL-1β, IL-1ra, and TGF-β1 by ELISA in Participant Plasma Samples

Three analytes were tested by ELISA; selected as the most significant changes in Branch's data (2006) and IL-1β to allow comparison with IL-1ra. The raw data are included as Appendix VII.

All samples were assayed in duplicate and the mean absorbance of the replicates at 450nm calculated. The mean absorbance of the replicates at 570nm was calculated and subtracted from the 450nm value to compensate for optical variation of the wells. Standard curves were constructed for each analyte and the slope used to calculate the analyte concentration in the sample using Microsoft Excel software (Figures 3.2-3.4). Final plasma concentration was determined by multiplying by any dilution factor of the plasma in the test method (Appendix VII).

### 3.4.1 Results of ELISA Measurement of Concentration of IL-1β, IL-1ra and TGF-β1 in Participant Plasma Samples

Fold change values were calculated by dividing the value of the analyte concentration in the 1 hour or 24 hour sample by that in the pre-test sample. Statistical analysis was performed using SPSS software version 17.0 (SPSS inc, Chicago, Illinois). The significance of the difference between means on normally distributed data was assessed using independent samples T test, normality of data was assessed using Kolmogorov-Smirnov test values >0.05 were assumed to indicate normality, and all data series in this study were found to have normal distribution.

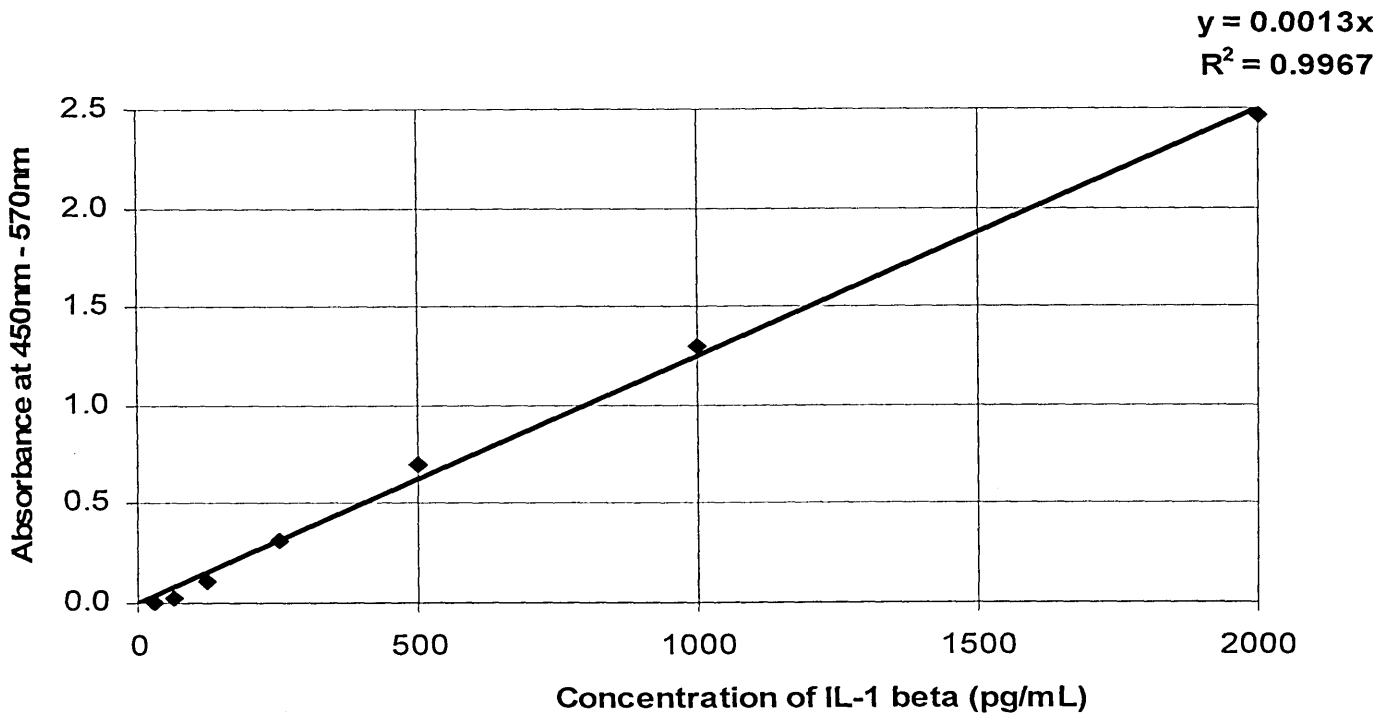



Figure 3.2 Standard curve, ELISA measurement of IL-1β. Concentration of standard on x-axis, absorbance on y-axis, showing formula for slope of curve, and regression line ($R^2$). Seven standard dilutions were prepared and tested in duplicate.

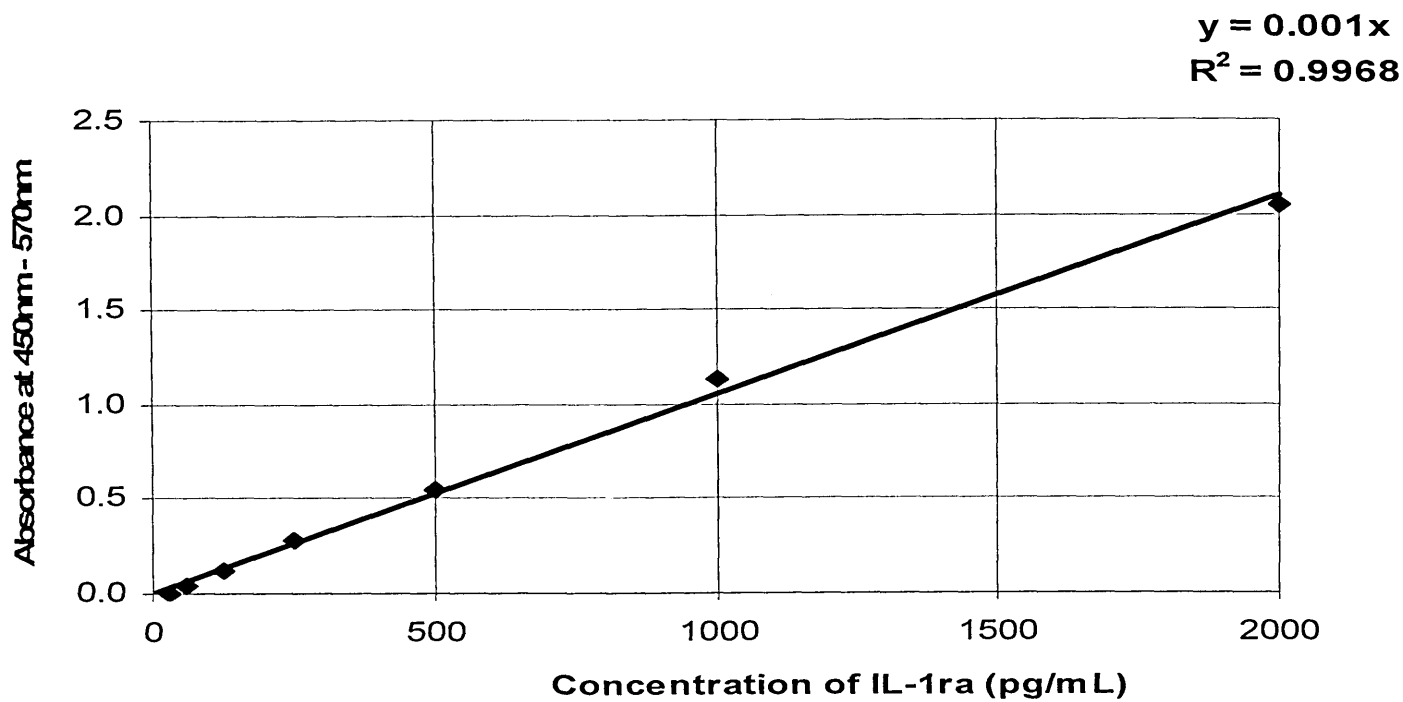



Figure 3.3 Standard curve, ELISA measurement of IL-1ra. Concentration of standard on x-axis, absorbance on y-axis, showing formula for slope of curve, and regression line ($R^2$). Seven standard dilutions were prepared and tested in duplicate.

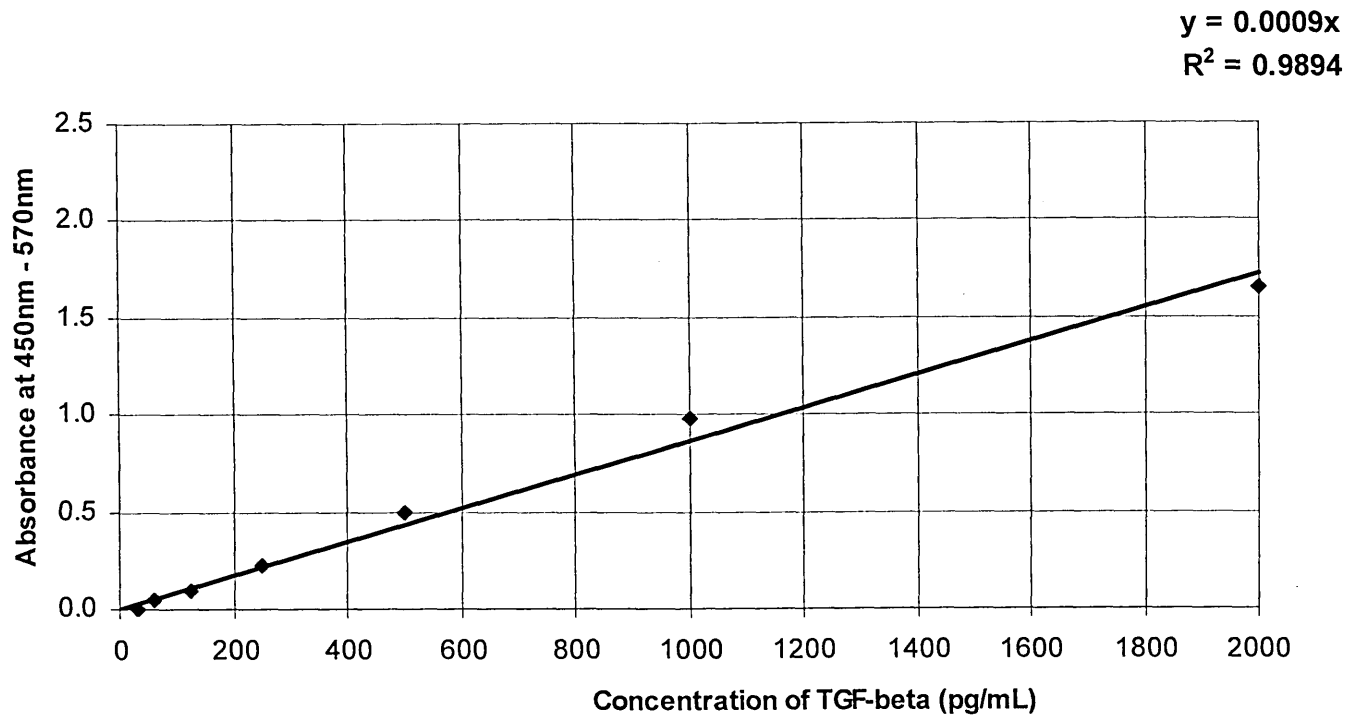



Figure 3.4 Standard curve, ELISA measurement of TGF-β1. Concentration of standard on x-axis, absorbance on y-axis, showing formula for slope of curve, and regression line ($R^2$). Seven standard dilutions were prepared and tested in duplicate.

### 3.4.2 The Results of Measurement of IL-1β Concentration in Participant Plasma Samples by ELISA

The results of ELISA assay of IL-1β concentration in the study samples are shown in Table 3.4. A summary of the results expressed as analyte concentration (Table 3.5) and fold change (Table 3.6) are included. Of the 17 participants in the D+ fetus group and 7 participants in the D- fetus group none had fold change in IL-1β concentration greater that 1.5, or below 0.5 in the 1 hour or 24 hour samples. There was no significant difference between means of fold change in D+ and D- fetus groups. Statistical analysis of IL-1β fold change data is shown in Table 3.6. Correlation in the fold change results obtained for each participant at 1 and 24 hours was strong in both D+ and D- fetus groups (Pearsons's correlation coefficient 0.714, p=0.002) the D- fetus group (0.696, p=0.072, therefore not significant in the D- fetus group n=7) (Figure 3.5). Graphical representations are included of positive and negative fold changes (Figure 3.6), and comparing the range of fold changes in each group (Figure 3.7)

### 3.4.3 The Results of Measurement of IL-1ra Concentration in Participant Plasma Samples by ELISA

The results of ELISA assay of IL-1ra concentration in study samples are shown in Table 3.7. A summary of the results expressed as analyte concentration are included (Table 3.8) and fold change (Table 3.9).IL-1ra concentrations through the time series are shown (Figures 3.8, and 3.9). One participant in the D+ fetus group (1 hour sample) and one in the D- fetus group (24 hour sample) had fold changes of IL-1ra of greater than 1.5 and none below 0.5. Of the two increases of greater than 1.5 fold, participant 7 in the D+ fetus group had a concentration of IL-1ra below, but within 1SD of the group mean before treatment. The participant in the D-fetus group had an IL-1ra concentration above, but within 1SD of the group mean. No significant difference between means of fold change in D+ and D- fetus groups was noted when calculated by independent T test (p=0.575 and

0.334 respectively. (Table 3.9). Correlation in the fold change results obtained for each participant at one and twenty four hours was strong in the D+ fetus group (Pearsons's correlation coefficient 0.739, p=0.001) but not the D- fetus group (0.417, p=0.355) (Figure 3.10). Graphical representations are included of positive and negative fold changes (Figure 3.11), and comparing range of fold change in each group (Figure 3.12).

### 3.4.4 The Results of Measurement of TGF-β1 Concentration in Participant Plasma Samples by ELISA

TGF-β1 ELISA results are shown in Table 3.10, and summarised in Table 3.11. Fold change analysis is in Table 3.12. ELISA results through the time series are included (Figures 3.13 and 3.14). In the D+ fetus group, 8/17 participants had fold increases in TGF-β1 in either the 1 hour (4) or 24 hour (4) samples, or both (3) and 8 had decreases below 0.5 in either the 1 hour (4), 24 hour (4) samples, or both (1). In the D- fetus group one participant had a fold increase in TGF-β1 greater than 1.5 at 1 hour. No significant difference between means of fold change in D+ and D- fetus groups was noted by independent T test (p=0.273 and 0.140 respectively).Correlation in the fold change results obtained with each participant at 1 and 24 hours was weak in the D+ fetus group (Pearsons's correlation coefficient 0.327, p=0.200) but stronger in the D- fetus group (0.778, p=0.04). Of the 8 participants having fold increases greater than 1.5, 7 had TGF-β1 concentrations below the group mean in the pre-anti-D sample, 2 of those being more than 1SD below the mean. Of the 9 participants in the D+ fetus group having fold decrease below 0.5, 9 had TGF-β1 concentrations above the group mean, one of which was more than 1SD above the mean. Graphical representations are included of positive and negative fold changes (Figures 3.15, 3.16), and comparing range of fold change in each group (Figure 3.17).

| | IL-1 beta ELISA (pg/mL) | | | | |
|---|---|---|---|---|---|
| | Sample | | | Fold change | |
| | Before anti-D | 1 hour | 24 hour | 1 hour | 24 hour |
| 1 | 26.125 | 24.458 | 36.125 | 0.94 | 1.38 |
| 2 | 33.625 | 31.958 | 25.292 | 0.95 | 0.75 |
| 3 | 31.125 | 31.958 | 28.625 | 1.03 | 0.92 |
| 4 | 31.958 | 26.125 | 31.125 | 0.82 | 0.97 |
| 5 | 28.625 | 26.958 | 29.458 | 0.94 | 1.03 |
| 6 | 38.625 | 29.458 | 30.292 | 0.76 | 0.78 |
| 7 | 41.125 | 23.625 | 23.625 | 0.57 | 0.57 |
| 8 | 30.292 | 29.458 | 31.125 | 0.97 | 1.03 |
| 9 | 39.458 | 32.792 | 36.958 | 0.83 | 0.94 |
| 10 | 28.625 | 27.792 | 28.625 | 0.97 | 1.00 |
| 11 | 27.792 | 33.625 | 31.125 | 1.21 | 1.12 |
| 12 | 26.958 | 28.625 | 31.125 | 1.06 | 1.15 |
| 13 | 26.125 | 24.458 | 27.792 | 0.94 | 1.06 |
| 14 | 30.269 | 25.653 | 27.961 | 0.85 | 0.92 |
| 15 | 26.423 | 27.192 | 22.576 | 1.03 | 0.85 |
| 16 | 29.500 | 27.961 | 28.730 | 0.95 | 0.97 |
| 17 | 29.500 | 24.884 | 27.192 | 0.84 | 0.92 |
| 18 | 24.115 | 24.115 | 26.423 | 1.00 | 1.10 |
| 19 | 27.192 | 30.269 | 37.192 | 1.11 | 1.37 |
| 20 | 37.961 | 31.807 | 45.653 | 0.84 | 1.20 |
| 21 | 29.500 | 32.576 | 32.576 | 1.10 | 1.10 |
| 22 | 34.115 | 16.423 | 15.653 | 0.48 | 0.46 |
| 23 | 14.115 | 15.653 | 14.115 | 1.11 | 1.00 |
| 24 | 12.576 | 12.576 | 14.115 | 1.00 | 1.12 |

Table 3.4 Analyte concentrations in pre-treatment, 1 hour and 24 hour post-treatment samples, and fold changes from pre-treatment and 1 and 24 hour samples of IL-1β detected by ELISA. Shading indicates participants with D+ fetuses.

| Group | Interval | Range of Fold change | Mean Fold change | SD | Significance of difference between means in D+ vs D- fetus groups (independent T test) |
|---|---|---|---|---|---|
| D+ fetus group n=17 | 1 hour | 0.57-1.11 | 0.93 | 0.14 | p= 0.806 |
| | 24 hours | 0.57-1.37 | 0.99 | 0.18 | p=0.846 |
| D- fetus group n=7 | 1 hour | 0.48-1.21 | 0.92 | 0.22 | |
| | 24 hours | 0.46-1.38 | 0.98 | 0.28 | |

Table 3.5 Summary of analyte concentration results for IL-1β measured by ELISA

| | IL-1β (pg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | D+ fetus group n-17 | | | D- fetus group n=7 | | |
| | Range | Mean | SD | Range | Mean | SD |
| Pre | 12.6-41.3 | 29.6 | 7.8 | 26.1-34.1 | 28.7 | 3.0 |
| 1 hour | 12.6-32.8 | 27.0 | 5.7 | 16.4-33.6 | 25.9 | 5.3 |
| 24 hour | 14.1-45.6 | 28.9 | 7.6 | 15.63-36.13 | 27.5 | 6.7 |

Table 3.6 Statistical analysis of IL-1β ELISA data, (SD standard deviation), showing no significant difference between means of D+ and D- fetus groups.

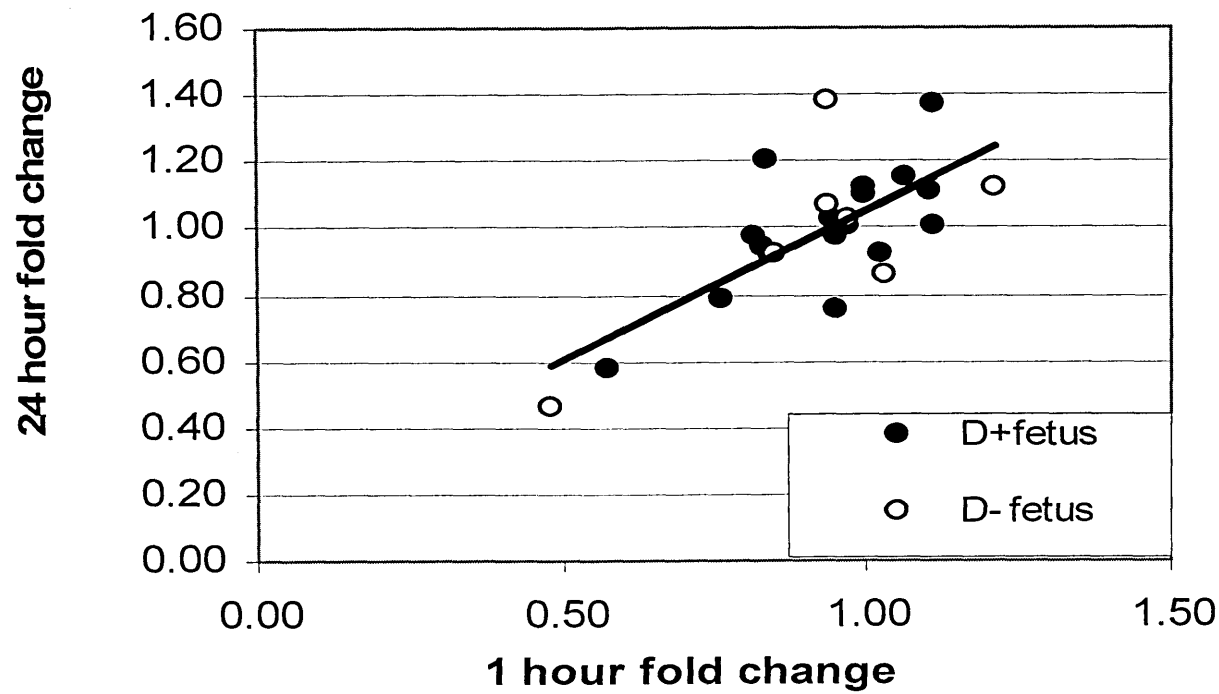



Figure 3.5 Correlation between fold changes detected by ELISA in IL-1β detected in 1 hour (x axis) and 24 hour (y axis) samples in D+ and D- fetus groups. Trend lines drawn by Microsoft Excel and for D+ and D- fetus groups coincide ($R^2$=0.509).

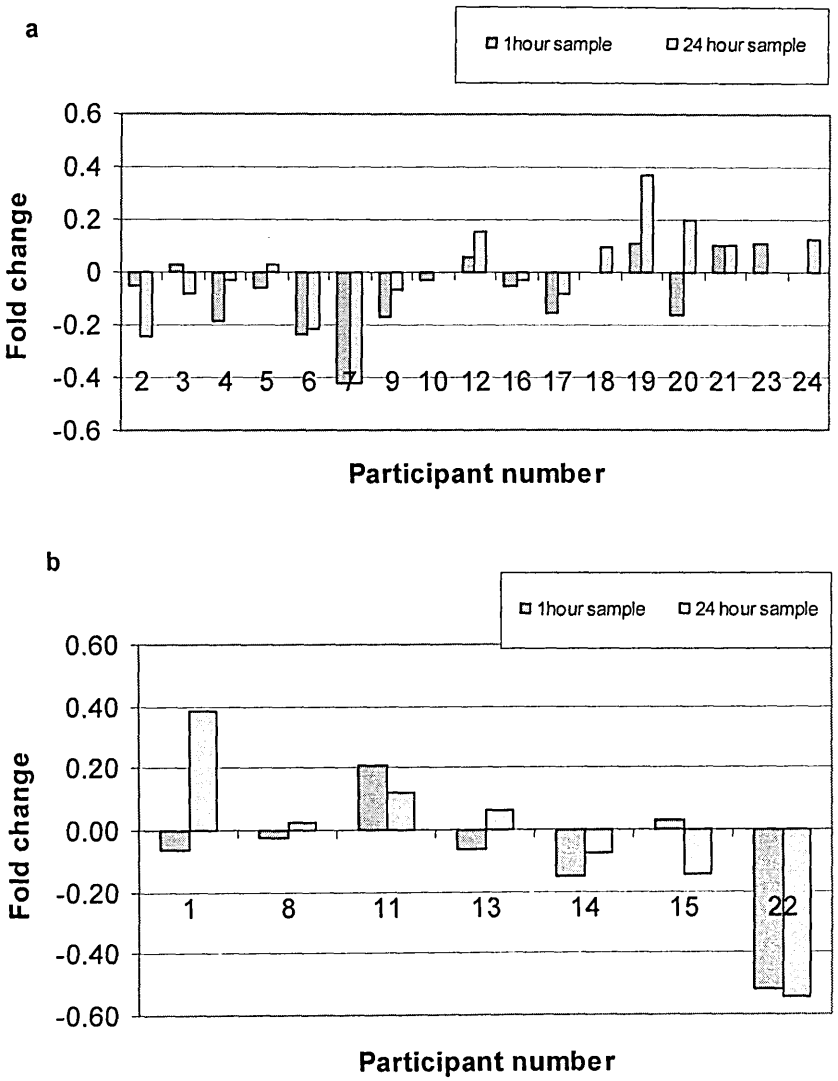



Figure 3.6 Distribution of positive and negative fold changes in IL-1β among 1 hour and 24 hour samples, in D+ fetus group (a) and D- fetus group (b). Fold change of 0 represents no change in this figure.

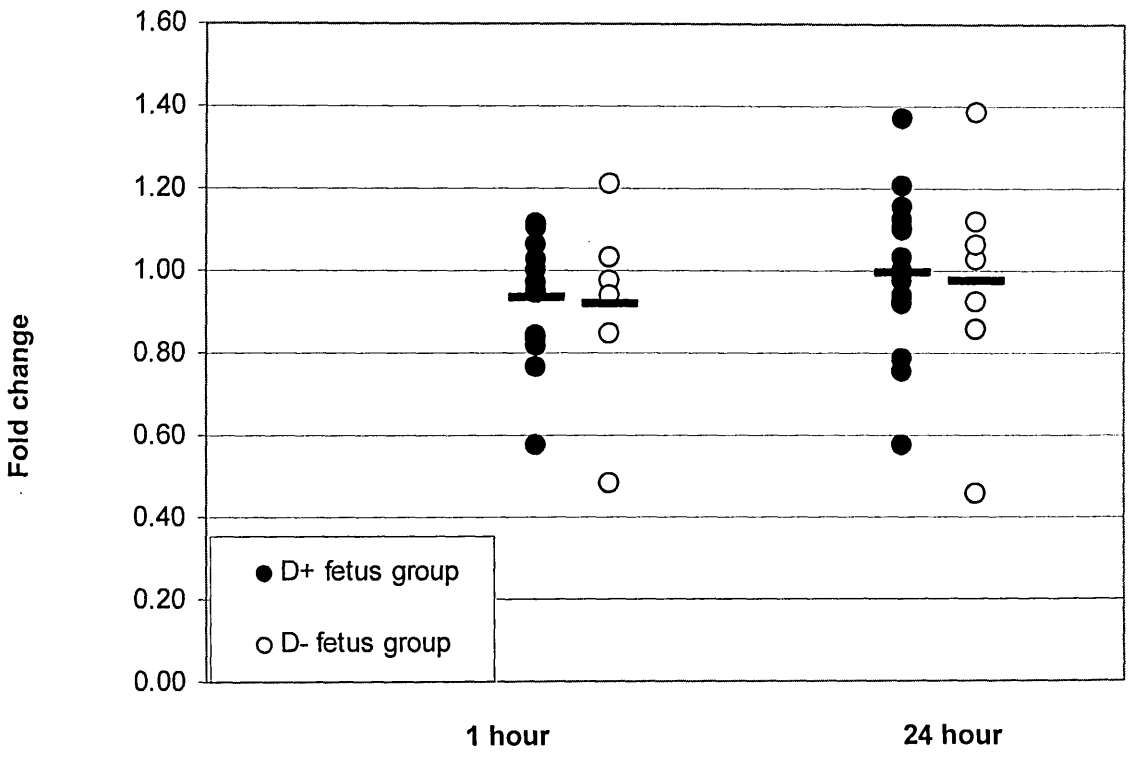



Figure 3.7 Fold change in IL-1β detected by ELISA in D+ and D- fetus groups in 1 hour and 24 hour samples. Fold change of 1 represents no change. Independent T test, significance of difference between means of D+ and D- fetus groups: 1hour p=0.806, 24 hours p=0.846

| | IL-1 ra ELISA (pg/mL) | | | | |
|---|---|---|---|---|---|
| | Sample | | | Fold change | |
| | Before anti-D | 1 hour | 24 hour | 1 hour | 24 hour |
| 1 | 226.50 | 201.00 | 206.00 | 0.89 | 0.91 |
| 2 | 683.00 | 692.00 | 536.00 | 1.01 | 0.78 |
| 3 | 326.00 | 245.50 | 283.00 | 0.75 | 0.87 |
| 4 | 322.00 | 324.50 | 317.50 | 1.01 | 0.99 |
| 5 | 777.00 | 858.00 | 799.00 | 1.10 | 1.03 |
| 6 | 586.50 | 649.00 | 742.00 | 1.11 | 1.27 |
| 7 | 260.00 | 435.50 | 363.00 | 1.68 | 1.40 |
| 8 | 735.50 | 762.00 | 895.50 | 1.04 | 1.22 |
| 9 | 454.00 | 252.50 | 351.50 | 0.56 | 0.77 |
| 10 | 387.00 | 275.50 | 297.50 | 0.71 | 0.77 |
| 11 | 688.00 | 842.50 | 1077.00 | 1.22 | 1.57 |
| 12 | 363.00 | 407.00 | 430.00 | 1.12 | 1.18 |
| 13 | 286.50 | 254.50 | 333.50 | 0.89 | 1.16 |
| 14 | 772.31 | 828.08 | 713.46 | 1.07 | 0.92 |
| 15 | 228.46 | 252.31 | 169.23 | 1.10 | 0.74 |
| 16 | 371.54 | 358.46 | 358.85 | 0.96 | 0.97 |
| 17 | 558.85 | 483.85 | 582.31 | 0.87 | 1.04 |
| 18 | 223.46 | 239.23 | 206.15 | 1.07 | 0.92 |
| 19 | 307.31 | 321.15 | 420.00 | 1.05 | 1.37 |
| 20 | 290.00 | 255.38 | 267.69 | 0.88 | 0.92 |
| 21 | 267.31 | 273.08 | 230.77 | 1.02 | 0.86 |
| 22 | 386.92 | 446.54 | 460.77 | 1.15 | 1.19 |
| 23 | 211.54 | 189.62 | 180.77 | 0.90 | 0.85 |
| 24 | 268.85 | 314.23 | 289.62 | 1.17 | 1.08 |

Table 3.7 Analyte concentrations in pre-treatment, 1 hour and 24 hour post-treatment samples, and fold changes from pre-treatment and 1 and 24 hour samples of IL-1ra detected by ELISA. Shading indicates participants with D+ fetuses.

| IL-1ra (pg/mL) | | | | | | |
|---|---|---|---|---|---|---|
| | D+ fetus group n=17 | | | D- fetus group n=7 | | |
| | Range | Mean | SD | Range | Mean | SD |
| Pre | 211.5-777.0 | 391.6 | 165.8 | 226.5-772.3 | 474.9 | 247.5 |
| 1 hour | 189.6-858.0 | 386.7 | 185.7 | 201.0-842.5 | 512.4 | 290.5 |
| 24 hour | 180.8-799.0 | 391.5 | 178.3 | 169.2-1077.0 | 550.8 | 351.7 |

Table 3.8 Summary of analyte concentration results for IL-1ra measured by ELISA

| Group | Interval | Range of Fold change | Mean Fold change | SD | Significance of difference between means in D+ vs D- fetus groups (independent T test) |
|---|---|---|---|---|---|
| D+ fetus group n=17 | 1 hour | 0.56-1.71 | 1.00 | 0.24 | p=0.575 |
| | 24 hours | 0.77-1.40 | 1.00 | 0.20 | p=0.334 |
| D- fetus group n=7 | 1 hour | 0.89-1.22 | 1.05 | 0.13 | |
| | 24 hours | 0.74-1.57 | 1.10 | 0.27 | |

Table 3.9 Statistical analysis of IL-1ra ELISA results, (Fold change fold change, SD standard deviation)

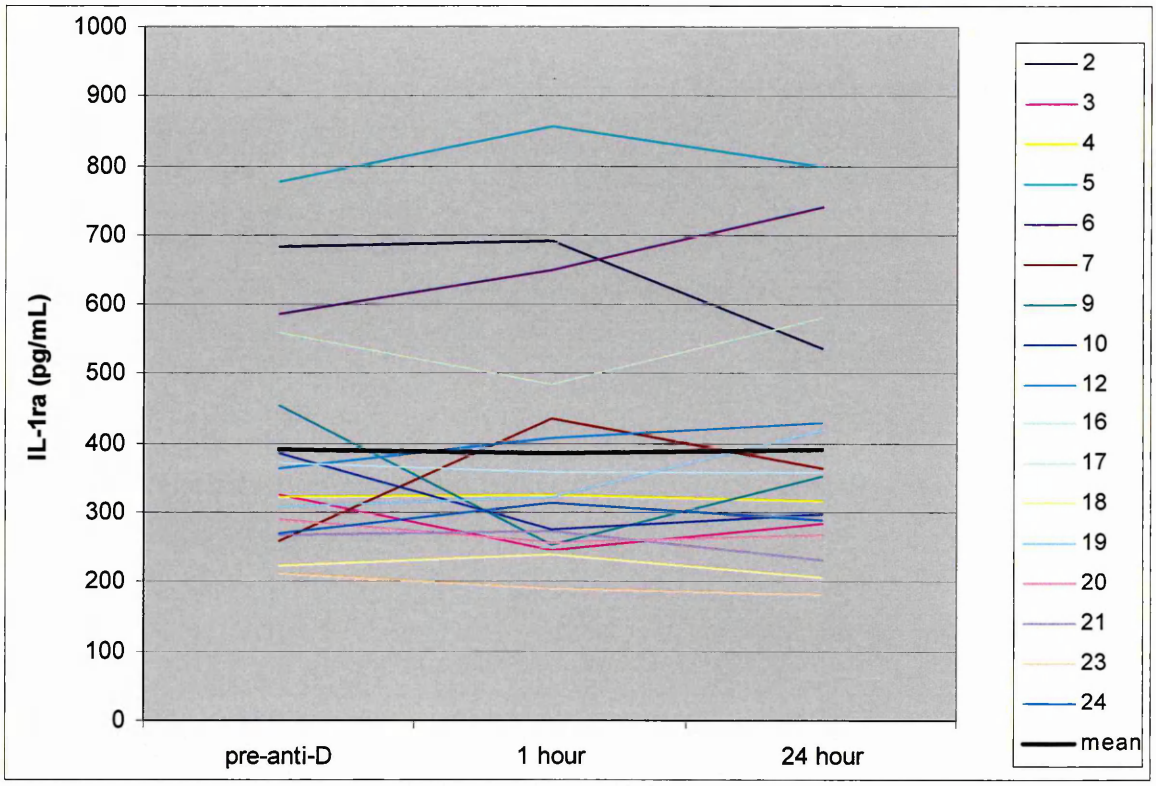



Figure 3.8 IL-1ra concentration measured by ELISA in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D+ fetus group (n=17). The mean line for the group is shown in black .

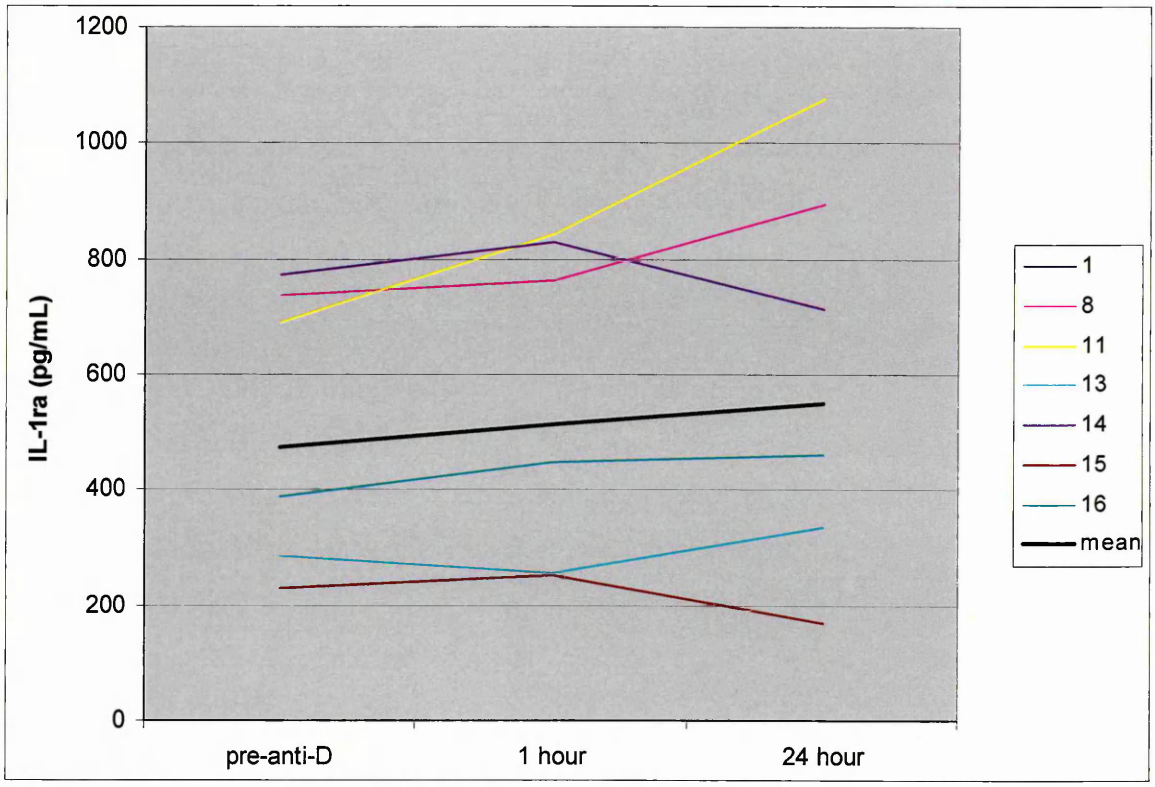



Figure 3.9 IL-1ra concentration measured by ELISA in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D- fetus group (n=7). The mean line for the group is shown in black.

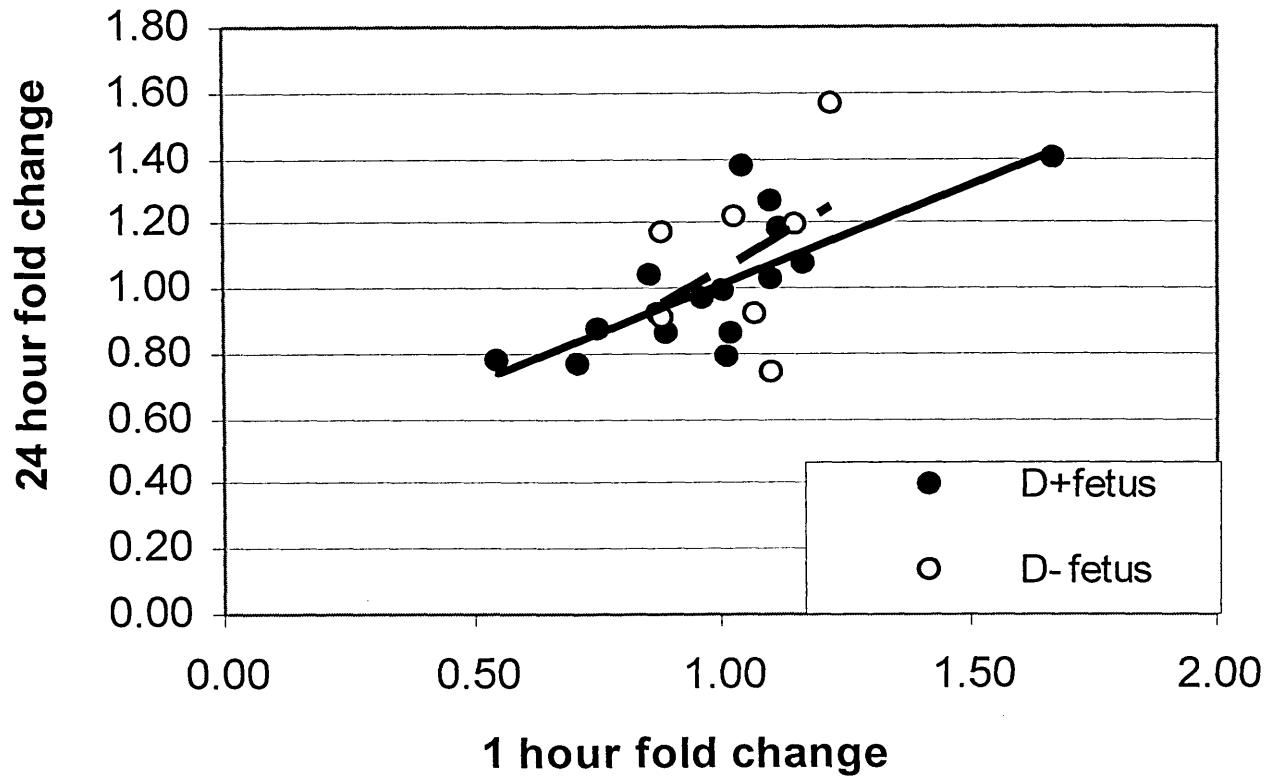
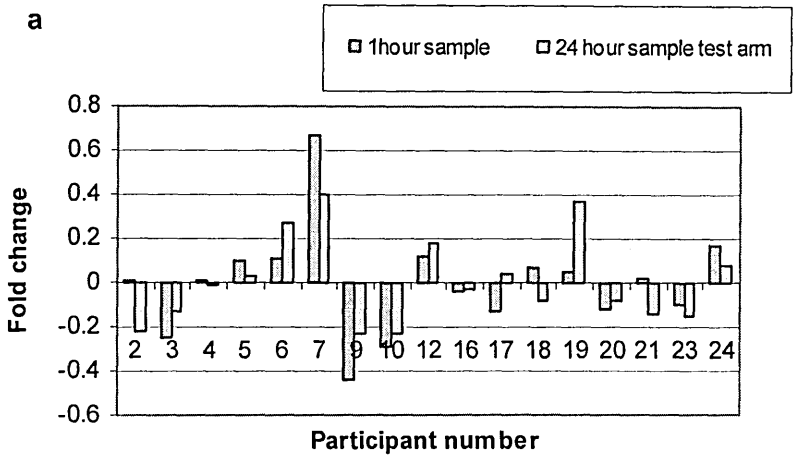



Figure 3.10. Correlation between fold changes detected by ELISA in IL-1ra detected in 1 hour (x axis) and 24 hour (y axis) samples in D+ and D- fetus groups. Trend lines (D+ fetus group solid line $R^2$=0.55, D- fetus group broken line $R^2$=0.17) drawn by Microsoft Excel



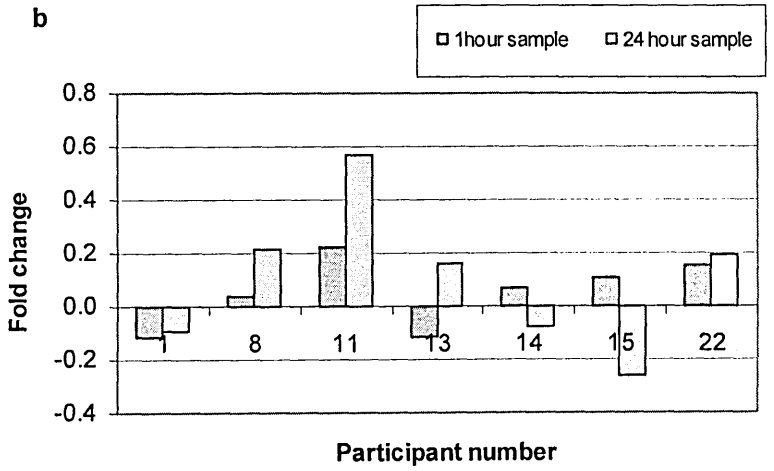




Figure 3.11 Distribution of positive and negative fold changes in IL-1ra detected by ELISA in 1 hour and 24 hour samples, in D+ fetus group (a) and D- fetus group (b). Fold change of 0 represents no change in this figure

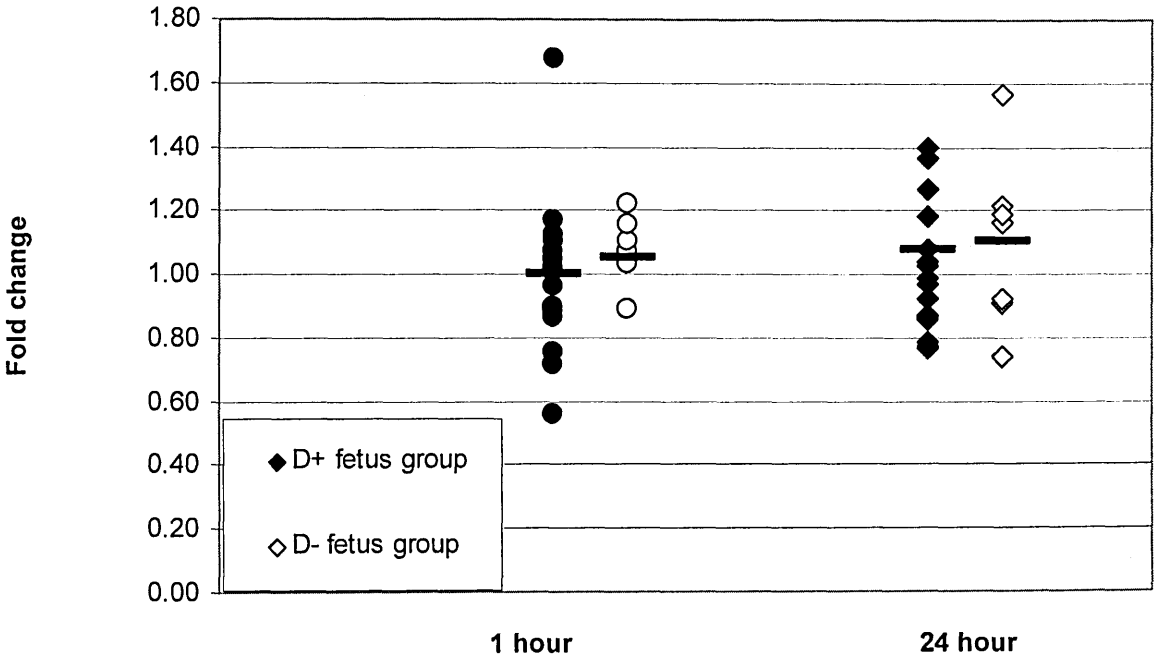



Figure 3.12 Fold change in IL-1ra detected by ELISA in the D+ and D- fetus groups in 1 hour and 24 hour samples. Fold change of 1 represents no change in this figure.

Independent T test, significance of difference between means of D+ and D- fetus groups: 1hour p=0.575, 24 hours p=0.534

| | TGF-β1 ELISA (pg/mL) | | | | |
|---|---|---|---|---|---|
| | Sample | | | Fold change | |
| | Before anti-D | 1 hour | 24 hour | 1 hour | 24 hour |
| 1 | 35266.67 | 47688.89 | 15400.00 | 1.35 | 0.44 |
| 2 | 48644.44 | 17488.89 | 3466.67 | 0.36 | 0.07 |
| 3 | 50044.44 | 22533.33 | 26911.11 | 0.45 | 0.54 |
| 4 | 51800.00 | 15577.78 | 60555.56 | 0.30 | 1.17 |
| 5 | 54644.44 | 48044.44 | 49622.22 | 0.88 | 0.91 |
| 6 | 79911.11 | 47088.89 | 12555.56 | 0.59 | 0.16 |
| 7 | 22222.22 | 13488.89 | 23622.22 | 0.61 | 1.06 |
| 8 | 73288.89 | 43555.56 | 50244.44 | 0.59 | 0.69 |
| 9 | 34866.67 | 19866.67 | 7600.00 | 0.57 | 0.22 |
| 10 | 27044.44 | 44022.22 | 47533.33 | 1.63 | 1.76 |
| 11 | 33155.56 | 55955.56 | 11444.44 | 1.69 | 0.35 |
| 12 | 11600.00 | 45622.22 | 29888.89 | 3.93 | 2.58 |
| 13 | 73822.22 | 42288.89 | 28666.67 | 0.57 | 0.39 |
| 14 | 65622.22 | 25111.11 | 23933.33 | 0.38 | 0.36 |
| 15 | 79511.11 | 51733.33 | 19288.89 | 0.65 | 0.24 |
| 16 | 52600.00 | 35600.00 | 12644.44 | 0.68 | 0.24 |
| 17 | 25066.67 | 39400.00 | No result | 1.57 | No Value |
| 18 | 10977.78 | 30400.00 | 21488.89 | 2.77 | 1.96 |
| 19 | 50622.22 | 21288.89 | 32555.56 | 0.42 | 0.64 |
| 20 | 37266.67 | 61733.33 | 43755.56 | 1.66 | 1.17 |
| 21 | 18355.56 | 17133.33 | 63733.33 | 0.93 | 3.47 |
| 22 | 71577.78 | 39644.44 | 32511.11 | 0.55 | 0.45 |
| 23 | 11044.44 | 41800.00 | 7022.22 | 3.78 | 0.64 |
| 24 | 29266.67 | 48888.89 | 23777.78 | 1.67 | 0.81 |

Table 3.10 Analyte concentrations in pre-treatment, 1 hour and 24 hour post-treatment samples, and fold changes from pre-treatment and 1 and 24 hour samples of TGF-β1 detected by ELISA. Shading indicates participants with D+ fetuses.

| | TGF-β1(pg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | D+ fetus group | | | D- fetus group | | |
| | Range | Mean | SD | Range | Mean | SD |
| Pre | 1044-79911 | 36234 | 19307 | 33155-79511 | 61749 | 19257 |
| 1 hour | 13488-61733 | 33520 | 14804 | 25111-55955 | 43711 | 9955 |
| 24 hour | 3466-63733 | 29171 | 18455 | 11444-50244 | 25927 | 12979 |

Table 3.11 Summary of analyte concentration results for TGF-β1 measured by ELISA

| Group | Interval | Range of Fold change | Mean Fold change | SD | Significance of difference between means in D+ vs D- fetus groups (independent T test) |
|---|---|---|---|---|---|
| D+ fetus group n=17 | 1 hour | 0.3-3.93 | 1.32 | 1.15 | p=0.273 |
| | 24 hours | 0.2-3.89 | 1.08 | 0.91 | p=0.140 |
| D- fetus group n=7 | 1 hour | 0.38-1.35 | 0.83 | 0.42 | |
| | 24 hours | 0.2-1.15 | 0.64 | 0.14 | |

Table 3.12 Statistical analysis of TGF-β1 ELISA results (SD, standard deviation)

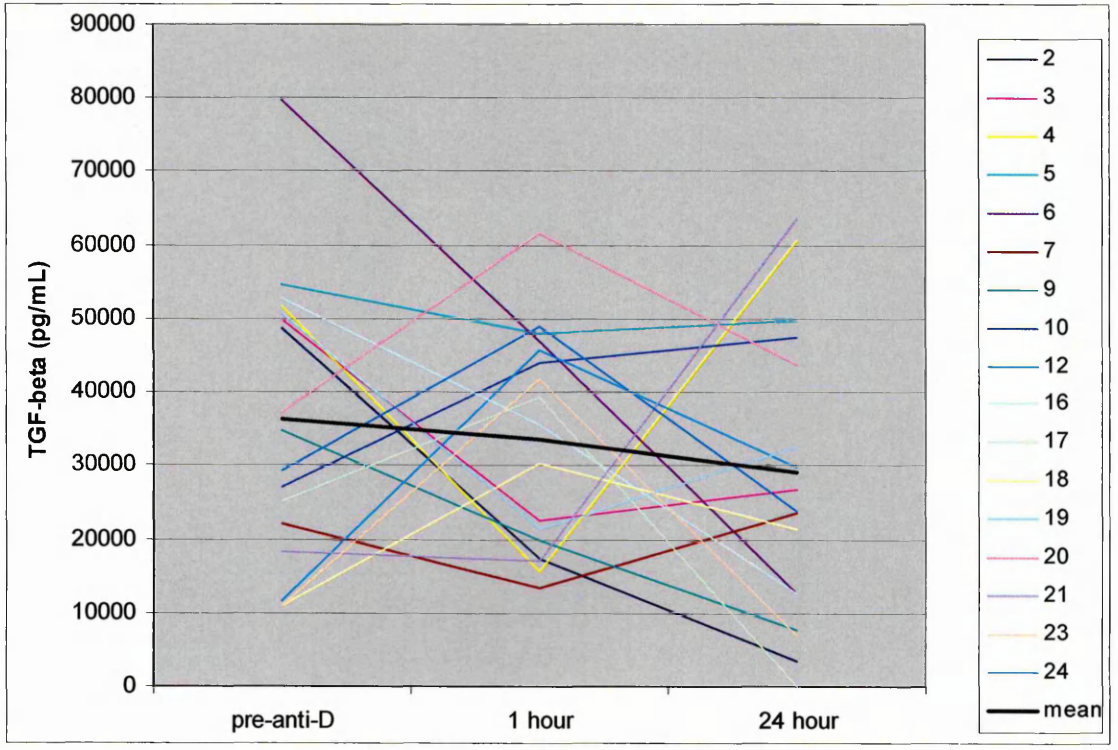



Figure 3.13 TGF-β1 concentration (pg/mL) measured by ELISA in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D+ fetus group (n=17). The mean line shown is calculated from entire cohort.

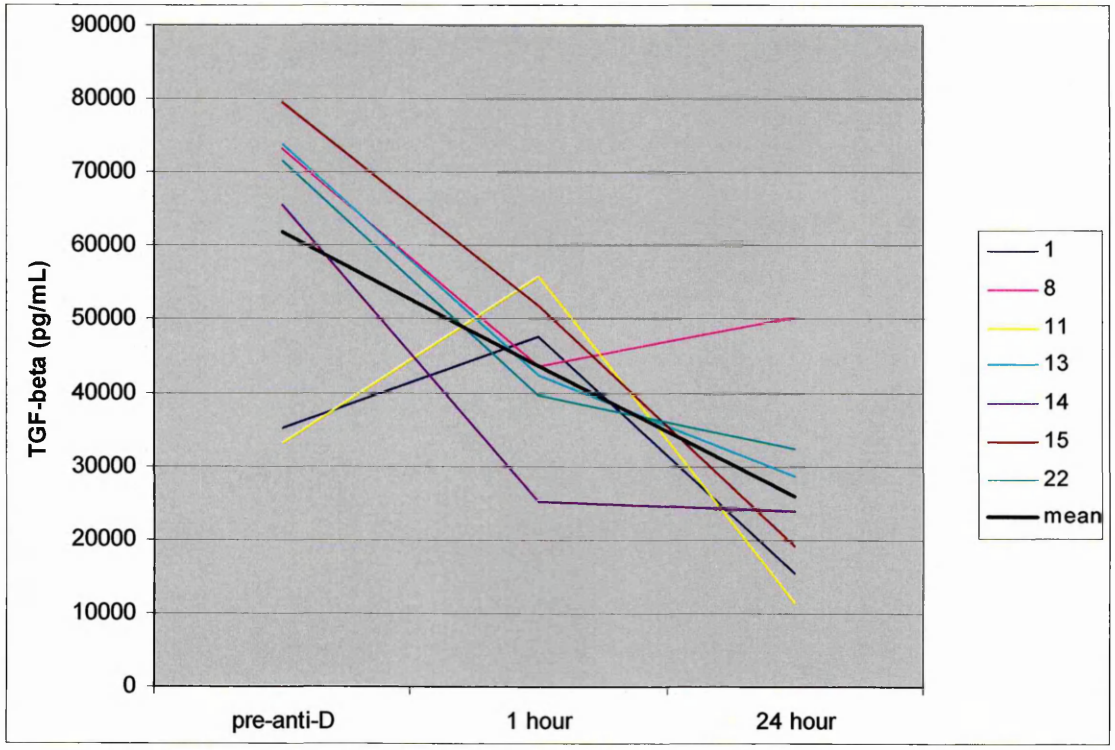



Figure 3.14 TGF-β1 concentration (pg/mL) measured by ELISA in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D- fetus group (n=7). The mean line shown is calculated from entire cohort.

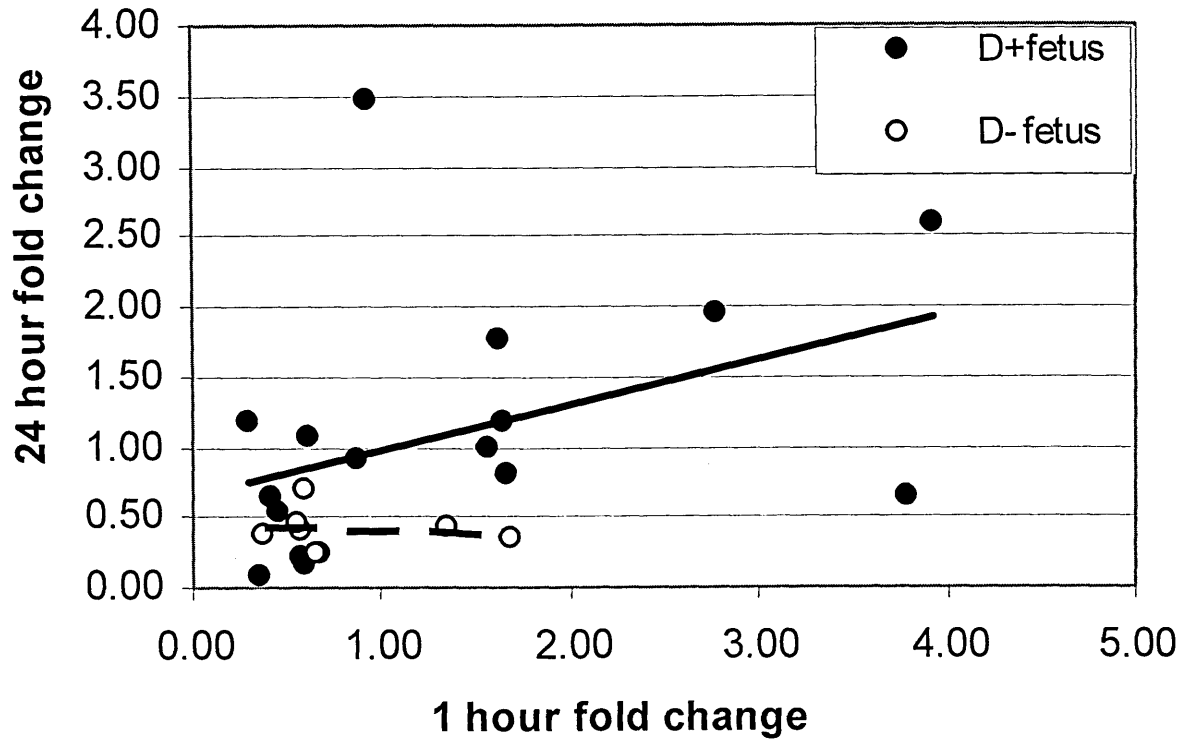



Figure 3.15. Correlation between fold changes detected by ELISA in TGF-β1 detected in 1 hour (x axis) and 24 hour (y axis) samples in D+ and D- fetus groups. Trend lines (D+ fetus group solid line, $R^2$= 0.11 D- fetus group broken line, $R^2$=0.02) drawn by Microsoft Excel.

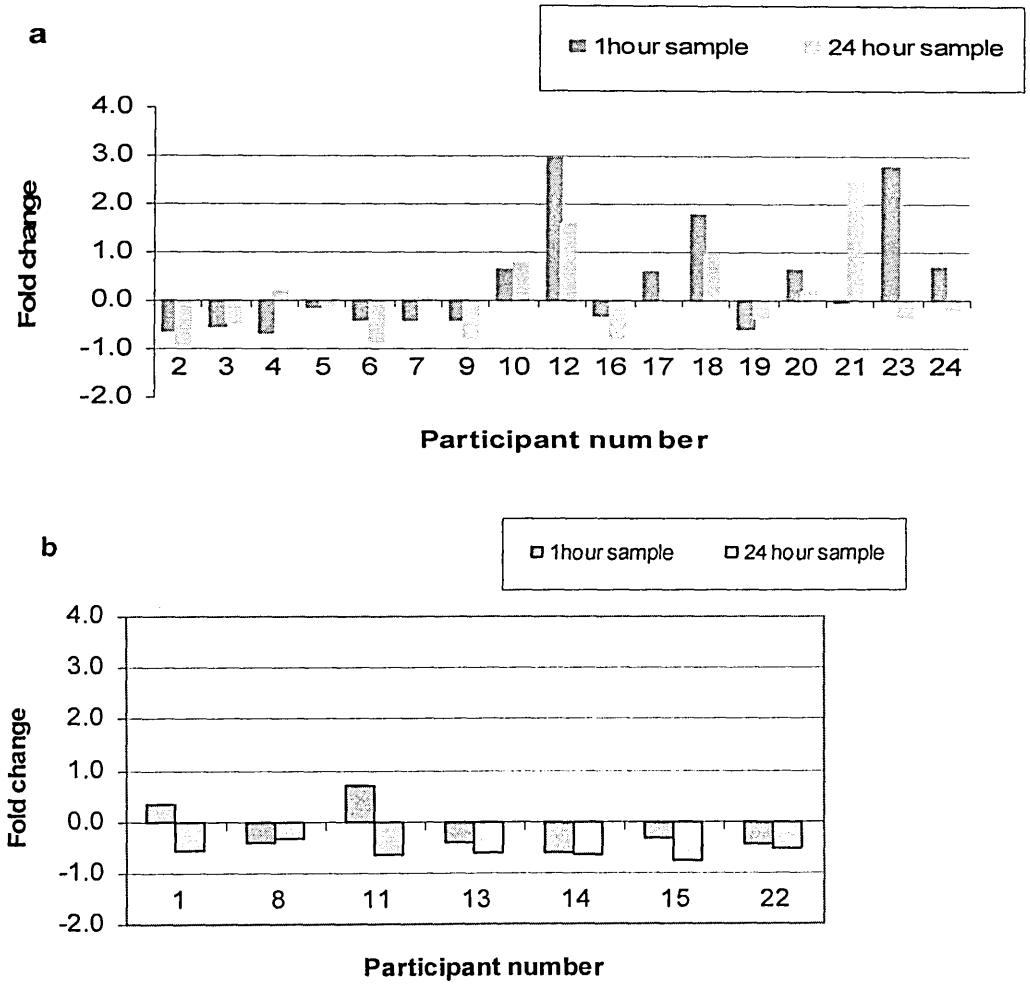



Figure 3.16 Distribution of positive and negative fold changes in TGF-β1 among 1 hour and 24 hour samples, in D- fetus group (a) and D- fetus group (b). Fold change of 0 represents no change.

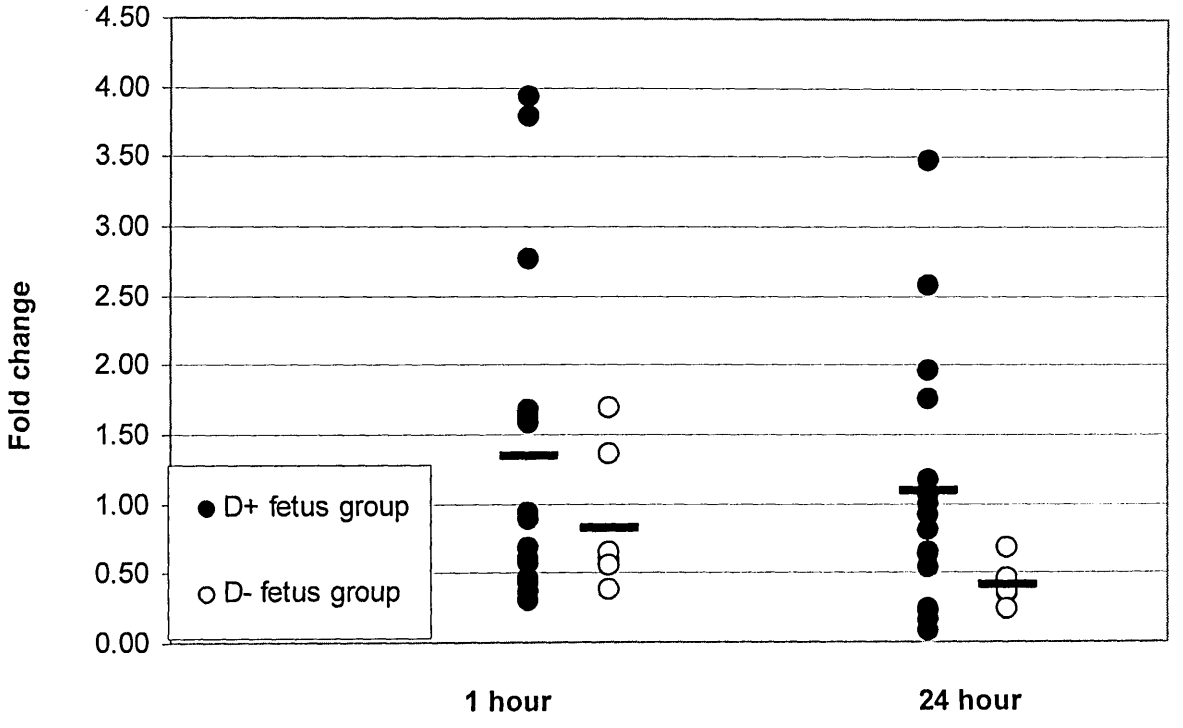



Figure 3.17 Fold change in TGF-β1 detected by ELISA in D+ and D- fetus groups in 1 hour and 24 hour samples, fold change of 1 represents no change. Independent T test, significance of difference between means of D+ and D- fetus groups: 1hour p=0.273, 24 hours p=0.140.

## 3.5 Results of RNA Extraction and RNA Assay for IL-1ra and TGF-β1 by qPCR

### 3.5.1 RNA extraction

The quantity and purity of extracted RNA was measured on each sample using a nanodrop 1000 instrument (Thermo Scientific Ltd) Light from a pulsed xenon lamp was passed through a 1μL sample of RNA in aqueous solution. The absorbance was measured at 230nm, and the concentration of RNA calculated by applying Beer-Lambert's law: (http//:nanodrop.com/library.nd-1000).

$$C=(Axe)b$$

Where

c= concentration of RNA (ng/μL)

A=absorbance

E=wavelength dependent extinction coefficient (40 for RNA)

B=path length

An example of the Nanodrop 1000 output is included ( Figure 3.18). Quantities of isolated RNA are listed in Appendix VIII.

### 3.5.2 Selection of analytes and endogenous controls

Based on ELISA results suggesting changes in the concentration of IL-1ra and TGF-β1 after treatment these two analytes were selected for analysis by qPCR.

Comparative qPCR to assess gene expression, as applied in this study, requires the use of an endogenous control gene, that is unlikely to vary during the study and against which study genes can be compared. Three widely used endogenous controls, GAPDH, Cyclophilin, and β actin were selected and assessed against cDNA isolated from 6 samples from the study along with the

study analytes. Mean CT and standard values were similar for all three genes; GAPDH had the lowest SD (Appendix IX) and was selected for use in the remainder of the study.

### 3.5.3 Validation of PCR response using cDNA dilution templates

The PCR response was validated with dilutions of pooled cDNA from the sample tested against each analyte and the endogenous control gene. An example of the amplification plot is included (Figure 3.19) and all results (Appendix X ). A plot of the response curves indicates good linearity and the gradients of the lines indicate high PCR performance, a slope of 3.3 representing 100% efficiency (Figure 3.20).

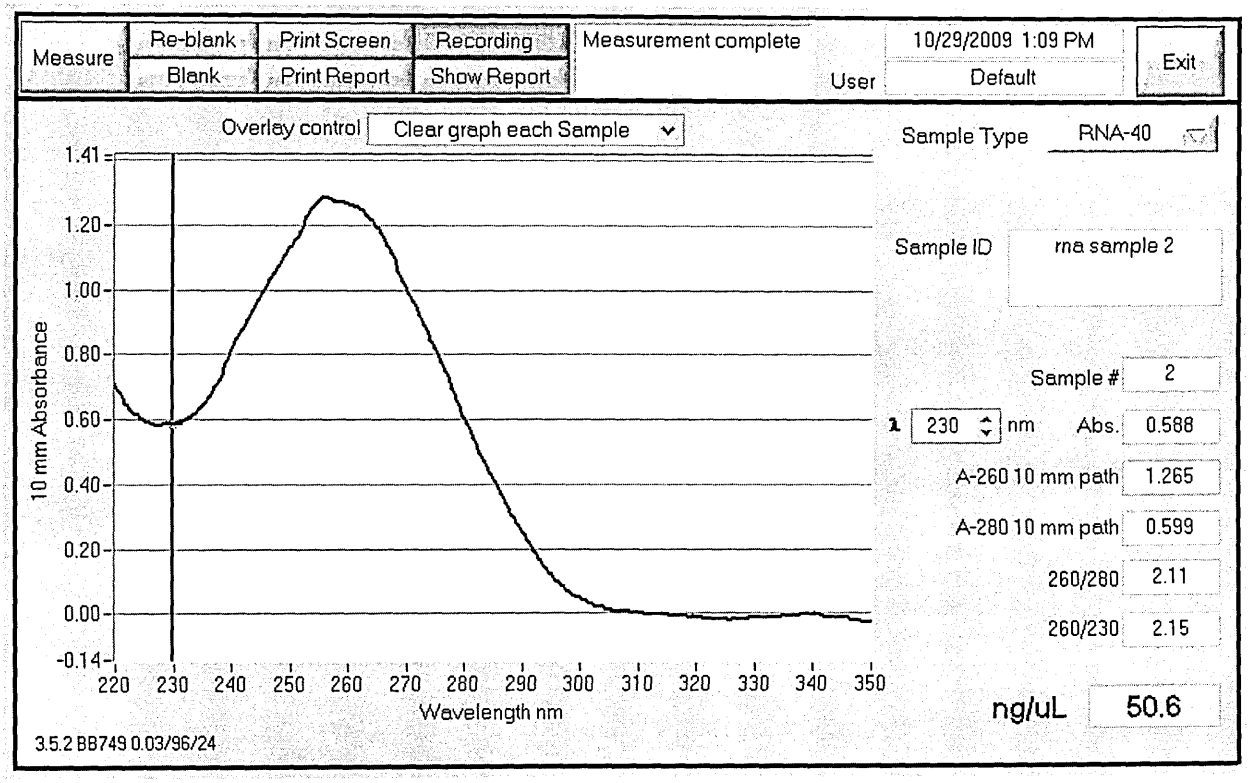



Figure 3.18 RNA quantification, Nanodrop output showing absorbance profile of extracted RNA. The concentration of RNA is calculated from the absorbance of light at 230 nm wavelength, the purity estimated from the ratio of absorbance at 260:280nm, ratios greater than 2 are considered to show high purity for RNA preparations

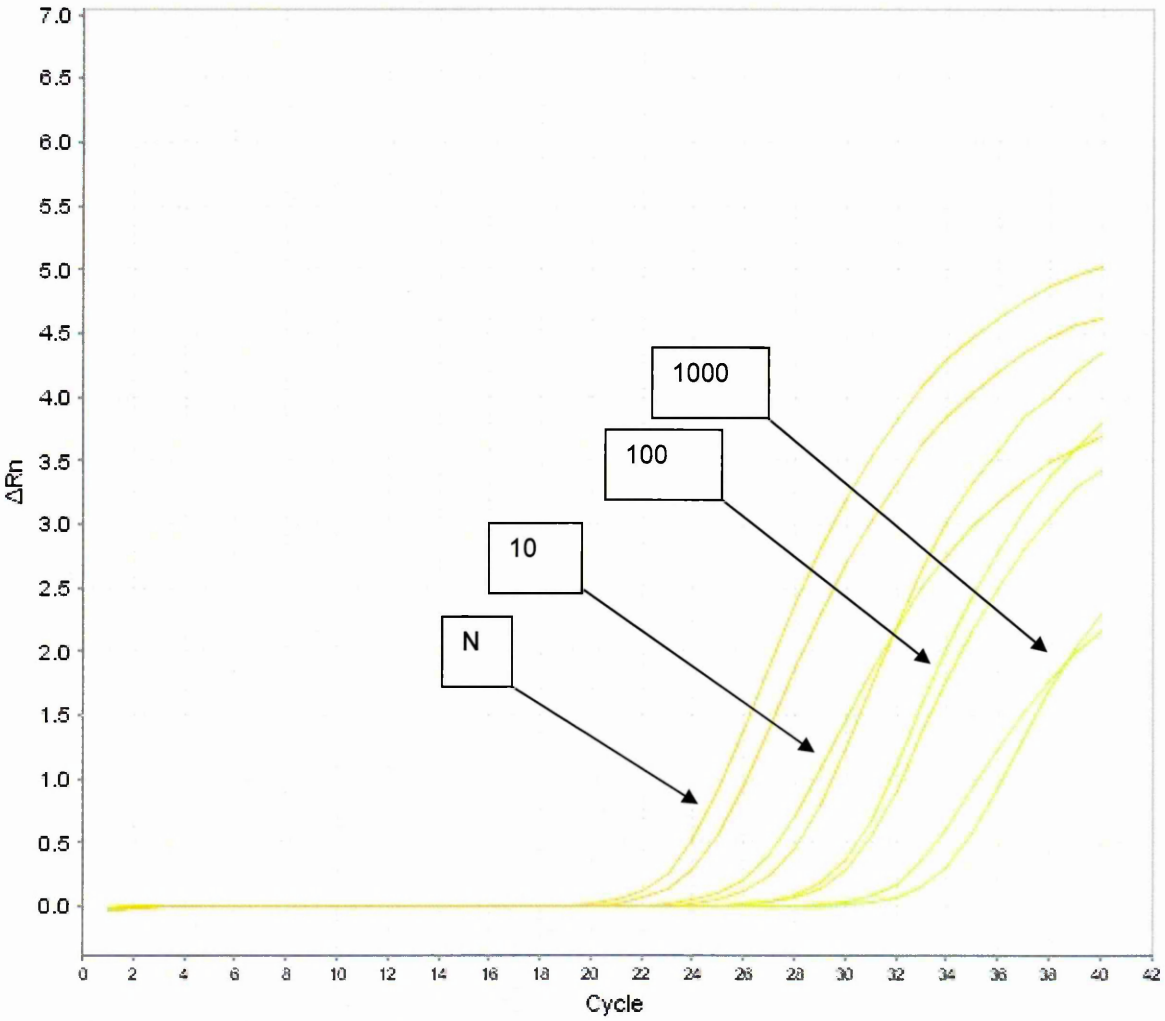



Figure 3.19 Determination of primer PCR efficiency by template dilution. Amplification plot for theTGF-β1 dilution template, the number of PCR cycles (x axis), fluorescence (y axis). The CT values at which fluorescence produced by amplification exceeds background is the point at which fluorescence exceeds a pre-determined threshold. Curves represent replicates of cDNA undiluted (N) and at dilutions of 1/10, 1/100, and 1/1000.

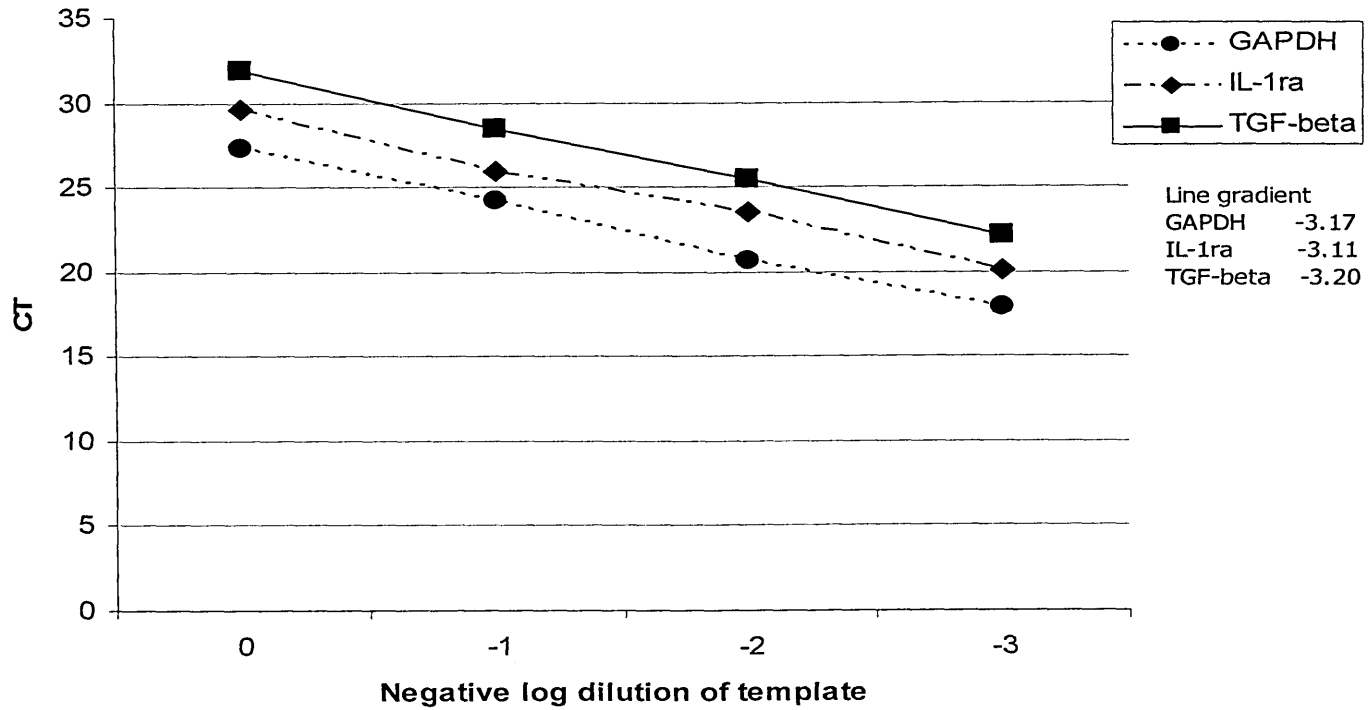



Figure 3.20 Determination of PCR efficiency by template dilution. Dilution template response curves, showing good linearity and line slopes close to -3.3 indicating high reaction efficiency.

### 3.5.4 qPCR Results IL-1ra

ΔCT values for IL-1ra for each participant are shown for the 3 time points in the study (Figures 3.21 and 3.22). In the D+ fetus group, five participants had fold change greater than 1.5 (one at 1, hour, four at 24 hours), in the D- fetus group two participants had fold changes >1.5 (one at 1 hour one at 24 hours), and one had a fold decrease < 0.5. Of the participants having a 1.5 fold or greater increase, one had a concentration of IL-1ra below the group mean in the pre-anti-D sample.

Summaries of ΔCT values, and fold change data are included (Tables 3.13, 3.14). No significant difference between means of fold change in D+ and D- fetus groups was noted. Correlation in the fold change results obtained with each participant was weak and not significant in the D+ and D- fetus groups (Pearsons's correlation coefficient 0.298, p=0.246, and 0.131, p=0.78) (Figure 3.23). Fold increases and decreases in IL-1ra are shown (Figure 3.24) and the distribution of fold changes in each test group are shown (Figure 3.25)

### 3.5.5 qPCR Results TGF-β1

ΔCT values for TGF-β1 for each participant are shown in time sequence (Figures 3.26 and 3.27). In the D+ fetus group, 5 participants had fold change greater than 1.5 (1 at 1 hour, 4 at 24 hours), in the D- fetus group no participants had fold changes >1.5. Three participants had fold decreases below <0.5. None of the participants with fold increase >1.5 had an initial ΔCT below the group mean.

Summaries of ΔCT values, and fold change data are included (Tables 3.15, 3.16). No significant difference between means of fold change in D+ and D- fetus groups was noted. Correlation in the fold change results obtained with each participant was weak and not significant in the D+ fetus group (Pearsons's correlation coefficient 0.408, p=0.104) and strong but not significant in the D- fetus group (0.840, p=0.18) (Figure 3.28). Fold increases and decreases in TGF-β1 are shown (Figure 3.29) and the distribution of fold changes in each test group are shown (Figure 3.30)

| | IL-1ra (ΔCT) | | | | | |
|---|---|---|---|---|---|---|
| | D+ fetus group | | | D- fetus group | | |
| | Range | Mean | SD | Range | Mean | SD |
| Pre | 1.12-3.95 | 2.60 | 0.65 | 0.91-3.14 | 2.33 | 0.73 |
| 1 hour | 2.02-3.71 | 2.73 | 0.47 | 0.31-3.66 | 2.65 | 1.10 |
| 24 hour | 0.91-3.36 | 2.52 | 0.80 | 1.26-4.29 | 2.51 | 0.91 |

Table 3.13 Summary of mRNA measurement results by qPCR for IL-1ra

| Group | Interval | Range of Fold change | Mean Fold change | SD | Significance of difference between means in D+ vs D- fetus groups (independent T test) |
|---|---|---|---|---|---|
| D+ fetus group n=17 | 1 hour | 0.46-2.23 | 0.99 | 0.44 | P=0.428 |
| | 24 hours | 0.45-4.14 | 1.29 | 0.97 | P=0.452 |
| D- fetus group n=7 | 1 hour | 0.36-1.53 | 0.84 | 0.35 | |
| | 24 hours | 0.27-1.69 | 1.00 | 0.45 | |

Table 3.14 Statistical analysis of IL-1ra qPCR results (SD standard deviation)

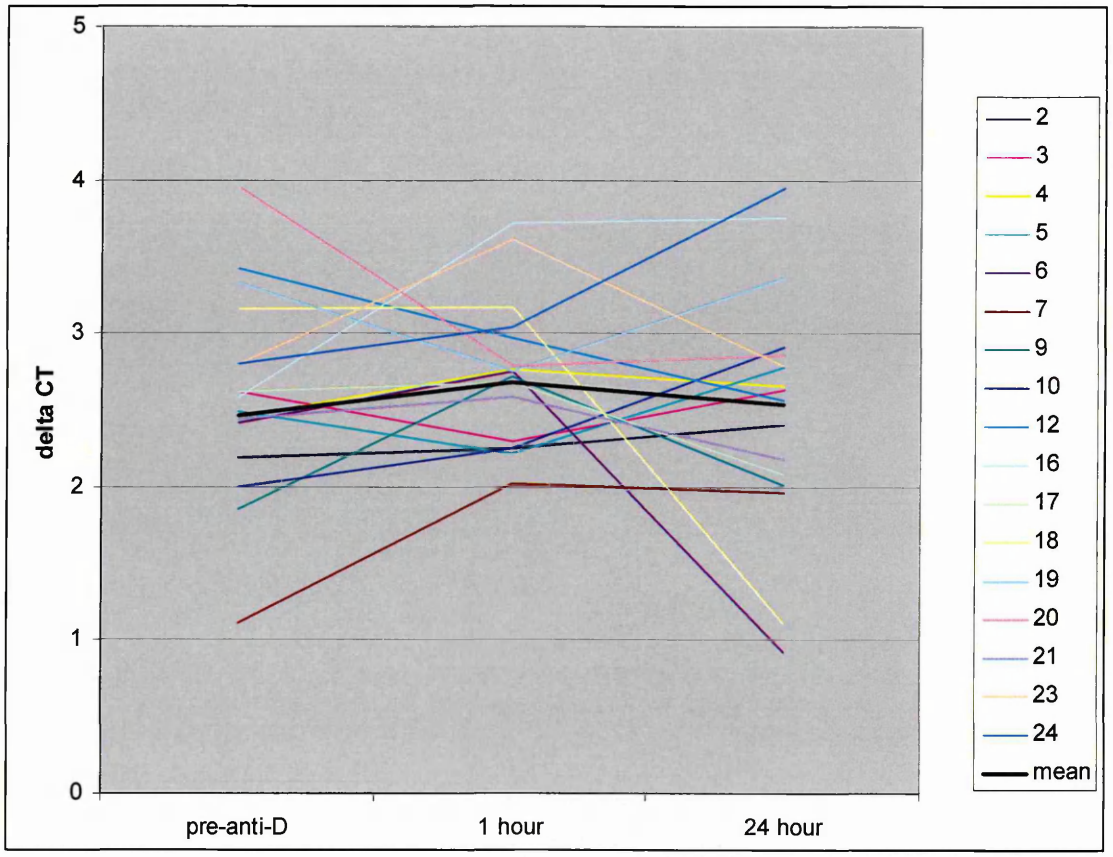



Figure 3.21 IL-1ra mRNA (ΔCT) measured by qPCR in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D+ fetus group (n=17). The mean line for the group is shown in black.

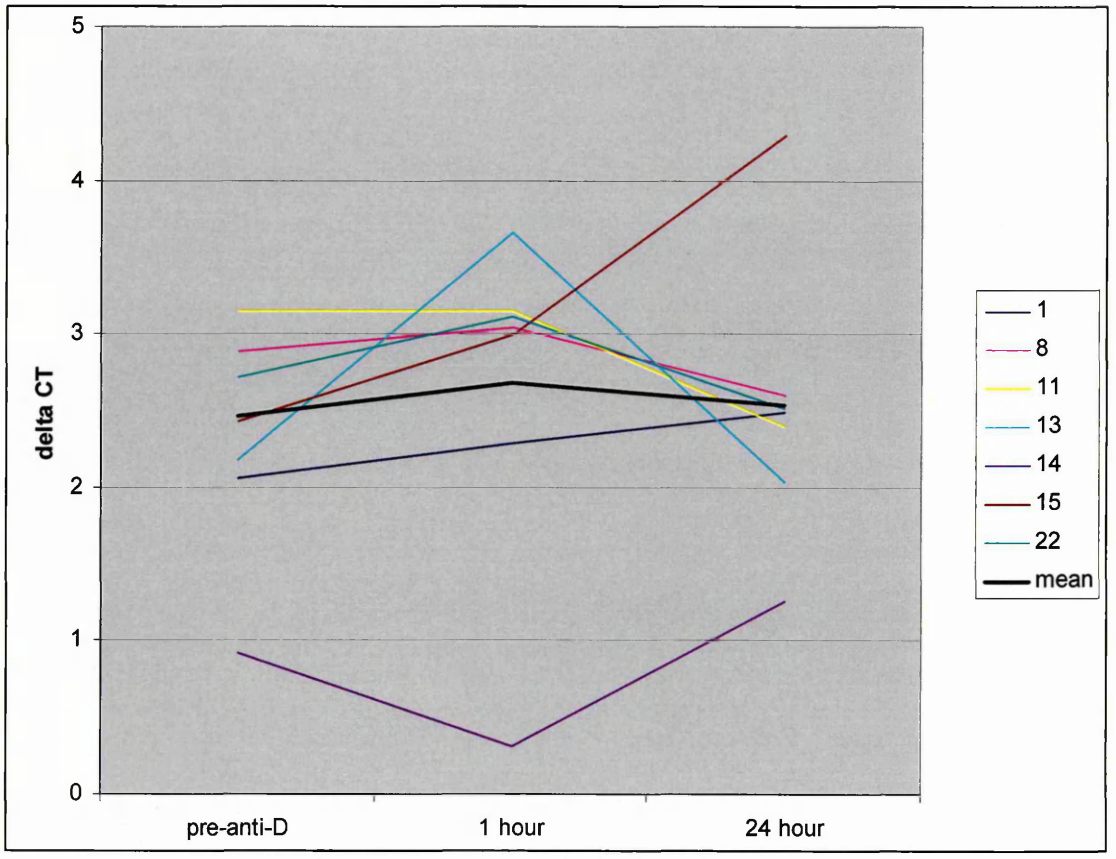



Figure 3.22 IL-1ra mRNA (ΔCT) measured by qPCR in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D- fetus group (n=7). The mean line shown is calculated from entire cohort.

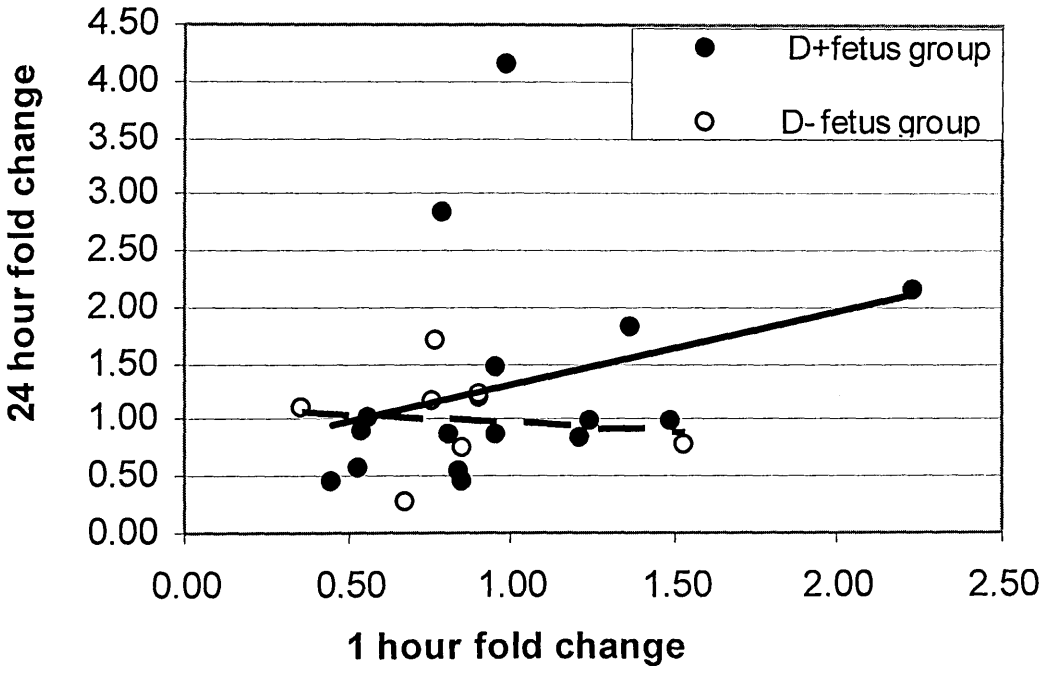



Figure 3.23 Correlation between fold changes detected by qPCR in IL-1ra detected in 1 hour (x-axis) and 24 hour (y-axis) samples in D+ and D- fetus groups. Trend lines (D+ fetus group solid line, $R^2$=0.09 D- fetus group broken line, $R^2$=0.02) drawn by Microsoft Excel

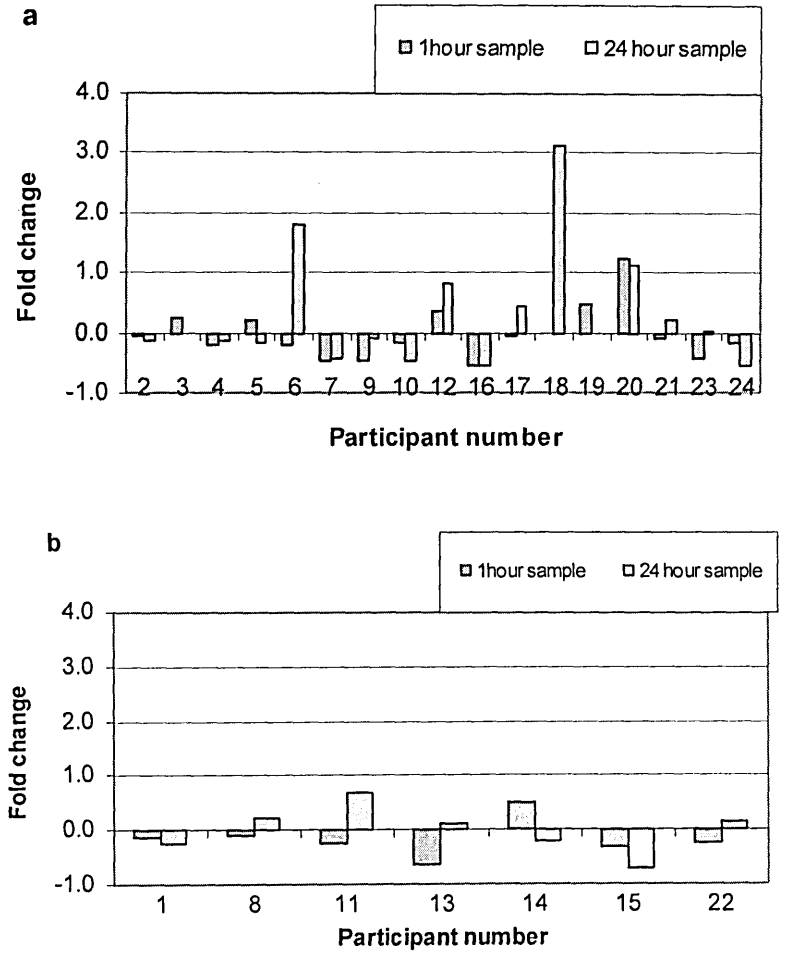



Figure 3.24 Distribution of positive and negative fold changes in IL-1ra among 1 hour and 24 hour samples, detected by qPCR in the D- fetus group. Fold change of 0 represents no change in this figure.

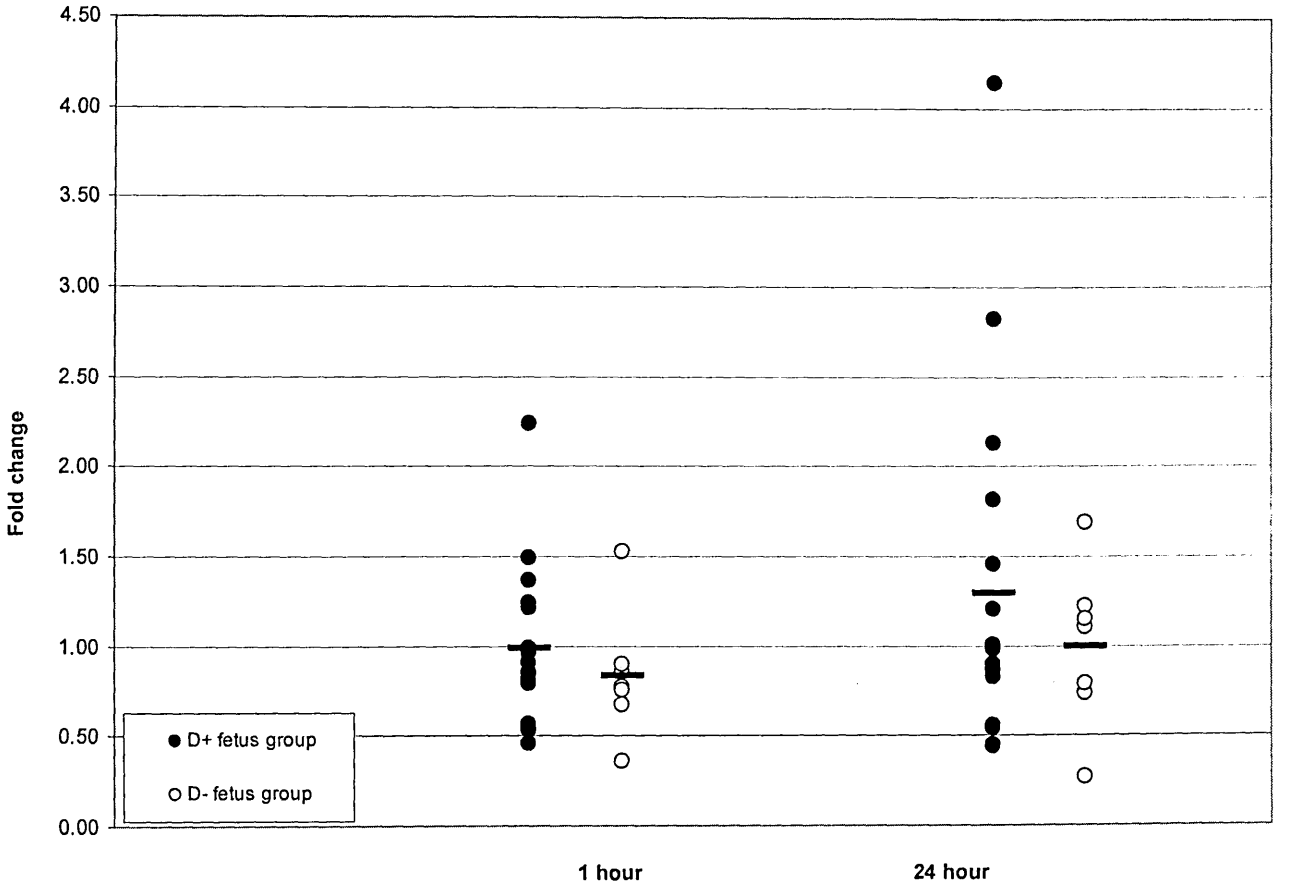



Figure 3.25 Fold change in IL-1ra detected by qPCR in the D+ and D- fetus groups in 1 hour and 24 hour samples. Fold change of 1 represents no change in this figure. The mean for each group is represented by a black line Independent T test, significance of difference between means of D+ and D- fetus groups: 1hour p=0.428, 24 hours p=0.452.

TGF-β1 (ΔCT)

| | D+ fetus group | | | D- fetus group | | |
|---|---|---|---|---|---|---|
| | Range | Mean | SD | Range | Mean | SD |
| Pre | 1.50-5.33 | 4.51 | 0.99 | 3.82-5.17 | 4.49 | 0.45 |
| 1 hour | 1.91-6.27 | 4.60 | 0.88 | 4.50-5.76 | 4.94 | 0.47 |
| 24 hour | 3.05-6.44 | 4.67 | 0.91 | 4.40-6.11 | 4.99 | 0.66 |

Table 3.15 Summary of mRNA measurement results by qPCR for TGF-β1

| Group | Interval | Range of Fold Change | Mean Fold change | SD | Significance of difference between means in D+ vs D- fetus groups (independent T test) |
|---|---|---|---|---|---|
| D+ fetus group n=17 | 1 hour | 0.52-2.05 | 1.0 | 0.36 | p=0.280 |
| | 24 hours | 0.06-3.8 | 1.25 | 1.01 | p=0.258 |
| D- fetus group n=7 | 1 hour | 0.43-1.21 | 0.82 | 0.35 | |
| | 24 hours | 0.27-1.37 | 0.79 | 0.36 | |

Table 3.16 Statistical analysis of TGF-β1 qPCR results,( SD standard deviation)

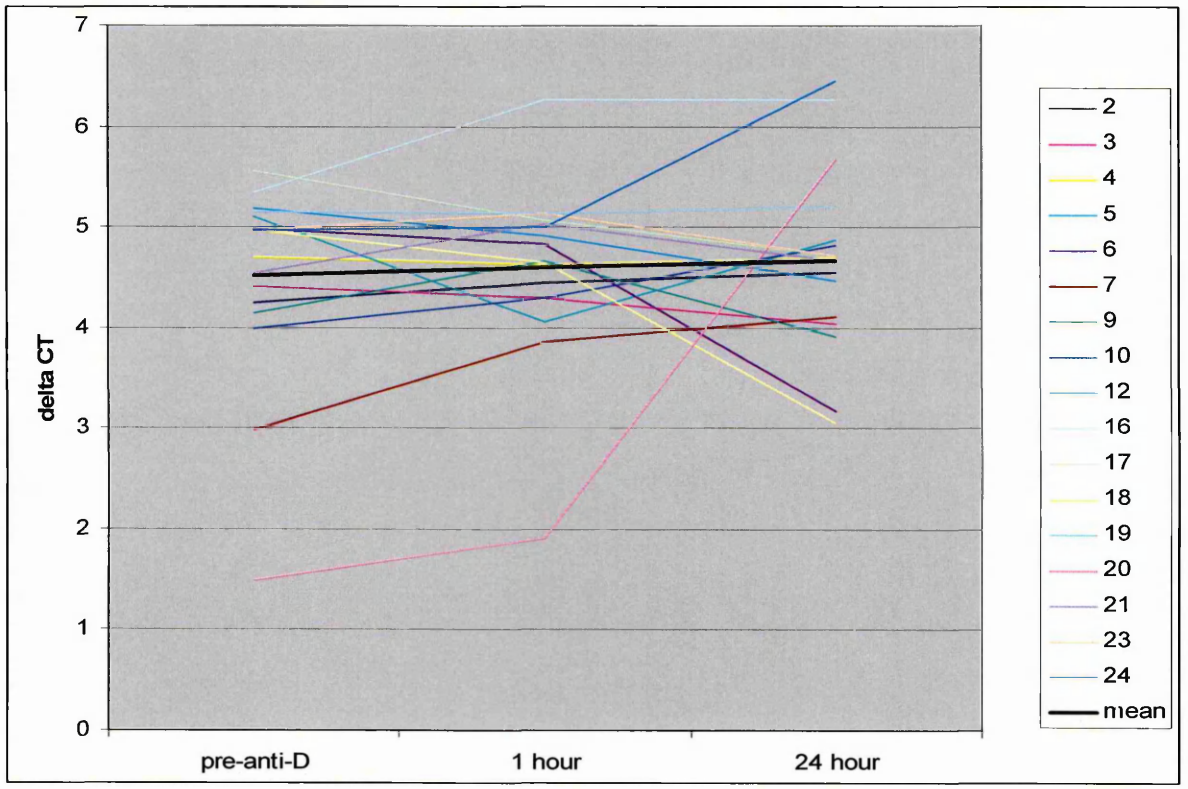



Figure 3.26 TGF-β1 mRNA (ΔCT) measured by qPCR in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D+ fetus group (n=17). The mean line shown is calculated from entire cohort.

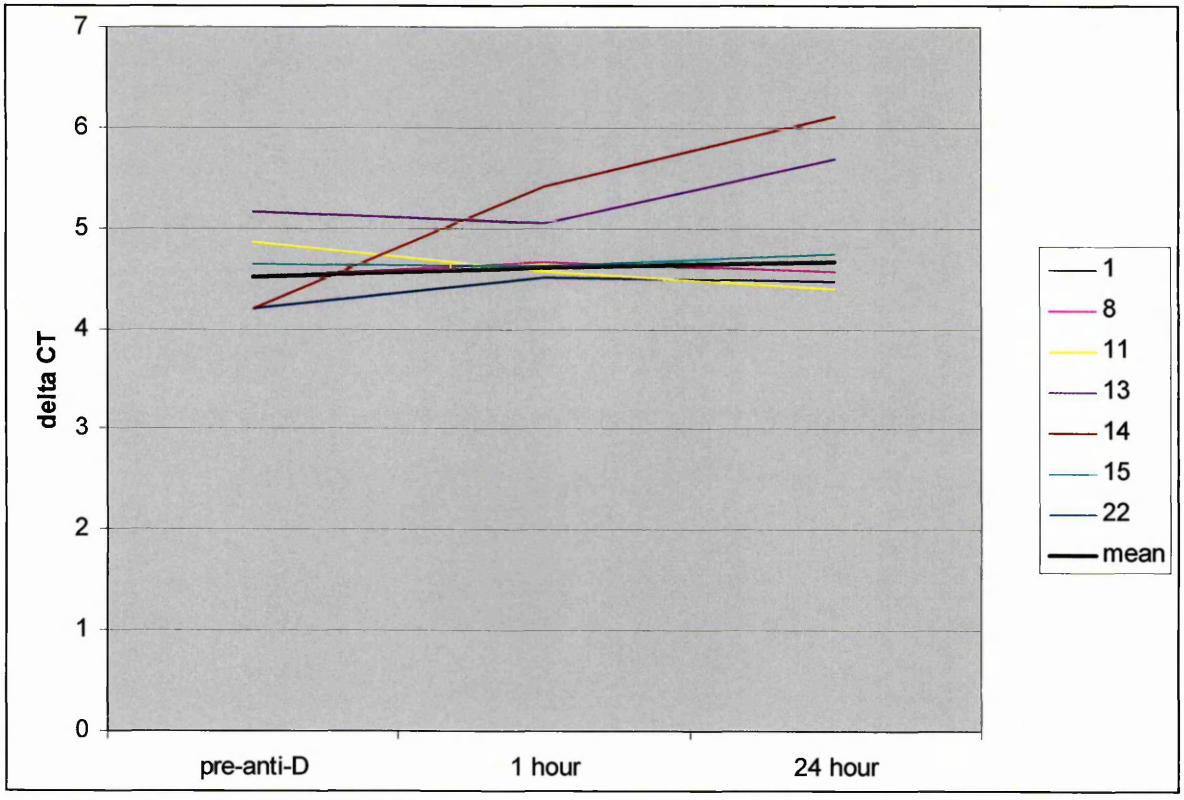



Figure 3.27 TGF-β1 mRNA (ΔCT) measured by qPCR in samples collected before anti-D administration and 1 hour and 24 hours afterwards, in D- fetus group (n=7). The mean line shown is calculated from entire cohort.

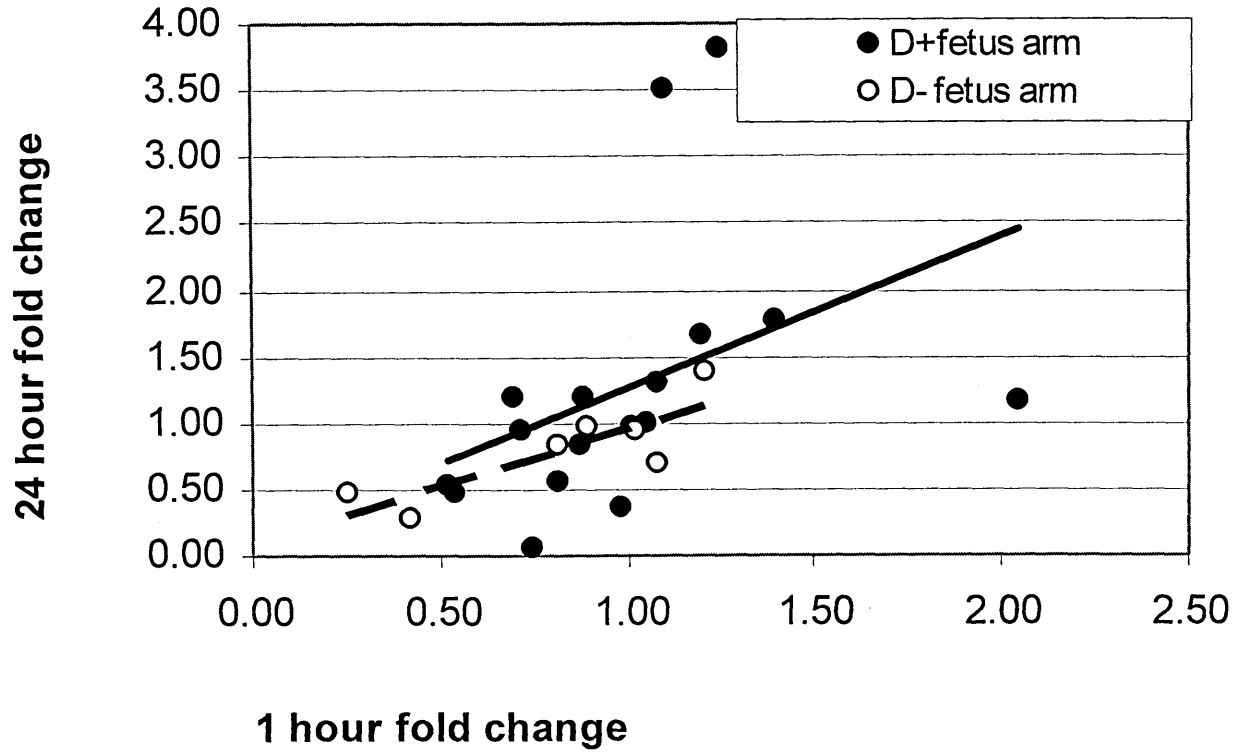
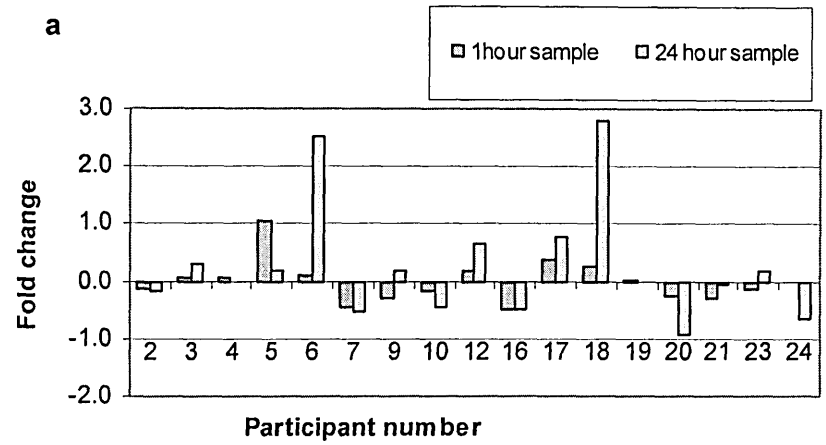



Figure 3.28 Correlation between fold changes detected by qPCR in TGF-β1 detected in 1 hour (x axis) and 24 hour (y axis) samples in D+ and D- fetus groups. Trend lines (D+ fetus group solid line, $R^2$=0.17 D- fetus group broken line, $R^2$=0.70) drawn by Microsoft Excel



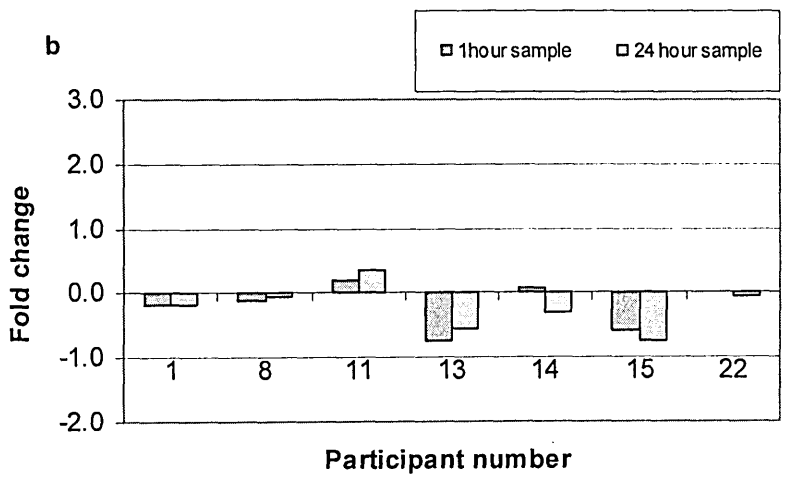




Figure 3.29 Distribution of positive and negative fold changes in TGF-β1 among 1 hour and 24 hour samples, detected by qPCR in the D- fetus group, fold change of 0 represents no change in this figure.

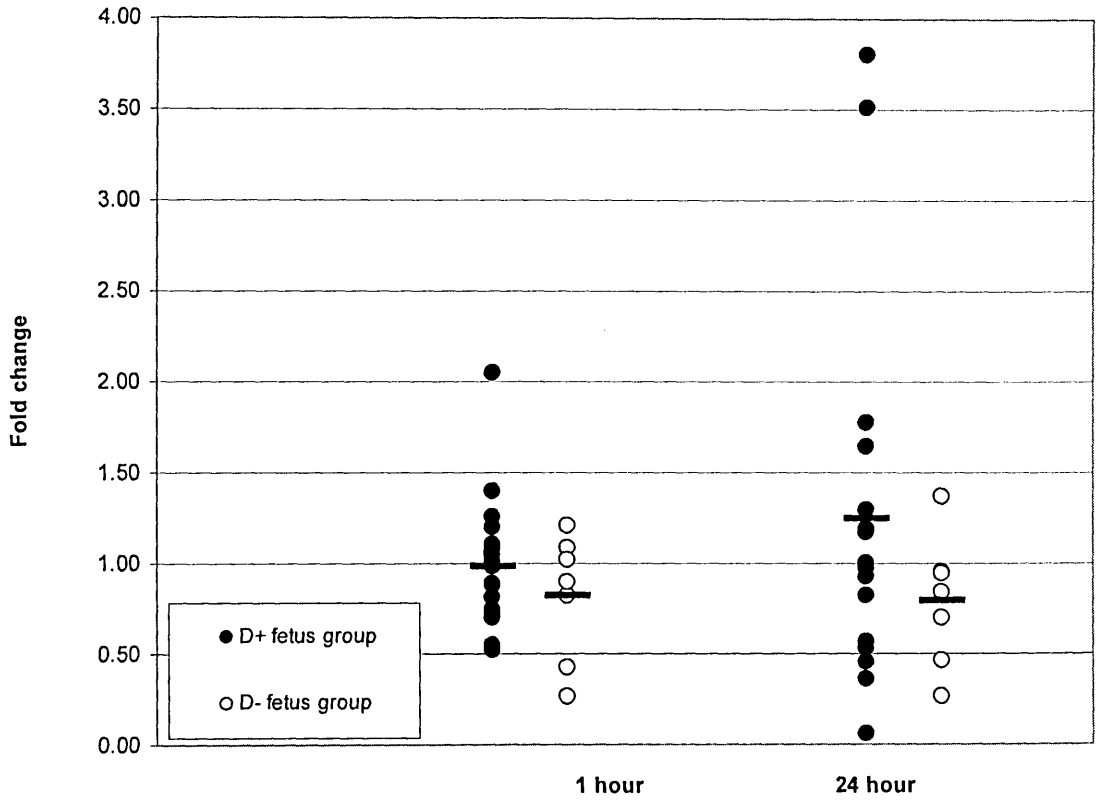



Figure 3.30 Fold change in TGF-β1 detected by qPCR in D+ and D- fetus groups in 1 hour and 24 hour samples, fold change of 1 represents no change in this figure. The mean for each group is represented by a black line
Independent T test, significance of difference between means of D+ and D- fetus groups: 1hour p=0.425, 24 hours p=0.452

### 3.6 Combined Results for ELISA and qPCR for IL-1ra and TGF-β1

For the two analytes included in the study, which have been assessed by both ELISA and qPCR, the results can be combined. This approach allows a more complete view of cytokine responses to anti-D prophylaxis, than can be achieved using either assay, and 1 and 24 hour samples in isolation. Using combined data allows assessment of secreted protein, and changes in DNA transcription to up-regulate expression in response to anti-D prophylaxis. The combined results are shown for 1 hour samples (Figures 3.31 and 3.32) and 24 hour samples (Figures 3.33 and 3.34)

If the ELISA and qPCR data in Table 3.17 are considered together for IL-1ra then 6/11 participants in the D+ fetus group have a fold change >1.5 (one detected by ELISA, five by qPCR), and 2/7 participants in the D- group have fold changes >1.5 (Two by qPCR, one of which was also detected by ELISA). Applying $Chi^2$ to this finding p=0.682 indicating that the difference between the groups is not significant (Table 3.17).

If the TGF-β1 data are considered in the same way 10/17 participants in the D+ fetus group have fold change increases>1.5 (5 detected by ELISA only, 2 by qPCR only, 3 by both techniques) and 1/7 participants in the D- fetus group (both ELISA and qPCR). Applying $Chi^2$ to this finding p=0.047 indicating that the difference between the groups is significant (Table 3.17). Combined results are summarized in table 3.18.

| | Group | ELISA | qPCR | Combined ELISA+qPCR | P |
|---|---|---|---|---|---|
| IL-1ra | D+ fetus group n=17 | 1 | 5 | 6 | 0.682 |
| | D- fetus group n=7 | 1 | 2 | 1 | |
| TGF-β1 | D+ fetus group n=17 | 8 | 5 | 10 | 0.047 |
| | D- fetus group n=7 | 1 | 0 | 1 | |

Table 3.17 Samples with fold change >1.5 detected by ELISA or qPCR .

p values calculated by $Chi^2$ for the frequency of fold change >1.5 detected by ELISA or qPCR in the D+ and D- fetus groups.

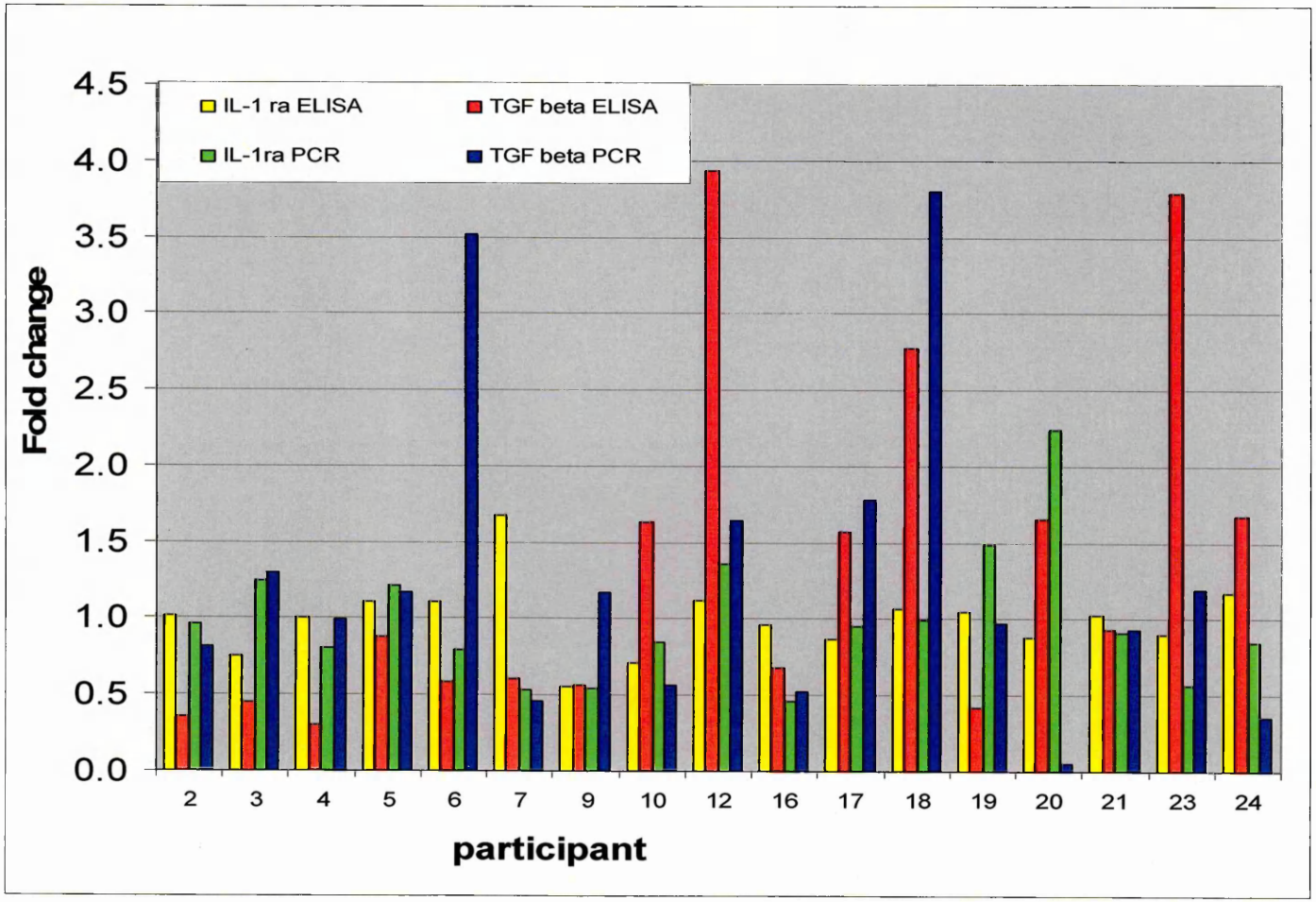



Figure 3.31 Fold change in 1 hour sample in IL-1ra and TGF-β1 detected by ELISA and qPCR in D+ fetus group of study

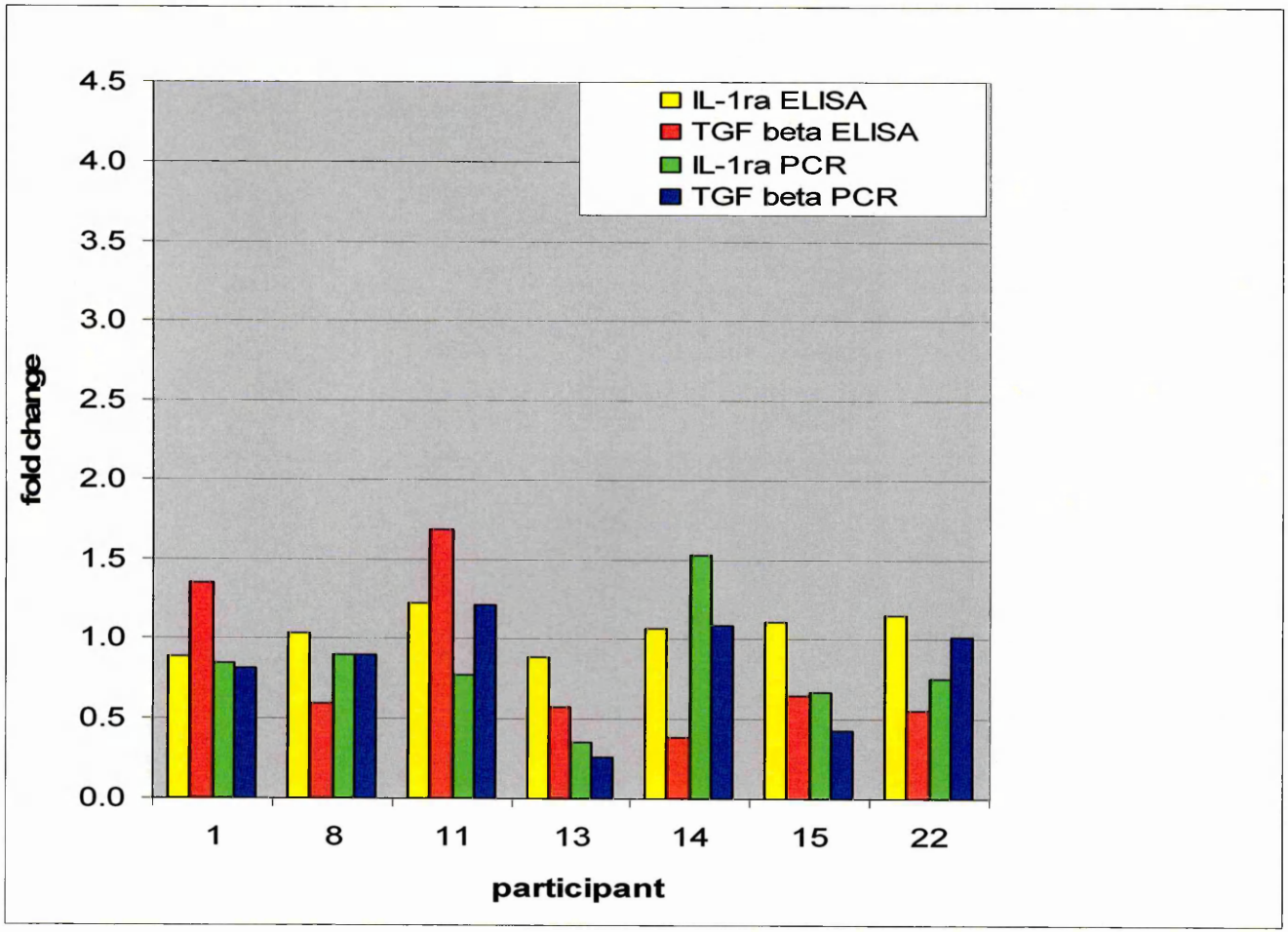



Figure 3.32 Fold change in 1 hour sample in IL-1ra and TGF-β1 detected by ELISA and qPCR in D- fetus group of study

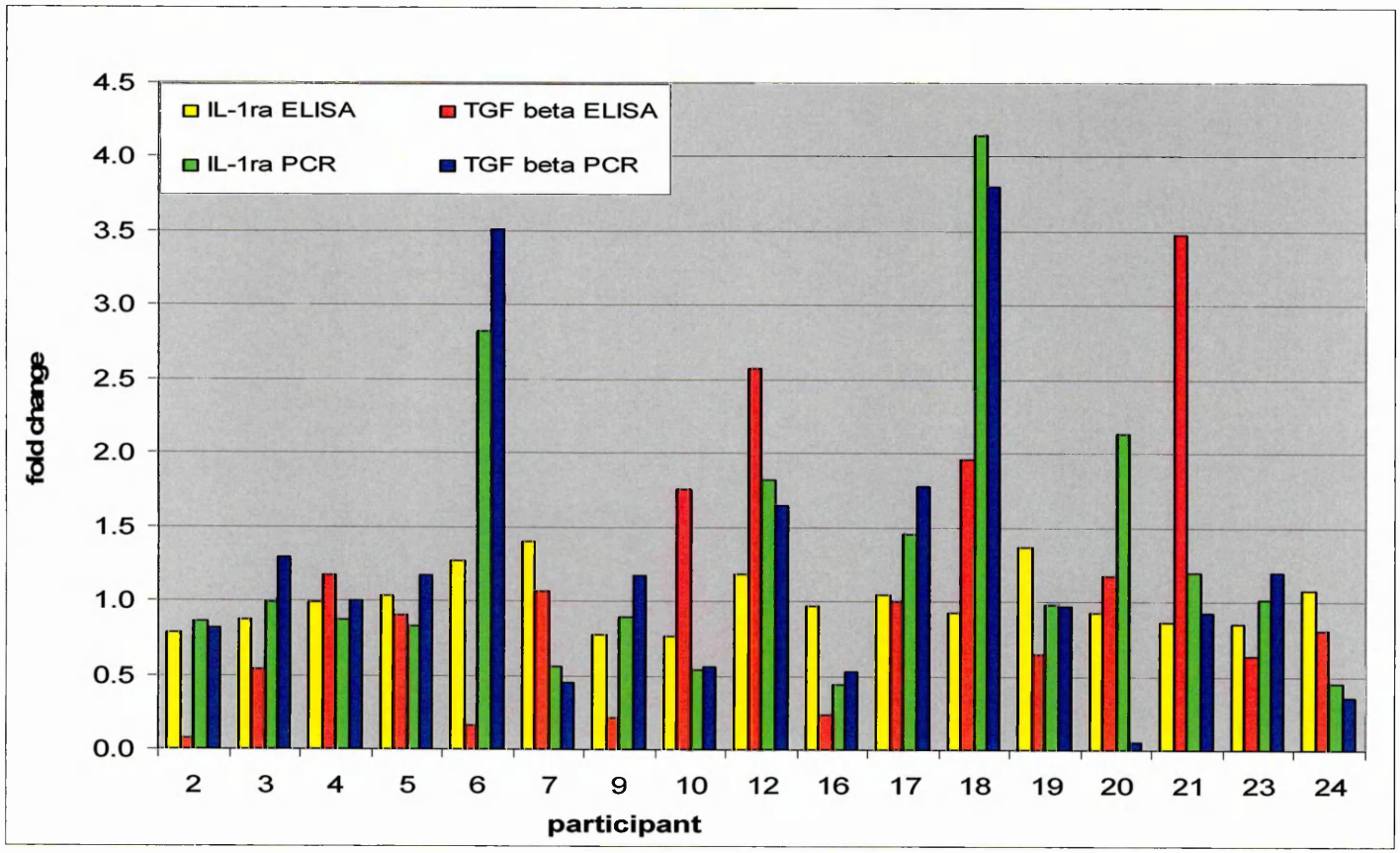



Figure 3.33 Fold change in 24 hour sample in IL-1ra and TGF-β1 detected by ELISA and qPCR in D+ fetus group of study

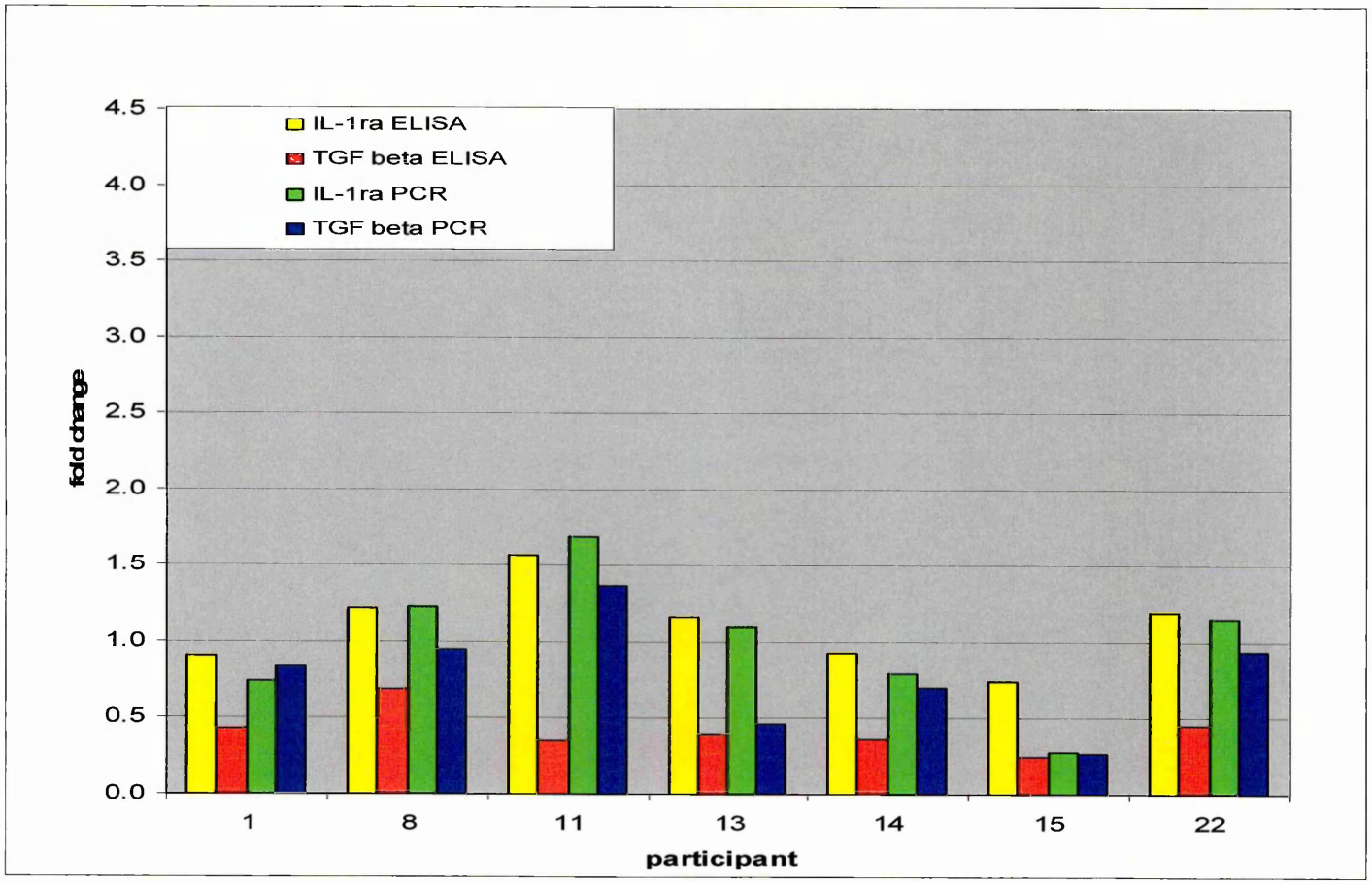



Figure 3.34 Fold change in 24 hour sample in IL-1ra and TGF-β1 detected by ELISA and qPCR in D- fetus group of study

| Test group | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Participant Number | IL-1 beta ELISA | | IL-1 ra ELISA | | TGF beta ELISA | | IL-1 ra qPCR | | TGF beta qPCR | |
| | 1 hour | 24 hour | 1 hour | 24 hour | 1 hour | 24 hour | 1 hour | 24 hour | 1 hour | 24 hour |
| 2 | 0.95 | 0.75 | 1.01 | 0.78 | 0.36 | 0.07 | 0.96 | 0.86 | 0.88 | 0.82 |
| 3 | 1.03 | 0.92 | 0.75 | 0.87 | 0.45 | 0.54 | 1.24 | 0.99 | 1.08 | 1.29 |
| 4 | 0.82 | 0.97 | 1.01 | 0.99 | 0.30 | 1.17 | 0.81 | 0.87 | 1.05 | 1.00 |
| 5 | 0.94 | 1.03 | 1.10 | 1.03 | 0.88 | 0.91 | 1.21 | 0.82 | 2.05 | 1.17 |
| 6 | 0.76 | 0.78 | 1.11 | 1.27 | 0.59 | 0.16 | 0.79 | 2.82 | 1.10 | 3.51 |
| 7 | 0.57 | 0.57 | 1.68 | 1.40 | 0.61 | 1.06 | 0.53 | 0.55 | 0.55 | 0.46 |
| 9 | 0.83 | 0.94 | 0.56 | 0.77 | 0.57 | 0.22 | 0.55 | 0.90 | 0.70 | 1.18 |
| 10 | 0.97 | 1.00 | 0.71 | 0.77 | 1.63 | 1.76 | 0.85 | 0.53 | 0.81 | 0.56 |
| 12 | 1.06 | 1.15 | 1.12 | 1.18 | 3.93 | 2.58 | 1.37 | 1.82 | 1.20 | 1.64 |
| 16 | 0.95 | 0.97 | 0.96 | 0.97 | 0.68 | 0.24 | 0.46 | 0.44 | 0.52 | 0.52 |
| 17 | 0.84 | 0.92 | 0.87 | 1.04 | 1.57 | 1.00 | 0.96 | 1.46 | 1.40 | 1.78 |
| 18 | 1.00 | 1.10 | 1.07 | 0.92 | 2.77 | 1.96 | 0.99 | 4.14 | 1.25 | 3.80 |
| 19 | 1.11 | 1.37 | 1.05 | 1.37 | 0.42 | 0.64 | 1.49 | 0.98 | 1.01 | 0.97 |
| 20 | 0.84 | 1.20 | 0.88 | 0.92 | 1.66 | 1.17 | 2.23 | 2.13 | 0.75 | 0.06 |
| 21 | 1.10 | 1.10 | 1.02 | 0.86 | 0.93 | 3.47 | 0.91 | 1.20 | 0.71 | 0.93 |
| 23 | 1.11 | 1.00 | 0.90 | 0.85 | 3.78 | 0.64 | 0.57 | 1.01 | 0.88 | 1.19 |
| 24 | 1.00 | 1.12 | 1.17 | 1.08 | 1.67 | 0.81 | 0.85 | 0.45 | 0.98 | 0.36 |
| Control group | | | | | | | | | | |
| 1 | 0.94 | 1.38 | 0.89 | 0.91 | 1.35 | 0.44 | 0.85 | 0.74 | 0.82 | 0.84 |
| 8 | 0.97 | 1.03 | 1.04 | 1.22 | 0.59 | 0.69 | 0.90 | 1.23 | 0.90 | 0.95 |
| 11 | 1.21 | 1.12 | 1.22 | 1.57 | 1.69 | 0.35 | 0.77 | 1.69 | 1.21 | 1.37 |
| 13 | 0.94 | 1.06 | 0.89 | 1.16 | 0.57 | 0.39 | 0.36 | 1.10 | 0.26 | 0.46 |
| 14 | 0.85 | 0.92 | 1.07 | 0.92 | 0.38 | 0.36 | 1.53 | 0.79 | 1.08 | 0.70 |
| 15 | 1.03 | 0.85 | 1.10 | 0.74 | 0.65 | 0.24 | 0.67 | 0.27 | 0.43 | 0.27 |
| 22 | 0.48 | 0.46 | 1.15 | 1.19 | 0.55 | 0.45 | 0.76 | 1.15 | 1.02 | 0.94 |

Table 3.18 .Illustrating fold change by analyte, and assay method in D+ and D- fetus groups. Colour codes used for fold change are included as a key below

| Fold change key |
|---|
| >3 |
| 2 to 3 |
| 1.5 to 2 |
| 1 to 1.5 |
| 0.75 to 1 |
| 0.4 to 0.75 |
| 0 to 0.4 |

# 4 Discussion

## 4.1 Acceptance and rejection of hypotheses

The objective of this study was to test two pairs of hypotheses

**Hypothesis 1**

$H_o$ There is no significant difference in cytokine expression in maternal circulation before and after DGG

and

$H_1$ There is a significant difference in cytokine expression in maternal circulation before and after DGG

Based on the combined data that in section 3.6 the null hypothesis is not rejected

**Hypothesis 2**

$H_o$ There is no significant difference in cytokine expression in maternal circulation after DGG in the presence or absence of D+ cells

and

$H_1$ There is a significant difference in cytokine expression in maternal circulation after DGG in the presence or absence of D+ cells.

The results of FMH measurement were insufficiently accurate to allow this hypothesis to be considered.

## 4.2 Methodology of the study

Three quantitative assays were applied as part of this study. The choices of techniques and analytes were made on the basis of economy, pragmatism and the objective of validating, and rigorously investigating changes in cytokine expression induced by anti-D administration in order to provide an insight into the mechanism of action of AMIS. Multiplex bead assays are the most recent of the applied techniques to be described, and have been considered to offer a rapid, cost effective approach to cytokine measurement (Young 2008). The cytokines chosen for assay by this technique were those considered most likely to show change, based on existing literature being incorporated into the study to increase the breadth and potentially the value of the research. Any of these analytes showing marked alteration in expression after treatment would have been included amongst those for further investigation by ELISA or RT-qPCR. In fact, the level of the analytes assayed by cytokine flow cytometry was below the minimum level of detectability. This is consistent with the findings of other authors (Branch 2006), and might be considered to indicate that treatment had no effect on these analytes. It should be considered that cytokines are capable of biological function at very low concentrations at the local cellular level, which may not be reflected in cytokine concentration in the circulation (Mantovani 2000).

ELISA is considered to be the standard technique for cytokine measurement offering high sensitivity, and was selected for analysis of 3 mediators of inflammation in this study. The technique is more time consuming, and more expensive that cytokine flow cytometry (Young 2008). The calculated analyte concentrations in participant's plasma were broadly consistent with those obtained by other authors (Makhseed 2000, Jonssen 2006, Branch 2006).

IL-1β was assayed by ELISA and cytokine flow cytometry. Concentrations of IL-1β were detected by ELISA up to 45.6pg/mL, which if reproduced by cytokine flow cytometry would have been above the concentrations of the 4$^{th}$ standard, however no IL-1β was detected by cytokine flow cytometry. Reasons offered for dissimilar performance of bead array assays when

compared to standard ELISA include: variations in specificity and avidity of monoclonal antibodies used in the technique and cross-reactions from the other reagents used in the multiplex test (Elshal 2006). Although detection of low levels of one analyte were seen to be less effective than ELISA, it is reasonable, based on the detection of standards to assume that none of the analytes assayed by flow cytometry; IFN-$\gamma$. IL-1$\beta$, IL-4, IL-6, IL-10, IL-12p70, IL-17A, TNF-$\alpha$ had radical increases in concentration during the 24 hours after treatment.

qPCR was included in the study to determine whether there was a change in cytokine gene transcription in circulating nucleated cells, indicating, the source of the cytokines in samples with elevated plasma concentration, as well as offering greater sensitivity than the measurement of plasma protein. qPCR has been employed in a semi-quantitative and more recently fully quantitative basis for cytokines and many other analytes (Bustin 2002). In this study the techniques for extraction of mRNA, reverse transcription to cDNA and the PCR process were found to be robust and straightforward using bespoke kits (Applied Biosystems Ltd), though the manual platform meant the processes were very time consuming. Replicate reproducibility and dilution template were good for IL-1ra, and TGF-$\beta$1. The tests kits were however relatively expensive and this precluded the analysis of other analytes in this study.

Poor correlation between results of ELISA and RT-qPCR were obtained, as expressed by fold change. This may relate to the biology of cytokine release, TGF-$\beta$1 is stored and secreted *in vivo* in a non-active form as a complex with Latency Associated Protein, as a result there is poor correlation between TGF-$\beta$1 transcription message levels and amounts of secreted protein, suggesting post transcriptional or translational regulation of TGF-$\beta$1 expression (Li 2006). Other authors have reported generally poor correlation between cytokine levels and fold change as detected by ELISA, RT-qPCR and cytokine flow cytometry, though studies of patterns of expression in longer time series using cultured and stimulated cell lines, with higher

concentrations of analytes in culture supernatant liquid have had better results (Favre 1997).

The use of multiple techniques, ELISA and qPCR for the assessment of cytokine expression, offers the opportunity to confirm the concentration of plasma protein with measurement of transcribed mRNA, in particular in *in vitro* or *ex vivo* experimental models in which protein synthesis and release are closely and temporally linked.

In an *in vivo* model such as that in the current study, in which the source of secreted cytokine, and the mechanism for release is not known, the link between plasma protein and transcribed mRNA may not be so strong, and an alternative approach to the use of multiple techniques may be considered.

The secretion of cytokines into the plasma may reflect the release of pre-sythesised and stored material in response to specific stimulus (e.g TGF-β1 in Th3 lymphocytes). In this case any increase in specific mRNA expression may occur after the plasma cytokine burst as protein is synthesised to replace secreted protein. Alternatively, cytokine secretion may be the result of *de novo* protein synthesis in nucleated cells following immunomodulatory signalling which typically would occur around 24 hours later. The timing of any cytokine burst in an *in vivo* model is therefore difficult to predict, and may vary between individuals. The brief nature of cytokine burst leaves the possibility that any time series without continuous monitoring, such as the present study, may fail to detect cytokine responses

For these reasons, in this study data, from ELISA and qPCR, as well as being considered separately were combined, with fold change values in D+ and D- fetus groups, greater than 1.5 detected by either technique at either time point were compared. Only by this approach were significant differences in TGF-β1 expression detected between D+ and D- fetus groups. This may indicate that in an in vivo model of investigation of anti-D prophylaxis neither ELISA or qPCR alone is sufficiently informative, or that in an extended study offering a

larger sample size with more sample points in the time series either or both techniques may provide significant findings.

One of the objectives of the study was to determine a link between the presence of fetal D+ cells and cytokines in response to anti-D injection. Two strategies were employed to achieve this: firstly determination of the fetal D+ group at delivery and allocation of participants to study arms based on fetal group, and secondly detection of circulating D+ cells in the maternal blood samples. No large FMH were detected (>4mL) in the study samples. These are most commonly encountered in the third trimester of pregnancy, particularly at the time of delivery (BCSH 2009). Poor results were obtained chiefly to high and variable background counts. This may be attributable to intrinsic failure of the techniques to acquire such high counts of events in clinical samples without accompanying background counts. This would confound the detection of low levels of D+ cells. Alternatively the high background may have been the result of contamination from consumables or reagents used, though high backgrounds were not particularly apparent in the validation exercise.

Without further investigation, particularly with a view to increasing data acquisition without exacerbating confounding high background counts, it may be that flow cytometry cannot reliably detect FMH at the minimum level required to elicit a maternal alloimmune response.

In planning this study, the use of acid elution (AE) for detecting FMH was considered and rejected. AE has a reputation for false positive results and for inaccuracy but is considered by some authors to be more sensitive than FC and may have been a useful addition to this study (BCSH 2009).

It has been suggested that molecular means are employed to detect FMH (Branch 2006). As naked fetal DNA is routinely detectable in maternal plasma from 18 weeks gestation in all pregnancies (Avent 2002) without any transfer of fetal cells to the maternal circulation it is hard to see how this strategy could be applied successfully.

## 4.3 Limitations of the Study

The results of this study can only be generalised to individuals, or groups, meeting the inclusion criteria, and undergoing the same treatment. In fact though anti-D prophylaxis is almost universal in the UK, and other developed countries at 28 weeks, much less typical is the choice of the intravenous administration route. Intramuscular administration is more widely practiced, but is unpopular amongst recipients as it is painful, and is notoriously poor and variable as a means to introduce anti-D immunoglobulin rapidly to the maternal circulation, especially when the dose is inadvertently delivered into fatty tissue (MacKenzie 2006).

The present study differs from that of Branch (2006) by the means of anti-D administration, the inclusion of a control group  the use of qPCR, and the elapsed time between treatment and administration (48 hours in Branch's study). The more rapid and predictable delivery of anti-D into the maternal circulation  prompted the use of a 1 hour and 24 hour sample regime. This appears to have been justified as fold changes in TGF-β1 were recorded in the D+ fetus arm 1 hour samples in ELISA (7/17) and qPCR (1/17) and the 24 hour samples ELISA (4/17) and qPCR(4/17). The selection of this sampling regime was based on previous indications of response times to anti-D (Branch 2006) and IVIg (Semple 2007), tempered by pragmatism in establishing a regime for sample collection consistent with recruiting a sufficiently large cohort for the study by making reasonable demands on participants attending for repeat sampling.

Cytokines have short circulating half lives. It is unlikely that FMH would occur spontaneously in the 24 hour period, from treatment to sampling, among the cohort so any cytokine response to the interaction of administered anti-D and fetal D+ cells is likely to result from fetal D+ cells already in the maternal circulation. In the absence of ongoing antigen supply, the cytokine response is likely to be a single short term event, in contrast to cytokine responses to

chronic conditions such as disease or pregnancy. It is impossible from the evidence of this study to determine whether more participants had cytokine responses which were not detected because they occurred before, outside the time points of the study. In Branch's study samples were taken 48 hours after anti-D was administered by intramuscular injection, as other authors have commented that may have resulted in failure to detect important responses (Semple 2007).

The decision to use the mothers of D- infants as the control group had significant benefits to the study. These women received exactly the same care as those in the D+ fetus arm, and it is reasonable to expect that they would, as a result have the same, if any, non-specific responses. This offers better study design that using a placebo control such as saline offered to another group of women (attendees at a glucose tolerance test clinic were considered for this control). In addition excellent blinding characteristics were established by this strategy as the fetal D group was unknown to participants and the investigators at the time of sampling, and at the time that laboratory assays were performed. The strategy for controlling the study did however prevent any control of the number of women in treatment and control arms. In the UK 40% of D- mothers deliver D- infants. It might be expected therefore that 9 or 10 of the cohort of 24 would form the control arm. In fact, only 7 (29.2%) did so, making the data from the control arm less easy to interpret, potentially reducing the statistical significance of the study findings. This finding of a lower than expected proportion of D- fetuses in this cohort may reflect the small sample size.

The inclusion criteria of the study were deliberately set very broadly. Any woman of 28-32 weeks gestation, receiving anti-D prophylaxis who was willing to participate was recruited. The strategy was adopted partly to avoid any discrimination as required for ethics approval, and to maximise generalisability of the findings; but also as a response to robust and frequent warnings from clinical colleagues who warned that recruitment would be problematic. No attempt to exclude women with pre-existing alloantibodies was made (in fact there were none) or any women suffering from infections. It is possible

therefore those cytokine responses may be due to changes other than the treatment under study.

**4.4 Findings of the study**

Studies on changes in cytokine profile after administration of exogenous IgG include those on IVIg and anti-D antibody when administered to treat ITP. Changes detected in the expression of inflammatory mediators in those studies are indicative of the need to investigate similar effects in AMIS, when used to prevent alloimmunisation to the RhD antigen, but caution must be exercised in comparing the findings. In particular the immunological processes associated with the underlying condition must be considered. IVIg is administered as an anti-inflammatory agent to treat autoimmune conditions such as ITP which occur in a pro-inflammatory immune environment. In contrast HDFN occurs in a Th2 dominated immune environment (Ragupathy 2001). The regulation of cytokines and their receptors and for FcR cannot therefore be directly compared.

In addition, the doses of anti-D administered in treating ITP (50-75μg/kg) are much higher than those in anti-D prophylaxis (300μg, typically 4μg/kg). Finally the quantity and supply of antigen is markedly different. In ITP, D antigen is present in constant excess on the patient's red cells, by contrast in anti-D prophylaxis, antigen dose from FMH is typically small and sporadic.

There is evidence that small doses of exogenous, specific IgG can cause powerful pathological responses. Anti-HLA IgG antibodies inadvertently administered in small quantities in blood products can elicit a powerful immune response, causing transfusion related acute lung injury (TRALI) an uncommon, but significant cause of post transfusion morbidity and mortality. Typically these reactions occur after the transfusion of plasma rich products, but have been recorded after transfusion of concentrated red cells containing less that 20mL of plasma. Approximately 95% of the IgG in Rhophyllac is of specificities other than RhD. (Rhophyllac package insert). The potential biological effects of this immunoglobulin are largely unexplored.

More directly comparable with the current study are the results of the investigation of Branch et al. (2006). In that study ELISA was used to measure

plasma cytokines, found TGF-β1 fold changes of >1.5 in 4/10 (40%) of participants at 48 hours after IM anti-D administration. These findings are comparable with those of the current study with 7/17 (41%) participants carrying D+ fetuses having similar fold change in TGF-β1 at 1 hour, and 4/17 (25%) at 24 hours. The lower proportion of TGF-β1 responses at 24 hours in the current study may result from more immediate dispersal of anti-D administered by the IV route.

It is important to note that a high proportion (7 of 8) of the participants having TGF-β1 fold changes of >1.5 had a starting antigen concentration below the group mean, though 7 out of 8 went on to exceed the group mean in either 1 hour or 24 hour samples. Similarly of those having a change below 0.5 all had starting concentrations above the group mean. It is possible therefore that some of these observations represent regression to the mean (Field 2003). The mean TGF-β1 concentration in the D- fetus arm is significantly higher than that in the D+ arm (difference between means of D+ and D- fetus group, p=0.007 independent T test). This observation might indicate that a suppressive process has been initiated by the maternal immune system even before anti-D is administered.

IL-1ra increases in the current study and Branch's are similar (1/17 at 1 hour and 24 hours, compared with 1/10 at 48 hours). IL-1ra has been reported to be more markedly elevated after anti-D administration to ITP patients (Semple 2002), and it has been suggested that short term IL-1ra responses may have been missed due to sample timing in Branch's study (Semple 2007). The current study includes a 1 hour samples (3 hour in the study of Semple 2002), and has not detected a significant increase in plasma IL-1ra in the D+ fetus arm. This may reflect a lack of IL-1ra response in this study though, 4/17 (24%) of participants in the D+ fetus had a >1.5 fold increase in IL-1ra mRNA, indicative of increased cytokine synthesis. A longer time series may have detected increased plasma IL-1ra levels.

If, as hypothesised, the change to cytokine profiles requires the presence of fetal D+ cells and exogenous anti-D it is unsurprising that not all participants

respond. Estimates vary of the proportion of women at 28 weeks who have circulating D+ cells from 10% to 45% (Woodrow 1996, Urbaniak 1999). The quantity of fetal D+ cells required to stimulate an antibody response is unclear, the quantity required to elicit a cytokine response in the presence of anti-D is untested.

When ELISA and RT-qPCR results are combined, the results of the current study indicate that the numbers of participants undergoing fold change increases in TGF-β1 is significant ($p=0.047$). Though IL-1ra fold change increases of similar magnitude were observed in some participants, the findings were not statistically significant ($p=0.682$).

This study offers new knowledge on the effects of anti-D immunoglobulin on cytokine profiles, in particular on TGF-β1. It is undeniable that the treatment has been overwhelmingly successful in reducing maternal alloimmunisation to the D antigen, and hence HDFN, though our lack of understanding of that mechanism has limited developments in safety, improved supply and our ability to translate the success into parallel areas of HDFN. The challenge of replacing anti-D retrieved from fractionated plasma, collected from volunteer blood donors with a monoclonal alternative has exercised researchers and the pharmaceutical industry for many years. The potential benefits are manifold, improving product safety and supply. Anti-D is regarded as a relatively safe product, but any blood product produced from pools of human plasma has the potential for widescale infection of recipients with pathogens which have escaped the screening and fractionation process. Anti-D fractionated from human donor plasma is currently available in adequate supply in the UK, and other developed countries, but the effect of the continuing global spread of vCJD on anti-D supply is uncertain, and is potentially catastrophic. The availability of a plentiful supply of effective synthetic anti-D would have a significant effect on management of HDFN.

It is striking in reviewing reports of studies into the efficacy of monoclonal IgG anti-D that the chief criterion for selection of clones for trial was the ability to clear D+ red cells from the circulation of D- volunteers (Beliard 2006). No

assessment of the effect of the antibodies on immune mediators including cytokines was made. None of the monoclonal or recombinant anti-D preparations so far trialled for prophylaxis have proved sufficiently reliable for routine clinical use, and to date no explanation for that failure has been obtained.

TGF-β1 is a powerful and versatile modulator of immunity, capable of impact at almost every stage of the humoral response (Li 2006). If, as this study suggests, its secretion is increased as a response to exogenous anti-D antibody then it may have a systemic role in AMIS. Understanding the role and mechanism of TGF-β1 induction by exogenous anti-D might affect the selection of monoclonal antibodies for future trials. Improved knowledge of specific characteristics of Fc effector regions might allow synthesis of IgG anti-D capable of eliciting the full range of effects of human donor derived product.

The success of anti-D prophylaxis in preventing RhD alloimmunisation has caused a shift in focus to HDFN caused by other antibodies, in particular anti-c, and anti-K. Alloimmunisation to red cells antigens other than RhD has been reported to occur in 0.15-1.1% of pregnant women (Koelewijn 2009). There is no strategy to prevent alloimmunisation to these antigens other than conservative transfusion, and no treatment to mitigate against antibodies stimulated by FMH. Though HDFN mediated by these antibodies is still less common and generally less severe than that caused by anti-D, fetal mortality occurs every year in the UK. In consequence understanding of the mechanism of AMIS may, in time, lead to prophylaxis for these antibodies as a routine.

Finally, transfusion scientists and clinicians are occasionally challenged by the management of the pregnancies of women who express qualitative variants of the RhD antigen. These women are capable of mounting an alloimmune response to red cells expressing normal D antigen, but their own cells, which outnumber fetal cells from FMH, bind administered exogenous anti-D administered for prophylaxis (Lubenko 1989). The issue of whether to administer anti-D, and at what dose has never been resolved. Understanding

the mechanism of action by which AMIS is effective would inform the management of these cases.

### 4.5 Recommendations for further research

It is perhaps inevitable that a small scale, practitioner-lead study with some findings of interest, concludes with a series of recommendations for further research.

This should in the first instance focus on addressing the limitations of the current study. In particular developing a more sensitive and reliable means of detecting and quantifying FMH to allow comparison with measured cytokine response. Recruiting a larger cohort, particularly in the control group, has the potential to improve statistical robustness, and resolve issues associated with regression to the mean in the current study. Expanding the range of analytes, in particular by RT-qPCR, and the sensitivity of the tests applied should be informative, and should be more so with a larger cohort. A better understanding of the link between FMH and cytokine response might be achieved by targeting women known to have undergone FMH. Recruiting a sufficiently large cohort and dealing with the additional variables of trauma and a wider range of gestational age would be problematic, though achievable. Such a cohort of women being treated as in-patients might also permit a more complete and extensive time series of samples to be obtained to detect brief cytokine spikes.

If the indications of cytokine mediated mechanisms in AMIS are confirmed by such studies, then focus might shift to assessment of monoclonal antibodies for clinical trial, and attempting to compare efficacy in clinical trial with any cytokine response elicited. If shifts in TGF-β1 expression can be demonstrated as a marker for successful AMIS, improving accessibility of cytokine assays might offer a diagnostic tool in timely confirmation that AMIS has been achieved in challenging cases.

## 4.6 Conclusion

Blood group serologists, interested clinicians, and some immunologists have for more than two generations explained the efficacy of AMIS through rapid clearance of opsonised red cells. It is understandable that the effect of reliable immunomodulation, and the evidence are linked in a causal manner without strong supporting evidence. Indeed questioning the mechanism of AMIS brings the risk of abandonment of a valuable, cost effective, and experimentally elegant therapy. It is only when the observer is armed with a sense of the bewildering complexity, the multiple effector mechanisms conferring insuring redundancy, and the exquisite specificity of the human immune system that improbability of such a simplistic explanation for the conundrum of AMIS is apparent.

The first clinical applications of AMIS were based on experiments made on observations made *in vivo,* which occurred when the science of immunology was emergent and largely uninformed at the molecular or cellular level. That the practice has been so spectacularly effective has made challenge of the assumptions, made at its inception, a heresy. Failure to make that challenge in the context of new knowledge is a dereliction of duty, and more importantly a failure to expand and extend the established practice of AMIS into therapies for existing candidates that are safer and more efficacious, as well as new treatments for those who currently have none.

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07 July 2008

Mr Mark Williams
Reference Service Manager
5 Longmeadows
Garforth
Leeds
LS25 2BR

Dear Mr Mark Williams

**Full title of study:** **The effect of anti-D immunoglobulin administration on serum cytokine profiles**
**REC reference number:** **08/H1311/22**

Thank you for your letter of 27 June 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

**Ethical review of research sites**

The Committee has designated this study as exempt from site-specific assessment (SSA). There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

**Conditions of the favourable opinion**

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| *Document* | *Version* | *Date* |
|---|---|---|
| Application | | 20 February 2008 |
| Investigator CV | | |
| Protocol | | 20 February 2008 |
| Covering Letter | | 20 February 2008 |
| Peer Review | 07/01/08 and 14/12/08 | |
| Statistician Comments | | |
| Participant Information Sheet: Clinic Test Arm | 3 | 26 June 2008 |
| Participant Information Sheet: Clinic Control Arm | 3 | 26 June 2008 |
| Participant Information Sheet: Admitted Test | 3 | 26 June 2008 |
| Participant Information Sheet: Admitted Control Arm | 3 | 26 June 2008 |
| Participant Consent Form | 2 | 26 June 2008 |
| Response to Request for Further Information | | |
| Supervisors CV | | |
| Participant Recruitment Leaflet | 2 | 26 June 2008 |

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**After ethical review**

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

| 08/H1311/22 | **Please quote this number on all correspondence** |
|---|---|

With the Committee's best wishes for the success of this project.

Yours sincerely

**Mr Andrew Coatesworth**
**Chair**

Email: Joanne.Holmes@York.NHS.UK

*Enclosures:* "After ethical review – guidance for researchers"

*Copy to:* *Mr Brian Littlejohn, Research Funding Coordinator, Sheffield Hall University, City Campus, Howard Street, Sheffield S1 1WB*

*Mr Mark Williams, Reference Service Manager, NHS Blood and Transplant, Red Cell Immunohaematology Laboratory, Bridge Path, Leeds, SL15 7TW*

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16/05/2008

Mr Mark Williams
NHS Blood and Transplant
5 Longmeadows
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Dear Mr Mark Williams

**Re: LTHT R&D Approval of EX08/8592: The effect of anti-D immunoglobulin administration on serum cytokine profiles**

I write with reference to the above research study. I can now confirm that this study has R&D approval and the study may proceed at The Leeds Teaching Hospitals NHS Trust (LTHT). This organisational level approval is given based on the information provided in the documents listed below.

As principal investigator you have responsibility for the design, management and reporting of the study. In undertaking this research you must comply with the requirements of the *Research Governance Framework for Health and Social Care* which is mandatory for all NHS employees. This document may be accessed on the Department of Health website at http://www.dh.gov.uk/research

R&D approval is therefore given on the understanding that you comply with the requirements of the *Framework* as listed in the attached sheet "Conditions of Approval".

If you have any queries about this approval please do not hesitate to contact the R&D Department on telephone 0113 392 2878.

**Indemnity Arrangements**

The Leeds Teaching Hospitals NHS Trust participates in the NHS risk pooling scheme administered by the NHS Litigation Authority 'Clinical Negligence Scheme for NHS Trusts' for: (i) medical professional and/or medical malpractice liability; and (ii) general liability. NHS Indemnity for negligent harm is extended to researchers with an employment contract (substantive or honorary) with the Trust. The Trust

only accepts liability for research activity that has been managerially approved by the R&D Department.

The Trust therefore accepts liability for the above research project and extends indemnity for negligent harm to cover you as principal investigator and the researchers listed on the R&D approval form provided that each member of the research team has an employment contract (substantive or honorary) with the Trust. Should there be any changes to the research team please ensure that you inform the R&D Department and that s/he obtains an employment contract with the Trust if required.

Yours sincerely

**Dr Dawn Lawson**
**R&D Manager**

**Approved documents**
The documents reviewed and approved are listed as follows

| *Document* | *Version* | *Date of document* |
|---|---|---|
| Protocol | | - |
| SSI Form | 5.5 | 24/01/08 |
| CMT Approval | | 17/01/08 |
| NHS REC Application Form | 5.5 | 24/01/08 |

## What would I have to do?

A sample of your blood would be taken before the injection of anti-D (we do that in any case) we would require an extra sample one hour afterwards and another the next day.

## How long would it take?

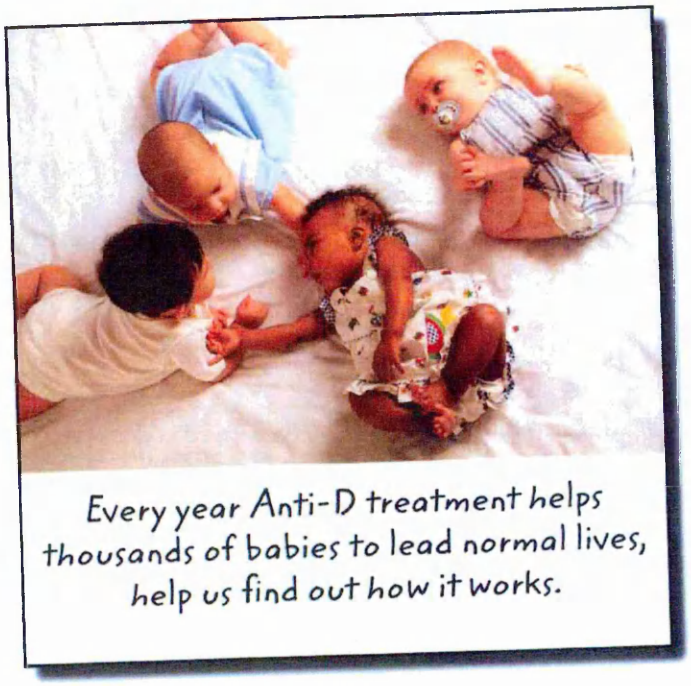
You would have to give a sample one hour after your injection, then just a few minutes the following day for your final sample. We would collect these samples at the Transfusion centre at Seacroft. We can pay travel expenses for this visit, or arrange a taxi.

## How do I join the trial?

If you would like to join the trial or need more information ring Mark Williams on 0113 214 8675.

Version 4 26/6/08

The Leeds NHS Teaching Hospitals
NHS Trust




Sheffield Hallam University

## What is anti-D?

It is a treatment given to women in pregnancy to protect their babies form haemolytic disease of the Newborn (HDN).

## What is HDN?

Without treatment some women form an antibody in their blood.
Sometimes that antibody can damage the baby's red cells leaving them anaemic.

## Who needs anti-D treatment?

Women whose blood group is Rh negative (your blood group is tested on the sample taken early in your pregnancy).

## Is it effective?

Very, HDN is now a rare disease.

## How does it work?

We know that anti-D stops mothers from forming anti-D, but we don't know how .

## How can we find out?

We're doing a study to measure chemicals (cytokines) in women's blood before and after they receive anti-D. This information could help us understand how anti-D works.

## Why does it matter?

The information we get might help to produce new treatments and improve existing ones.

## Can I help?

You can, by giving us samples of your blood before and after you receive anti-D. We'll also be taking some samples from women who don't receive anti-D (Rh positive).

***Research Participant Information Sheet***

***The effect of anti-D immunoglobulin administration on serum cytokine levels***

*We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.*

*Anti-D is given to pregnant women who are Rh (Rhesus) negative to prevent babies in current and subsequent pregnancies becoming ill. The treatment is both safe and effective, it has been given to many thousands of women, but we still do not have some of the information we need about how anti-D works.*

*We cannot promise the study will help you but the information we get may help to prevent haemolytic disease in babies.*

*In this study we need to measure the levels of substances (cytokines) in the blood, which are part of the immune system. We will measure cytokine levels before and after anti-D is given. In addition we need to measure these same substances in some women who do not receive anti-D.*

*If you agree to participate we will take a sample of blood just before you receive anti-D, and take another sample 1 hour afterwards. About 24 hours after your injection is given we will collect one further sample. The second and third samples would be collected by nurses at the Blood Transfusion Centre at Seacroft Hospital. We can pay travel expenses, or arrange a local taxi to get you to and from the centre.*

*Participation in the study will not affect the treatment that you or your baby receives.*

*It is up to you to decide whether to participate. If you do we will describe the study and go through this information sheet which we will then give to you at your 28 week clinic appointment. We will then ask you to sign a consent show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive. If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal.*

*If you have a concern about any aspect of this study, you should ask to speak to the researcher who will do his best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.*

*All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised).*

*All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by York Hospitals Research Ethics Committee.*

*Mark Williams, Leeds Blood Centre, Bridle Path Leeds, LS15 7TW, Tel 0113 214 8675*
*26/6/08 version ct3.0*

Centre Number: EX08/8592

Study Number 08/H1311/22

Patient identification number

## Consent Form

Title of Project: The effect of anti-D immunoglobulin administration on serum cytokine profiles

Name of Researcher: Mark Williams

Please initial box

1, I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2, I understand that the participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3, I understand that relevant sections of my medical notes and data collected during my study may be looked at by the researcher, from regulatory authorities, or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records ☐

4, I agree to take part in the above study ☐

| Name of patient | Date | Signature |
|---|---|---|
| Name of person taking consent | Date | Signature |

When completed 1 copy for patient, 1 for researcher site file, 1 (original) for medical notes

Appendix V Results of FMH estimation on pre-treatment samples

| rticipant umber | Events in positive region Total | Events in negative region Total | % in positive region | corrected vol | Fetal D group |
|---|---|---|---|---|---|
| 1 | 829 | 571 | 0.0258 | 0.566568 | D- |
| 2 | 1186 | 546 | 0.064 | 1.40544 | D+ |
| 3 | 856 | 263 | 0.0593 | 1.302228 | D+ |
| 4 | 506 | 578 | -0.0072 | -0.158112 | D+ |
| 5 | 770 | 649 | 0.0121 | 0.265716 | D+ |
| 6 | 215 | 39 | 0.0176 | 0.386496 | D+ |
| 7 | 521 | 853 | -0.0332 | -0.729072 | D+ |
| 8 | 799 | 677 | 0.0122 | 0.267912 | D- |
| 9 | 954 | 931 | 0.0023 | 0.050508 | D+ |
| 10 | 879 | 864 | 0.0015 | 0.03294 | D+ |
| 11 | 1161 | 989 | 0.0172 | 0.377712 | D- |
| 12 | 525 | 336 | 0.0189 | 0.415044 | D+ |
| 13 | 357 | 437 | -0.008 | -0.17568 | D- |
| 14 | 831 | 975 | -0.0144 | -0.316224 | D- |
| 15 | 857 | 409 | 0.0448 | 0.983808 | D- |
| 16 | 755 | 620 | 0.0135 | 0.29646 | D+ |
| 17 | 561 | 360 | 0.0201 | 0.441396 | D+ |
| 18 | 939 | 849 | 0.009 | 0.19764 | D+ |
| 19 | 1130 | 1330 | -0.02 | -0.4392 | D+ |
| 20 | 911 | 629 | 0.0282 | 0.619272 | D+ |
| 21 | 301 | 264 | 0.0037 | 0.081252 | D+ |
| 22 | 543 | 585 | -0.0042 | -0.092232 | D- |
| 23 | 1045 | 874 | 0.0171 | 0.375516 | D+ |
| 24 | 421 | 267 | 0.0154 | 0.338184 | D+ |

FMH results sorted by fetal RhD group

| D-fetus | | D+ Fetus | |
|---|---|---|---|
| FMH value (mL) | participant no | FMH value (mL) | participant no |
| 0.566568 | 1 | 1.40544 | 2 |
| 0.267912 | 8 | 1.302228 | 3 |
| 0.377712 | 11 | 0 | 4 |
| 0 | 13 | 0.265716 | 5 |
| 0 | 14 | 0.386496 | 6 |
| 0.983808 | 15 | 0 | 7 |
| 0 | 22 | 0.050508 | 9 |
| | | 0.03294 | 10 |
| | | 0.415044 | 12 |
| | | 0.29646 | 16 |
| | | 0.441396 | 17 |
| | | 0.19764 | 18 |
| | | 0 | 19 |
| | | 0.619272 | 20 |
| | | 0.081252 | 21 |
| | | 0.375516 | 23 |
| | | 0.338184 | 24 |
| 0.313714 | Mean | 0.365182 | Mean |
| 0.368421 | Standard deviation | 0.415867 | Standard Deviation |

Appendix VI Cytokine Flow Cytometry data

| Sample ID | Std01 | Std02 | Std03 | Std04 | Std05 | Std06 | Std07 | Std08 | Std09 | Std10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 18.16 | 43.31 | 81.41 | 159.45 | 311.4 | 608.43 | 1229.59 | 2566.6 |
| Human IL-1b | 0 | 10.35 | 20.52 | 39.85 | 74.44 | 153.15 | 332.79 | 632.56 | 1240.64 | 2466.98 |
| Human IL-4 | 0 | 9.58 | 21.61 | 38.3 | 77.63 | 160.31 | 323.29 | 608.45 | 1224.58 | 2541.08 |
| Human IL-10 | 0 | 9.62 | 21.81 | 39.24 | 73.48 | 160.22 | 332.12 | 615.98 | 1203.12 | 2545.89 |
| Human IL-6 | 0 | 9.63 | 21.44 | 39.57 | 74.76 | 159.23 | 330.66 | 607.62 | 1224.22 | 2533.38 |
| Human IL-12p70 | 0 | 8.54 | 23.26 | 38.94 | 77.69 | 157.89 | 318.31 | 609.01 | 1239.13 | 2538.82 |
| Human IL-17A | 0 | 0 | 16.38 | 37.88 | 82.38 | 162.41 | 322.27 | 603.91 | 1202.06 | 2588.68 |
| Human TNF | 0 | 10.3 | 19.41 | 41.55 | 75.54 | 159.35 | 316.77 | 623.47 | 1243.99 | 2499.41 |

| Sample ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 0 | 0 | 17.9 | 0 | 0 | 0 | 0 | 0 |
| Human IL-1b | 0 | 0 | 0 | 0 | 2.74 | 0 | 0 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 0 | 0 | 2.44 | 0 | 0 | 0 | 0 | 0 |
| Human IL-10 | 2.35 | 2.64 | 0 | 0 | 3.41 | 2.26 | 2.02 | 2.35 | 3.02 | 1.87 |
| Human IL-6 | 1.69 | 1.6 | 0 | 0 | 3.19 | 2.29 | 1.96 | 2.04 | 1.96 | 1.27 |
| Human IL-12p70 | 0 | 1.87 | 0 | 0 | 3.52 | 0 | 0 | 0 | 1.65 | 0 |
| Human IL-17A | 0 | 0 | 0 | 0 | 18.36 | 0 | 0 | 0 | 0 | 0 |
| Human TNF | 0 | 1.42 | 0 | 0 | 2.36 | 0 | 0 | 0 | 0 | 0 |

Appendix VI Cytokine Flow Cytometry data

| Sample ID | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-1b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-10 | 1.87 | 0 | 1.42 | 1.42 | 1.66 | 0 | 0 | 1.54 | 2.07 | 1.76 |
| Human IL-6 | 2.33 | 3.7 | 0 | 1.87 | 0 | 0 | 1.27 | 1.6 | 0 | 0 |
| Human IL-12p70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-17A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human TNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Sample ID | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 11.74 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-1b | 0 | 0 | 1.72 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 1.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-10 | 1.87 | 2.45 | 3.51 | 2.74 | 1.87 | 2.45 | 0 | 0 | 0 | 0 |
| Human IL-6 | 0 | 2.37 | 3.53 | 6.58 | 1.27 | 1.5 | 0 | 0 | 0 | 0 |
| Human IL-12p70 | 0 | 1.8 | 2.41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-17A | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human TNF | 0 | 0 | 1.34 | 0 | 0 | 1.1 | 0 | 0 | 0 | 0 |

Appendix VI Cytokine Flow Cytometry data

| Sample ID | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-1b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-10 | 0 | 1.87 | 2.16 | 0 | 0 | 0 | 0 | 1.42 | 0 | 0 |
| Human IL-6 | 0 | 1.39 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.36 |
| Human IL-12p70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-17A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human TNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Sample ID | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-1b | 0 | 0 | 0 | 0 | 1.49 | 2.34 | 1.17 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 0 | 0 | 1.28 | 0 | 0 | 0 | 0 | 0 |
| Human IL-10 | 0 | 0 | 0 | 0 | 2.45 | 0 | 0 | 0 | 0 | 0 |
| Human IL-6 | 3.02 | 0 | 0 | 0 | 2.21 | 0 | 0 | 0 | 0 | 0 |
| Human IL-12p70 | 0 | 0 | 0 | 0 | 2.42 | 0 | 0 | 0 | 0 | 0 |
| Human IL-17A | 0 | 0 | 0 | 0 | 12.39 | 0 | 0 | 0 | 0 | 0 |
| Human TNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Appendix VI Cytokine Flow Cytometry data

| Sample ID | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11.74 |
| Human IL-1b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.34 |
| Human IL-10 | 0 | 0 | 0 | 0 | 0 | 1.66 | 0 | 1.71 | 2.35 | 3.02 |
| Human IL-6 | 0 | 0 | 0 | 0 | 0 | 2.61 | 0 | 0 | 1.87 | 1.6 |
| Human IL-12p70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2.53 |
| Human IL-17A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 12.39 |
| Human TNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Sample ID | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 |
|---|---|---|---|---|---|---|---|---|---|---|
| Human IFN-g | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-1b | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-10 | 0 | 0 | 0 | 0 | 0 | 0 | 1.28 | 1.42 | 0 | 0 |
| Human IL-6 | 0 | 0 | 0 | 0 | 2.45 | 1.5 | 0 | 0 | 0 | 0 |
| Human IL-12p70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human IL-17A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Human TNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Appendix VI Cytokine Flow Cytometry data

| Sample ID | 71 | 72 |
|---|---|---|
| Human IFN-g | 9.92 | 0 |
| Human IL-1b | 0 | 0 |
| Human IL-4 | 1.5 | 0 |
| Human IL-10 | 3.12 | 0 |
| Human IL-6 | 2.49 | 0 |
| Human IL-12p70 | 2.12 | 0 |
| Human IL-17A | 0 | 0 |
| Human TNF | 0 | 0 |

Appendix VII ELISA results

IL-1β ELISA plate 1

| Standard - pg/mL | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ |
|---|---|---|---|---|---|---|---|---|
| 2000 | 2.371 | 2.471 | 0.042 | 0.046 | 2.329 | 2.425 | 2.3770 | 2.3325 |
| 1000 | 1.472 | 1.483 | 0.044 | 0.043 | 1.428 | 1.440 | 1.4340 | 1.3895 |
| 500 | 0.88 | 0.847 | 0.047 | 0.051 | 0.833 | 0.796 | 0.8145 | 0.7700 |
| 250 | 0.481 | 0.48 | 0.046 | 0.057 | 0.435 | 0.423 | 0.4290 | 0.3845 |
| 125 | 0.251 | 0.263 | 0.048 | 0.06 | 0.203 | 0.203 | 0.2030 | 0.1585 |
| 62.5 | 0.171 | 0.173 | 0.059 | 0.06 | 0.112 | 0.113 | 0.1125 | 0.0680 |
| 31.2 | 0.124 | 0.081 | 0.058 | 0.058 | 0.066 | 0.023 | 0.0445 | 0.0000 |
| 0 | 0.0723 | 0.063 | 0.082 | 0.072 | -0.010 | -0.009 | -0.0094 | -0.0539 |

**Study Samples**

| Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - p |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.066 | 0.066 | 0.044 | 0.044 | 0.022 | 0.022 | 0.0220 | 0.0314 | 26.12 |
| 2 | 0.067 | 0.067 | 0.047 | 0.047 | 0.020 | 0.020 | 0.0200 | 0.0294 | 24.45 |
| 3 | 0.084 | 0.084 | 0.050 | 0.050 | 0.034 | 0.034 | 0.0340 | 0.0434 | 36.12 |
| 4 | 0.084 | 0.084 | 0.053 | 0.053 | 0.031 | 0.031 | 0.0310 | 0.0404 | 33.62 |
| 5 | 0.087 | 0.087 | 0.058 | 0.058 | 0.029 | 0.029 | 0.0290 | 0.0384 | 31.95 |
| 6 | 0.077 | 0.077 | 0.056 | 0.056 | 0.021 | 0.021 | 0.0210 | 0.0304 | 25.29 |
| 7 | 0.087 | 0.087 | 0.059 | 0.059 | 0.028 | 0.028 | 0.0280 | 0.0374 | 31.12 |
| 8 | 0.089 | 0.089 | 0.060 | 0.060 | 0.029 | 0.029 | 0.0290 | 0.0384 | 31.95 |
| 9 | 0.070 | 0.070 | 0.045 | 0.045 | 0.025 | 0.025 | 0.0250 | 0.0344 | 28.62 |
| 10 | 0.083 | 0.083 | 0.054 | 0.054 | 0.029 | 0.029 | 0.0290 | 0.0384 | 31.95 |
| 11 | 0.066 | 0.066 | 0.044 | 0.044 | 0.022 | 0.022 | 0.0220 | 0.0314 | 26.12 |
| 12 | 0.071 | 0.071 | 0.043 | 0.043 | 0.028 | 0.028 | 0.0280 | 0.0374 | 31.12 |

| IL-1β plate1 Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - p |
|---|---|---|---|---|---|---|---|---|---|
| 13 | 0.082 | 0.082 | 0.057 | 0.057 | 0.025 | 0.025 | 0.0250 | 0.0344 | 28.62 |
| 14 | 0.081 | 0.081 | 0.058 | 0.058 | 0.023 | 0.023 | 0.0230 | 0.0324 | 26.95 |
| 15 | 0.082 | 0.082 | 0.056 | 0.056 | 0.026 | 0.026 | 0.0260 | 0.0354 | 29.45 |
| 16 | 0.101 | 0.101 | 0.064 | 0.064 | 0.037 | 0.037 | 0.0370 | 0.0464 | 38.62 |
| 17 | 0.075 | 0.075 | 0.049 | 0.049 | 0.026 | 0.026 | 0.0260 | 0.0354 | 29.45 |
| 18 | 0.079 | 0.079 | 0.052 | 0.052 | 0.027 | 0.027 | 0.0270 | 0.0364 | 30.29 |
| 19 | 0.077 | 0.077 | 0.053 | 0.053 | 0.056 | 0.024 | 0.0400 | 0.0494 | 41.12 |
| 20 | 0.074 | 0.074 | 0.055 | 0.055 | 0.019 | 0.019 | 0.0190 | 0.0284 | 23.62 |
| 21 | 0.084 | 0.084 | 0.065 | 0.065 | 0.019 | 0.019 | 0.0190 | 0.0284 | 23.62 |
| 22 | 0.087 | 0.087 | 0.060 | 0.060 | 0.027 | 0.027 | 0.0270 | 0.0364 | 30.29 |
| 23 | 0.075 | 0.075 | 0.049 | 0.049 | 0.026 | 0.026 | 0.0260 | 0.0354 | 29.45 |
| 24 | 0.078 | 0.078 | 0.050 | 0.050 | 0.028 | 0.028 | 0.0280 | 0.0374 | 31.12 |
| 25 | 0.086 | 0.086 | 0.048 | 0.048 | 0.038 | 0.038 | 0.0380 | 0.0474 | 39.45 |
| 26 | 0.087 | 0.087 | 0.057 | 0.057 | 0.030 | 0.030 | 0.0300 | 0.0394 | 32.79 |
| 27 | 0.082 | 0.082 | 0.047 | 0.047 | 0.035 | 0.035 | 0.0350 | 0.0444 | 36.95 |
| 28 | 0.081 | 0.081 | 0.056 | 0.056 | 0.025 | 0.025 | 0.0250 | 0.0344 | 28.62 |
| 29 | 0.075 | 0.075 | 0.051 | 0.051 | 0.024 | 0.024 | 0.0240 | 0.0334 | 27.79 |
| 30 | 0.090 | 0.090 | 0.065 | 0.065 | 0.025 | 0.025 | 0.0250 | 0.0344 | 28.62 |
| 31 | 0.076 | 0.076 | 0.052 | 0.052 | 0.024 | 0.024 | 0.0240 | 0.0334 | 27.79 |
| 32 | 0.092 | 0.092 | 0.061 | 0.061 | 0.031 | 0.031 | 0.0310 | 0.0404 | 33.62 |
| 33 | 0.070 | 0.070 | 0.042 | 0.042 | 0.028 | 0.028 | 0.0280 | 0.0374 | 31.12 |
| 34 | 0.073 | 0.073 | 0.050 | 0.050 | 0.023 | 0.023 | 0.0230 | 0.0324 | 26.95 |
| 35 | 0.076 | 0.076 | 0.051 | 0.051 | 0.025 | 0.025 | 0.0250 | 0.0344 | 28.62 |
| 36 | 0.074 | 0.074 | 0.046 | 0.046 | 0.028 | 0.028 | 0.0280 | 0.0374 | 31.12 |
| 37 | 0.078 | 0.078 | 0.056 | 0.056 | 0.022 | 0.022 | 0.0220 | 0.0314 | 26.12 |
| 38 | 0.077 | 0.077 | 0.057 | 0.057 | 0.020 | 0.020 | 0.0200 | 0.0294 | 24.45 |
| 39 | 0.079 | 0.079 | 0.055 | 0.055 | 0.024 | 0.024 | 0.0240 | 0.0334 | 27.79 |

**IL-1beta ELISA plate 2**

| Standard - pg/mL | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean orrected$A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ |
|---|---|---|---|---|---|---|---|---|
| 2000 | 2.614 | 2.622 | 0.085 | 0.07 | 2.529 | 2.552 | 2.5405 | 2.4690 |
| 1000 | 1.251 | 1.602 | 0.066 | 0.055 | 1.185 | 1.547 | 1.3660 | 1.2945 |
| 500 | 0.728 | 0.908 | 0.05 | 0.052 | 0.678 | 0.856 | 0.7670 | 0.6955 |
| 250 | 0.388 | 0.467 | 0.047 | 0.051 | 0.341 | 0.416 | 0.3785 | 0.3070 |
| 125 | 0.225 | 0.24 | 0.046 | 0.048 | 0.179 | 0.192 | 0.1855 | 0.1140 |
| 62.5 | 0.134 | 0.151 | 0.04 | 0.047 | 0.094 | 0.104 | 0.0990 | 0.0275 |
| 31.2 | 0.12 | 0.122 | 0.049 | 0.05 | 0.071 | 0.072 | 0.0715 | 0.0000 |
| 0 | 0.051 | 0.056 | 0.044 | 0.046 | 0.007 | 0.010 | 0.0085 | -0.0630 |

**Study Samples**

| Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - |
|---|---|---|---|---|---|---|---|---|---|
| 40 | 0.087 | 0.087 | 0.057 | 0.057 | 0.030 | 0.030 | 0.0300 | 0.0394 | 30.26 |
| 41 | 0.070 | 0.070 | 0.046 | 0.046 | 0.024 | 0.024 | 0.0240 | 0.0334 | 25.65 |
| 42 | 0.083 | 0.083 | 0.056 | 0.056 | 0.027 | 0.027 | 0.0270 | 0.0364 | 27.96 |
| 43 | 0.086 | 0.086 | 0.061 | 0.061 | 0.025 | 0.025 | 0.0250 | 0.0344 | 26.42 |
| 44 | 0.080 | 0.080 | 0.054 | 0.054 | 0.026 | 0.026 | 0.0260 | 0.0354 | 27.19 |
| 45 | 0.087 | 0.087 | 0.067 | 0.067 | 0.020 | 0.020 | 0.0200 | 0.0294 | 22.57 |
| 46 | 0.096 | 0.096 | 0.067 | 0.067 | 0.029 | 0.029 | 0.0290 | 0.0384 | 29.50 |
| 47 | 0.096 | 0.096 | 0.069 | 0.069 | 0.027 | 0.027 | 0.0270 | 0.0364 | 27.96 |
| 48 | 0.093 | 0.093 | 0.065 | 0.065 | 0.028 | 0.028 | 0.0280 | 0.0374 | 28.73 |
| 49 | 0.068 | 0.068 | 0.039 | 0.039 | 0.029 | 0.029 | 0.0290 | 0.0384 | 29.50 |
| 50 | 0.066 | 0.066 | 0.043 | 0.043 | 0.023 | 0.023 | 0.0230 | 0.0324 | 24.88 |
| 51 | 0.079 | 0.079 | 0.053 | 0.053 | 0.026 | 0.026 | 0.0260 | 0.0354 | 27.19 |
| 52 | 0.075 | 0.075 | 0.053 | 0.053 | 0.022 | 0.022 | 0.0220 | 0.0314 | 24.11 |

| IL-1β plate2 Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - p |
|---|---|---|---|---|---|---|---|---|---|
| 53 | 0.076 | 0.076 | 0.054 | 0.054 | 0.022 | 0.022 | 0.0220 | 0.0314 | 24.115 |
| 54 | 0.087 | 0.087 | 0.062 | 0.062 | 0.025 | 0.025 | 0.0250 | 0.0344 | 26.423 |
| 55 | 0.084 | 0.084 | 0.058 | 0.058 | 0.026 | 0.026 | 0.0260 | 0.0354 | 27.192 |
| 56 | 0.092 | 0.092 | 0.062 | 0.062 | 0.030 | 0.030 | 0.0300 | 0.0394 | 30.269 |
| 57 | 0.079 | 0.079 | 0.040 | 0.040 | 0.039 | 0.039 | 0.0390 | 0.0484 | 37.192 |
| 58 | 0.085 | 0.085 | 0.045 | 0.045 | 0.040 | 0.040 | 0.0400 | 0.0494 | 37.961 |
| 59 | 0.077 | 0.077 | 0.045 | 0.045 | 0.032 | 0.032 | 0.0320 | 0.0414 | 31.807 |
| 60 | 0.094 | 0.094 | 0.044 | 0.044 | 0.050 | 0.050 | 0.0500 | 0.0594 | 45.653 |
| 61 | 0.088 | 0.088 | 0.059 | 0.059 | 0.029 | 0.029 | 0.0290 | 0.0384 | 29.500 |
| 62 | 0.094 | 0.094 | 0.061 | 0.061 | 0.033 | 0.033 | 0.0330 | 0.0424 | 32.576 |
| 63 | 0.096 | 0.096 | 0.063 | 0.063 | 0.033 | 0.033 | 0.0330 | 0.0424 | 32.576 |
| 64 | 0.103 | 0.103 | 0.068 | 0.068 | 0.035 | 0.035 | 0.0350 | 0.0444 | 34.115 |
| 65 | 0.055 | 0.055 | 0.043 | 0.043 | 0.012 | 0.012 | 0.0120 | 0.0214 | 16.423 |
| 66 | 0.051 | 0.051 | 0.040 | 0.040 | 0.011 | 0.011 | 0.0110 | 0.0204 | 15.653 |
| 67 | 0.049 | 0.049 | 0.040 | 0.040 | 0.009 | 0.009 | 0.0090 | 0.0184 | 14.115 |
| 68 | 0.055 | 0.055 | 0.044 | 0.044 | 0.011 | 0.011 | 0.0110 | 0.0204 | 15.653 |
| 69 | 0.052 | 0.052 | 0.043 | 0.043 | 0.009 | 0.009 | 0.0090 | 0.0184 | 14.115 |
| 70 | 0.058 | 0.058 | 0.051 | 0.051 | 0.007 | 0.007 | 0.0070 | 0.0164 | 12.576 |
| 71 | 0.054 | 0.054 | 0.047 | 0.047 | 0.007 | 0.007 | 0.0070 | 0.0164 | 12.576 |
| 72 | 0.061 | 0.061 | 0.052 | 0.052 | 0.009 | 0.009 | 0.0090 | 0.0184 | 14.115 |

IL-1ra ELISA plate 1

| Standard - pg/mL | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ |
|---|---|---|---|---|---|---|---|---|
| 2000 | 2.118 | 2.283 | 0.012 | 0.109 | 2.106 | 2.174 | 2.1400 | 2.0510 |
| 1000 | 1.253 | 1.34 | 0.079 | 0.085 | 1.174 | 1.255 | 1.2145 | 1.1255 |
| 500 | 0.693 | 0.716 | 0.074 | 0.066 | 0.619 | 0.650 | 0.6345 | 0.5455 |
| 250 | 0.395 | 0.455 | 0.06 | 0.064 | 0.335 | 0.391 | 0.3630 | 0.2740 |
| 125 | 0.253 | 0.252 | 0.047 | 0.052 | 0.206 | 0.200 | 0.2030 | 0.1140 |
| 62.5 | 0.182 | 0.18 | 0.046 | 0.054 | 0.136 | 0.126 | 0.1310 | 0.0420 |
| 31.2 | 0.154 | 0.139 | 0.053 | 0.062 | 0.101 | 0.077 | 0.0890 | 0.0000 |
| 0 | 0.107 | 0.09 | 0.041 | 0.052 | 0.066 | 0.038 | 0.0520 | -0.0370 |

**Study Samples**

| Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - ng/mL |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.299 | 0.356 | 0.047 | 0.051 | 0.252 | 0.305 | 0.2785 | 0.2265 | 226.5 |
| 2 | 0.309 | 0.312 | 0.057 | 0.058 | 0.252 | 0.254 | 0.2530 | 0.2010 | 201.0 |
| 3 | 0.320 | 0.313 | 0.057 | 0.060 | 0.263 | 0.253 | 0.2580 | 0.2060 | 206.0 |
| 4 | 0.803 | 0.777 | 0.047 | 0.063 | 0.756 | 0.714 | 0.7350 | 0.6830 | 683.0 |
| 5 | 0.804 | 0.804 | 0.060 | 0.060* | 0.744 | 0.744 | 0.7440 | 0.6920 | 692.0 |
| 6 | 0.643 | 0.674 | 0.074 | 0.067 | 0.569 | 0.607 | 0.5880 | 0.5360 | 536.0 |
| 7 | 0.458 | 0.421 | 0.063 | 0.060 | 0.395 | 0.361 | 0.3780 | 0.3260 | 326.0 |
| 8 | 0.361 | 0.375 | 0.068 | 0.073 | 0.293 | 0.302 | 0.2975 | 0.2455 | 245.5 |
| 9 | 0.378 | 0.388 | 0.049 | 0.047 | 0.329 | 0.341 | 0.3350 | 0.2830 | 283.0 |
| 10 | 0.420 | 0.444 | 0.057 | 0.059 | 0.363 | 0.385 | 0.3740 | 0.3220 | 322.0 |
| 11 | 0.419 | 0.441 | 0.046 | 0.061 | 0.373 | 0.380 | 0.3765 | 0.3245 | 324.5 |
| 12 | 0.401 | 0.438 | 0.042 | 0.058 | 0.359 | 0.380 | 0.3695 | 0.3175 | 317.5 |
| 13 | 0.820 | 0.968 | 0.063 | 0.067 | 0.757 | 0.901 | 0.8290 | 0.7770 | 777.0 |

| IL-1ra plate 1 Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - ng/mL |
|---|---|---|---|---|---|---|---|---|---|
| 14 | 0.908 | 1.045 | 0.060 | 0.073 | 0.848 | 0.972 | 0.9100 | 0.8580 | 858.0 |
| 15 | 0.910 | 0.928 | 0.068 | 0.068 | 0.842 | 0.860 | 0.8510 | 0.7990 | 799.0 |
| 16 | 0.742 | 0.679 | 0.073 | 0.071 | 0.669 | 0.608 | 0.6385 | 0.5865 | 586.5 |
| 17 | 0.759 | 0.738 | 0.050 | 0.045 | 0.709 | 0.693 | 0.7010 | 0.6490 | 649.0 |
| 18 | 0.832 | 0.867 | 0.055 | 0.056 | 0.777 | 0.811 | 0.7940 | 0.7420 | 742.0 |
| 19 | 0.642 | 0.624 | 0.058 | 0.056 | 0.056 | 0.568 | 0.3120 | 0.2600 | 260.0 |
| 20 | 0.493 | 0.585 | 0.053 | 0.050 | 0.440 | 0.535 | 0.4875 | 0.4355 | 435.5 |
| 21 | 0.478 | 0.478 | 0.063 | 0.063* | 0.415 | 0.415 | 0.4150 | 0.3630 | 363.0 |
| 22 | 0.847 | 0.836 | 0.054 | 0.054 | 0.793 | 0.782 | 0.7875 | 0.7355 | 735.5 |
| 23 | 0.897 | 0.843 | 0.061 | 0.051 | 0.836 | 0.792 | 0.8140 | 0.7620 | 762.0 |
| 24 | 1.037 | 0.990 | 0.067 | 0.065 | 0.970 | 0.925 | 0.9475 | 0.8955 | 895.5 |
| 25 | 0.542 | 0.557 | 0.043 | 0.044 | 0.499 | 0.513 | 0.5060 | 0.4540 | 454.0 |
| 26 | 0.360 | 0.345 | 0.049 | 0.047 | 0.311 | 0.298 | 0.3045 | 0.2525 | 252.5 |
| 27 | 0.441 | 0.462 | 0.051 | 0.045 | 0.390 | 0.417 | 0.4035 | 0.3515 | 351.5 |
| 28 | 0.486 | 0.486 | 0.047 | 0.047* | 0.439 | 0.439 | 0.4390 | 0.3870 | 387.0 |
| 29 | 0.431 | 0.328 | 0.044 | 0.060 | 0.387 | 0.268 | 0.3275 | 0.2755 | 275.5 |
| 30 | 0.475 | 0.333 | 0.051 | 0.058 | 0.424 | 0.275 | 0.3495 | 0.2975 | 297.5 |
| 31 | 0.805 | 0.805 | 0.065 | 0.065* | 0.740 | 0.740 | 0.7400 | 0.6880 | 688.0 |
| 32 | 1.034 | 0.921 | 0.082 | 0.084 | 0.952 | 0.837 | 0.8945 | 0.8425 | 842.5 |
| 33 | 1.135 | 1.213 | 0.043 | 0.047 | 1.092 | 1.166 | 1.1290 | 1.0770 | 1077.0 |
| 34 | 0.464 | 0.463 | 0.052 | 0.045 | 0.412 | 0.418 | 0.4150 | 0.3630 | 363.0 |
| 35 | 0.503 | 0.495 | 0.040 | 0.040 | 0.463 | 0.455 | 0.4590 | 0.4070 | 407.0 |
| 36 | 0.523 | 0.518 | 0.038 | 0.039 | 0.485 | 0.479 | 0.4820 | 0.4300 | 430.0 |
| 37 | 0.390 | 0.380 | 0.047 | 0.046 | 0.343 | 0.334 | 0.3385 | 0.2865 | 286.5 |
| 38 | 0.367 | 0.337 | 0.044 | 0.047 | 0.323 | 0.290 | 0.3065 | 0.2545 | 254.5 |
| 39 | 0.431 | 0.438 | 0.046 | 0.052 | 0.385 | 0.386 | 0.3855 | 0.3335 | 333.5 |

IL-1ra ELISA plate 2

| Standard - pg/mL | A450 #1 | A450 #2 | A570 #1 | A570 #2 | Corrected A450-A570 #1 | Corrected A450-A570 #1 | Mean Corrected A450-A570 | Net Corrected A450-A570 | |
|---|---|---|---|---|---|---|---|---|---|
| 2000 | 2.614 | 2.622 | 0.085 | 0.07 | 2.529 | 2.552 | 2.5405 | 2.4690 | |
| 1000 | 1.251 | 1.602 | 0.066 | 0.055 | 1.185 | 1.547 | 1.3660 | 1.2945 | |
| 500 | 0.728 | 0.908 | 0.05 | 0.052 | 0.678 | 0.856 | 0.7670 | 0.6955 | |
| 250 | 0.388 | 0.467 | 0.047 | 0.051 | 0.341 | 0.416 | 0.3785 | 0.3070 | |
| 125 | 0.225 | 0.24 | 0.046 | 0.048 | 0.179 | 0.192 | 0.1855 | 0.1140 | |
| 62.5 | 0.134 | 0.151 | 0.04 | 0.047 | 0.094 | 0.104 | 0.0990 | 0.0275 | |
| 31.2 | 0.12 | 0.122 | 0.049 | 0.05 | 0.071 | 0.072 | 0.0715 | 0.0000 | |
| 0 | 0.051 | 0.056 | 0.044 | 0.046 | 0.007 | 0.010 | 0.0085 | -0.0630 | |

| Study Sample | A570 #1 | A570 #2 | A570 #1 | A570 #2 | Corrected A450-A570 #1 | Corrected A450-A570 #1 | Mean Corrected A450-A570 | Net Corrected A450-A570 | Antigen - ng/mL |
|---|---|---|---|---|---|---|---|---|---|
| 40 | 1.050 | 1.083 | 0.051 | 0.057 | 0.999 | 1.026 | 1.0125 | 1.0040 | 772.3 |
| 41 | 1.150 | 1.142 | 0.055 | 0.067 | 1.095 | 1.075 | 1.0850 | 1.0765 | 828.1 |
| 42 | 0.979 | 1.014 | 0.056 | 0.065 | 0.923 | 0.949 | 0.9360 | 0.9275 | 713.5 |
| 43 | 0.346 | 0.371 | 0.048 | 0.058 | 0.298 | 0.313 | 0.3055 | 0.2970 | 228.5 |
| 44 | 0.390 | 0.385 | 0.047 | 0.055 | 0.343 | 0.330 | 0.3365 | 0.3280 | 252.3 |
| 45 | 0.292 | 0.266 | 0.047 | 0.054 | 0.245 | 0.212 | 0.2285 | 0.2200 | 169.2 |
| 46 | 0.576 | 0.514 | 0.051 | 0.056 | 0.525 | 0.458 | 0.4915 | 0.4830 | 371.5 |
| 47 | 0.562 | 0.507 | 0.056 | 0.064 | 0.506 | 0.443 | 0.4745 | 0.4660 | 358.5 |
| 48 | 0.511 | 0.541 | 0.052 | 0.050 | 0.459 | 0.491 | 0.4750 | 0.4665 | 358.8 |
| 49 | 0.798 | 0.788 | 0.059 | 0.057 | 0.739 | 0.731 | 0.7350 | 0.7265 | 558.8 |
| 50 | 0.703 | 0.697 | 0.063 | 0.062 | 0.640 | 0.635 | 0.6375 | 0.6290 | 483.8 |
| 51 | 0.820 | 0.829 | 0.060 | 0.058 | 0.760 | 0.771 | 0.7655 | 0.7570 | 582.3 |

| IL-1ra plate 2 Sample | A450 #1 | A450 #2 | A570 #1 | A570 #2 | Corrected A450-A570 #1 | Corrected A450-A570 #1 | Mean Corrected A450-A570 | Net Corrected A450-A570 | |
|---|---|---|---|---|---|---|---|---|---|
| 52 | 0.357 | 0.372 | 0.065 | 0.066 | 0.292 | 0.306 | 0.2990 | 0.2905 | 223.5 |
| 53 | 0.394 | 0.374 | 0.068 | 0.061 | 0.326 | 0.313 | 0.3195 | 0.3110 | 239.2 |
| 54 | 0.319 | 0.336 | 0.054 | 0.048 | 0.265 | 0.288 | 0.2765 | 0.2680 | 206.2 |
| 55 | 0.478 | 0.473 | 0.066 | 0.069 | 0.412 | 0.404 | 0.4080 | 0.3995 | 307.3 |
| 56 | 0.484 | 0.467 | 0.053 | 0.046 | 0.431 | 0.421 | 0.4260 | 0.4175 | 321.2 |
| 57 | 0.615 | 0.613 | 0.059 | 0.060 | 0.556 | 0.553 | 0.5545 | 0.5460 | 420.0 |
| 58 | 0.437 | 0.449 | 0.057 | 0.058 | 0.380 | 0.391 | 0.3855 | 0.3770 | 290.0 |
| 59 | 0.411 | 0.381 | 0.056 | 0.055 | 0.355 | 0.326 | 0.3405 | 0.3320 | 255.4 |
| 60 | 0.443 | 0.400 | 0.066 | 0.064 | 0.377 | 0.336 | 0.3565 | 0.3480 | 267.7 |
| 61 | 0.414 | 0.393 | 0.048 | 0.047 | 0.366 | 0.346 | 0.3560 | 0.3475 | 267.3 |
| 62 | 0.398 | 0.429 | 0.048 | 0.052 | 0.350 | 0.377 | 0.3635 | 0.3550 | 273.1 |
| 63 | 0.394 | 0.358 | 0.068 | 0.067 | 0.326 | 0.291 | 0.3085 | 0.3000 | 230.8 |
| 64 | 0.659 | 0.454 | 0.044 | 0.046 | 0.615 | 0.408 | 0.5115 | 0.5030 | 386.9 |
| 65 | 0.621 | 0.662 | 0.050 | 0.055 | 0.571 | 0.607 | 0.5890 | 0.5805 | 446.5 |
| 66 | 0.669 | 0.657 | 0.052 | 0.059 | 0.617 | 0.598 | 0.6075 | 0.5990 | 460.8 |
| 67 | 0.341 | 0.318 | 0.050 | 0.042 | 0.291 | 0.276 | 0.2835 | 0.2750 | 211.5 |
| 68 | 0.301 | 0.304 | 0.044 | 0.051 | 0.257 | 0.253 | 0.2550 | 0.2465 | 189.6 |
| 69 | 0.291 | 0.293 | 0.045 | 0.052 | 0.246 | 0.241 | 0.2435 | 0.2350 | 180.8 |
| 70 | 0.468 | 0.351 | 0.043 | 0.060 | 0.425 | 0.291 | 0.3580 | 0.3495 | 268.8 |
| 71 | 0.486 | 0.483 | 0.060 | 0.075 | 0.426 | 0.408 | 0.4170 | 0.4085 | 314.2 |
| 72 | 0.424 | 0.424 | 0.039 | 0.039 | 0.385 | 0.385 | 0.3850 | 0.3765 | 289.6 |

| TGF-β ELISA plate 1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Standard - pg/mL | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ |
| 2000 | 1.726 | 1.792 | 0.065 | 0.043 | 1.680 | 1.738 | 1.7090 | 1.6500 |
| 1000 | 1.032 | 1.132 | 0.055 | 0.045 | 0.989 | 1.087 | 1.0380 | 0.9790 |
| 500 | 0.566 | 0.646 | 0.048 | 0.044 | 0.520 | 0.600 | 0.5600 | 0.5010 |
| 250 | 0.302 | 0.363 | 0.038 | 0.041 | 0.255 | 0.312 | 0.2835 | 0.2245 |
| 125 | 0.203 | 0.221 | 0.049 | 0.046 | 0.154 | 0.159 | 0.1565 | 0.0975 |
| 62.5 | 0.143 | 0.161 | 0.05 | 0.048 | 0.094 | 0.114 | 0.1040 | 0.0450 |
| 31.2 | 0.103 | 0.114 | 0.046 | 0.043 | 0.048 | 0.070 | 0.0590 | 0.0000 |
| 0 | 0.07 | 0.078 | 0.041 | 0.042 | 0.014 | 0.026 | 0.0200 | -0.0390 |

**Experimental Samples**

| Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - pg/mL |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.888 | 0.822 | 0.041 | 0.042 | 0.847 | 0.780 | 0.8135 | 0.7935 | 35266.7 |
| 2 | 1.192 | 1.090 | 0.043 | 0.053 | 1.149 | 1.037 | 1.0930 | 1.0730 | 47688.9 |
| 3 | 0.428 | 0.395 | 0.044 | 0.046 | 0.384 | 0.349 | 0.3665 | 0.3465 | 15400.0 |
| 4 | 1.212 | 1.116 | 0.048 | 0.051 | 1.164 | 1.065 | 1.1145 | 1.0945 | 48644.4 |
| 5 | 0.493 | 0.442 | 0.054 | 0.054 | 0.439 | 0.388 | 0.4135 | 0.3935 | 17488.9 |
| 6 | 0.156 | 0.146 | 0.052 | 0.054 | 0.104 | 0.092 | 0.0980 | 0.0780 | 3466.7 |
| 7 | 1.253 | 1.153 | 0.058 | 0.056 | 1.195 | 1.097 | 1.1460 | 1.1260 | 50044.4 |
| 8 | 0.573 | 0.573 | 0.044 | 0.048 | 0.529 | 0.525 | 0.5270 | 0.5070 | 22533.3 |
| 9 | 0.689 | 0.644 | 0.042 | 0.040 | 0.647 | 0.604 | 0.6255 | 0.6055 | 26911.1 |
| 10 | 1.220 | 1.251 | 0.051 | 0.049 | 1.169 | 1.202 | 1.1855 | 1.1655 | 51800.0 |
| 11 | 0.419 | 0.418 | 0.049 | 0.047 | 0.370 | 0.371 | 0.3705 | 0.3505 | 15577.8 |
| 12 | 1.402 | 1.467 | 0.053 | 0.051 | 1.349 | 1.416 | 1.3825 | 1.3625 | 60555.6 |

| TGF-β plate 1 Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - pg/mL |
|---|---|---|---|---|---|---|---|---|---|
| 13 | 1.269 | 1.345 | 0.059 | 0.056 | 1.210 | 1.289 | 1.2495 | 1.2295 | 54644.4 |
| 14 | 1.049 | 1.271 | 0.060 | 0.058 | 0.989 | 1.213 | 1.1010 | 1.0810 | 48044.4 |
| 15 | 1.177 | 1.210 | 0.058 | 0.056 | 1.119 | 1.154 | 1.1365 | 1.1165 | 49622.2 |
| 16 | 1.934 | 1.839 | 0.069 | 0.068 | 1.865 | 1.771 | 1.8180 | 1.7980 | 79911.1 |
| 17 | 1.129 | 1.112 | 0.041 | 0.041 | 1.088 | 1.071 | 1.0795 | 1.0595 | 47088.9 |
| 18 | 0.357 | 0.337 | 0.045 | 0.044 | 0.312 | 0.293 | 0.3025 | 0.2825 | 12555.6 |
| 19 | 1.086 | 1.032 | 0.048 | 0.048 | 0.056 | 0.984 | 0.5200 | 0.5000 | 22222.2 |
| 20 | 0.377 | 0.361 | 0.047 | 0.044 | 0.330 | 0.317 | 0.3235 | 0.3035 | 13488.9 |
| 21 | 0.604 | 0.608 | 0.056 | 0.053 | 0.548 | 0.555 | 0.5515 | 0.5315 | 23622.2 |
| 22 | 1.719 | 1.738 | 0.060 | 0.059 | 1.659 | 1.679 | 1.6690 | 1.6490 | 73288.9 |
| 23 | 1.052 | 1.059 | 0.056 | 0.055 | 0.996 | 1.004 | 1.0000 | 0.9800 | 43555.6 |
| 24 | 1.189 | 1.234 | 0.062 | 0.060 | 1.127 | 1.174 | 1.1505 | 1.1305 | 50244.4 |
| 25 | 0.871 | 0.822 | 0.041 | 0.043 | 0.830 | 0.779 | 0.8045 | 0.7845 | 34866.7 |
| 26 | 0.517 | 0.504 | 0.044 | 0.043 | 0.473 | 0.461 | 0.4670 | 0.4470 | 19866.7 |
| 27 | 0.245 | 0.230 | 0.046 | 0.047 | 0.199 | 0.183 | 0.1910 | 0.1710 | 7600.0 |
| 28 | 0.685 | 0.665 | 0.045 | 0.048 | 0.640 | 0.617 | 0.6285 | 0.6085 | 27044.4 |
| 29 | 1.081 | 1.052 | 0.057 | 0.055 | 1.024 | 0.997 | 1.0105 | 0.9905 | 44022.2 |
| 30 | 1.174 | 1.105 | 0.048 | 0.052 | 1.126 | 1.053 | 1.0895 | 1.0695 | 47533.3 |
| 31 | 0.863 | 0.775 | 0.053 | 0.053 | 0.810 | 0.722 | 0.7660 | 0.7460 | 33155.6 |
| 32 | 1.382 | 1.301 | 0.061 | 0.064 | 1.321 | 1.237 | 1.2790 | 1.2590 | 55955.6 |
| 33 | 0.297 | 0.340 | 0.045 | 0.037 | 0.252 | 0.303 | 0.2775 | 0.2575 | 11444.4 |
| 34 | 0.324 | 0.329 | 0.049 | 0.042 | 0.275 | 0.287 | 0.2810 | 0.2610 | 11600.0 |
| 35 | 1.082 | 1.102 | 0.049 | 0.042 | 1.033 | 1.060 | 1.0465 | 1.0265 | 45622.2 |
| 36 | 0.727 | 0.737 | 0.039 | 0.040 | 0.688 | 0.697 | 0.6925 | 0.6725 | 29888.9 |
| 37 | 1.722 | 1.742 | 0.052 | 0.050 | 1.670 | 1.692 | 1.6810 | 1.6610 | 73822.2 |
| 38 | 1.017 | 1.024 | 0.046 | 0.052 | 0.971 | 0.972 | 0.9715 | 0.9515 | 42288.9 |
| 39 | 0.761 | 0.696 | 0.060 | 0.067 | 0.701 | 0.629 | 0.6650 | 0.6450 | 28666.7 |

| TGF-beta ELISA plate 2 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Standard - pg/mL | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ |
| 2000 | 1.856 | 1.8 | 0.046 | 0.054 | 1.810 | 1.746 | 1.7780 | 1.7020 |
| 1000 | 1.052 | 1.029 | 0.043 | 0.045 | 1.009 | 0.984 | 0.9965 | 0.9205 |
| 500 | 0.656 | 0.604 | 0.046 | 0.046 | 0.610 | 0.558 | 0.5840 | 0.5080 |
| 250 | 0.351 | 0.364 | 0.047 | 0.051 | 0.304 | 0.313 | 0.3085 | 0.2325 |
| 125 | 0.231 | 0.238 | 0.049 | 0.062 | 0.182 | 0.176 | 0.1790 | 0.1030 |
| 62.5 | 0.161 | 0.155 | 0.049 | 0.047 | 0.112 | 0.108 | 0.1100 | 0.0340 |
| 31.2 | 0.133 | 0.118 | 0.055 | 0.044 | 0.078 | 0.074 | 0.0760 | 0.0000 |
| 0 | 0.09 | 0.091 | 0.056 | 0.052 | 0.034 | 0.039 | 0.0365 | -0.0395 |

**Study Samples**

| Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - pg/mL |
|---|---|---|---|---|---|---|---|---|---|
| 40 | 1.528 | 1.587 | 0.044 | 0.045 | 1.484 | 1.542 | 1.5130 | 1.4765 | 65622.22 |
| 41 | 0.630 | 0.656 | 0.042 | 0.041 | 0.588 | 0.615 | 0.6015 | 0.5650 | 25111.11 |
| 42 | 0.617 | 0.616 | 0.042 | 0.041 | 0.575 | 0.575 | 0.5750 | 0.5385 | 23933.33 |
| 43 | 1.886 | 1.862 | 0.050 | 0.047 | 1.836 | 1.815 | 1.8255 | 1.7890 | 79511.11 |
| 44 | 1.273 | 1.254 | 0.068 | 0.058 | 1.205 | 1.196 | 1.2005 | 1.1640 | 51733.33 |
| 45 | 0.535 | 0.528 | 0.059 | 0.063 | 0.476 | 0.465 | 0.4705 | 0.4340 | 19288.89 |
| 46 | 1.278 | 1.274 | 0.054 | 0.058 | 1.224 | 1.216 | 1.2200 | 1.1835 | 52600.00 |
| 47 | 0.874 | 0.921 | 0.052 | 0.068 | 0.822 | 0.853 | 0.8375 | 0.8010 | 35600.00 |
| 48 | 0.369 | 0.365 | 0.048 | 0.044 | 0.321 | 0.321 | 0.3210 | 0.2845 | 12644.44 |
| 49 | 0.611 | 0.674 | 0.042 | 0.042 | 0.569 | 0.632 | 0.6005 | 0.5640 | 25066.67 |
| 50 | 0.978 | 0.963 | 0.055 | 0.040 | 0.923 | 0.923 | 0.9230 | 0.8865 | 39400.00 |
| 51 | | | | | 0.000 | 0.000 | 0.0000 | | #VALUE! |
| 52 | 0.322 | 0.334 | 0.044 | 0.045 | 0.278 | 0.289 | 0.2835 | 0.2470 | 10977.78 |

| TGF=β plate 2 Sample | $A_{570}$ #1 | $A_{570}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen - pg/mL |
|---|---|---|---|---|---|---|---|---|---|
| 53 | 0.761 | 0.804 | 0.065 | 0.059 | 0.696 | 0.745 | 0.7205 | 0.6840 | 30400.00 |
| 54 | 0.567 | 0.588 | 0.054 | 0.061 | 0.513 | 0.527 | 0.5200 | 0.4835 | 21488.89 |
| 55 | 1.236 | 1.240 | 0.059 | 0.066 | 1.177 | 1.174 | 1.1755 | 1.1390 | 50622.22 |
| 56 | 0.566 | 0.543 | 0.039 | 0.039 | 0.527 | 0.504 | 0.5155 | 0.4790 | 21288.89 |
| 57 | 0.845 | 0.782 | 0.051 | 0.038 | 0.794 | 0.744 | 0.7690 | 0.7325 | 32555.56 |
| 58 | 0.923 | 0.915 | 0.042 | 0.046 | 0.881 | 0.869 | 0.8750 | 0.8385 | 37266.67 |
| 59 | 1.430 | 1.517 | 0.046 | 0.050 | 1.384 | 1.467 | 1.4255 | 1.3890 | 61733.33 |
| 60 | 1.387 | 0.756 | 0.049 | 0.052 | 1.338 | 0.704 | 1.0210 | 0.9845 | 43755.56 |
| 61 | 0.497 | 0.506 | 0.053 | 0.051 | 0.444 | 0.455 | 0.4495 | 0.4130 | 18355.56 |
| 62 | 0.459 | 0.474 | 0.049 | 0.040 | 0.410 | 0.434 | 0.4220 | 0.3855 | 17133.33 |
| 63 | 1.469 | 1.586 | 0.057 | 0.057 | 1.412 | 1.529 | 1.4705 | 1.4340 | 63733.33 |
| 64 | 1.734 | 1.647 | 0.044 | 0.043 | 1.690 | 1.604 | 1.6470 | 1.6105 | 71577.78 |
| 65 | 0.964 | 0.987 | 0.047 | 0.047 | 0.917 | 0.940 | 0.9285 | 0.8920 | 39644.44 |
| 66 | 0.804 | 0.819 | 0.043 | 0.044 | 0.761 | 0.775 | 0.7680 | 0.7315 | 32511.11 |
| 67 | 0.326 | 0.322 | 0.039 | 0.039 | 0.287 | 0.283 | 0.2850 | 0.2485 | 11044.44 |
| 68 | 1.028 | 1.016 | 0.043 | 0.047 | 0.985 | 0.969 | 0.9770 | 0.9405 | 41800.00 |
| 69 | 0.234 | 0.238 | 0.040 | 0.043 | 0.194 | 0.195 | 0.1945 | 0.1580 | 7022.22 |
| 70 | 0.762 | 0.711 | 0.041 | 0.042 | 0.721 | 0.669 | 0.6950 | 0.6585 | 29266.67 |
| 71 | 1.171 | 1.215 | 0.051 | 0.062 | 1.120 | 1.153 | 1.1365 | 1.1000 | 48888.89 |
| 72 | 0.607 | 0.616 | 0.038 | 0.042 | 0.569 | 0.574 | 0.5715 | 0.5350 | 23777.78 |

**PGE-2 ELISA plate 2**

| Standard - pg/mL | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ |
|---|---|---|---|---|---|---|---|---|
| 2500 | 0.125 | 0.127 | 0.042 | 0.044 | 0.083 | 0.083 | 0.0830 | 12.9283 |
| 1250 | 0.165 | 0.292 | 0.041 | 0.051 | 0.124 | 0.241 | 0.1825 | 28.4268 |
| 625 | 0.266 | 0.28 | 0.044 | 0.048 | 0.222 | 0.232 | 0.2270 | 35.3583 |
| 312 | 0.403 | 0.337 | 0.045 | 0.051 | 0.358 | 0.286 | 0.3220 | 50.1558 |
| 156 | 0.444 | 0.438 | 0.06 | 0.062 | 0.384 | 0.376 | 0.3800 | 59.1900 |
| 78 | 0.538 | 0.51 | 0.055 | 0.055 | 0.483 | 0.455 | 0.4690 | 73.0530 |
| 39 | 0.595 | 0.539 | 0.053 | 0.06 | 0.542 | 0.479 | 0.5105 | 79.5171 |
| 0 | 0.642 | 0.534 | 0.056 | 0.059 | 0.586 | 0.475 | 0.5305 | 82.6324 |

**Study Samples**

| Sample | $A_{450}$ #1 | $A_{450}$ #2 | $A_{570}$ #1 | $A_{570}$ #2 | Corrected $A_{450}$-$A_{570}$ #1 | Corrected $A_{450}$-$A_{570}$ #1 | Mean Corrected $A_{450}$-$A_{570}$ | Net Corrected $A_{450}$-$A_{570}$ | Antigen concentration pg/mL |
|---|---|---|---|---|---|---|---|---|---|
| 40 | 0.491 | 0.543 | 0.047 | 0.048 | 0.444 | 0.495 | 0.4695 | 73.1308 | -3245.68 |
| 41 | 0.459 | 0.420 | 0.046 | 0.048 | 0.413 | 0.372 | 0.3925 | 61.1371 | 5909.87 |
| 42 | 0.445 | 0.443 | 0.051 | 0.051 | 0.394 | 0.392 | 0.3930 | 61.2150 | 5850.42 |
| 43 | 0.478 | 0.515 | 0.046 | 0.052 | 0.432 | 0.463 | 0.4475 | 69.7040 | -629.81 |
| 44 | 0.470 | 0.578 | 0.067 | 0.056 | 0.403 | 0.522 | 0.4625 | 72.0405 | -2413.36 |
| 45 | 0.491 | 0.528 | 0.063 | 0.064 | 0.428 | 0.464 | 0.4460 | 69.4704 | -451.45 |
| 46 | 0.460 | 0.531 | 0.056 | 0.056 | 0.404 | 0.475 | 0.4395 | 68.4579 | 321.42 |
| 47 | 0.521 | 0.640 | 0.060 | 0.061 | 0.461 | 0.579 | 0.5200 | 80.9969 | -9250.29 |
| 48 | 0.540 | 0.655 | 0.047 | 0.048 | 0.493 | 0.607 | 0.5500 | 85.6698 | -12817.39 |
| 49 | 0.499 | 0.429 | 0.050 | 0.053 | 0.449 | 0.376 | 0.4125 | 64.2523 | 3531.80 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 50 | 0.546 | 0.688 | 0.054 | 0.061 | 0.492 | 0.627 | 0.5595 | 87.1495 | -13946.97 |
| 51 | 0.560 | 0.780 | 0.053 | 0.062 | 0.507 | 0.718 | 0.6125 | 95.4050 | -20248.84 |
| 52 | 0.582 | 0.652 | 0.064 | 0.064 | 0.518 | 0.588 | 0.5530 | 86.1371 | -13174.10 |
| **PGE-2 plate 2 Sample** | **$A_{450}$ #1** | **$A_{450}$ #2** | **$A_{570}$ #1** | **$A_{570}$ #2** | **Corrected $A_{450}$-$A_{570}$ #1** | **Corrected $A_{450}$-$A_{570}$ #1** | **Mean Corrected $A_{450}$-$A_{570}$** | **Net Corrected $A_{450}$-$A_{570}$** | Antigen concentration pg/mL |
| 53 | 0.609 | 0.753 | 0.061 | 0.074 | 0.548 | 0.679 | 0.6135 | 95.5607 | -20367.75 |
| 54 | 0.532 | 0.626 | 0.054 | 0.059 | 0.478 | 0.567 | 0.5225 | 81.3863 | -9547.55 |
| 55 | 0.574 | 0.660 | 0.067 | 0.065 | 0.507 | 0.595 | 0.5510 | 85.8255 | -12936.29 |
| 56 | 0.468 | 0.614 | 0.048 | 0.046 | 0.420 | 0.568 | 0.4940 | 76.9470 | -6158.81 |
| 57 | 0.455 | 0.806 | 0.055 | 0.048 | 0.400 | 0.758 | 0.5790 | 90.1869 | -16265.58 |
| 58 | 0.453 | 0.737 | 0.058 | 0.054 | 0.395 | 0.683 | 0.5390 | 83.9564 | -11509.46 |
| 59 | 0.467 | 0.642 | 0.058 | 0.059 | 0.409 | 0.583 | 0.4960 | 77.2586 | -6396.62 |
| 60 | 0.522 | 0.705 | 0.065 | 0.064 | 0.457 | 0.641 | 0.5490 | 85.5140 | -12698.49 |
| 61 | 0.503 | 0.689 | 0.047 | 0.056 | 0.456 | 0.633 | 0.5445 | 84.8131 | -12163.42 |
| 62 | 0.533 | 0.595 | 0.063 | 0.053 | 0.470 | 0.542 | 0.5060 | 78.8162 | -7585.65 |
| 63 | 0.595 | 0.562 | 0.061 | 0.061 | 0.534 | 0.501 | 0.5175 | 80.6075 | -8953.04 |
| 64 | 0.497 | 0.768 | 0.059 | 0.052 | 0.438 | 0.716 | 0.5770 | 89.8754 | -16027.78 |
| 65 | 0.460 | 0.559 | 0.060 | 0.056 | 0.400 | 0.503 | 0.4515 | 70.3271 | -1105.42 |
| 66 | 0.471 | 0.623 | 0.062 | 0.050 | 0.409 | 0.573 | 0.4910 | 76.4798 | -5802.10 |
| 67 | 0.425 | 0.774 | 0.057 | 0.054 | 0.368 | 0.720 | 0.5440 | 84.7352 | -12103.97 |
| 68 | 0.394 | 0.394 | 0.051 | 0.053 | 0.343 | 0.341 | 0.3420 | 53.2710 | 11914.48 |
| 69 | 0.444 | 0.719 | 0.051 | 0.058 | 0.393 | 0.661 | 0.5270 | 82.0872 | -10082.62 |
| 70 | 0.455 | 0.624 | 0.050 | 0.051 | 0.405 | 0.573 | 0.4890 | 76.1682 | -5564.29 |
| 71 | 0.536 | 0.695 | 0.058 | 0.082 | 0.478 | 0.613 | 0.5455 | 84.9688 | -12282.33 |
| 72 | 0.491 | 0.533 | 0.047 | 0.050 | 0.444 | 0.483 | 0.4635 | 72.1963 | -2532.26 |

Appendix VIII RNA quantification data ng/mL

| | | | | | |
|---|---|---|---|---|---|
| 1.1 | 98.6 | 1.2 | 87.2 | 1.3 | 11.6 |
| 2.1 | 101.3 | 2.2 | 81.2 | 2.3 | 102.5 |
| 3.1 | 82.6 | 3.2 | 66.1 | 3.3 | 96.6 |
| 4.1 | 74.5 | 4.2 | 64.4 | 4.3 | 79.6 |
| 5.1 | 66.6 | 5.2 | 52.9 | 5.3 | 35.9 |
| 6.1 | 114.4 | 6.2 | 70.5 | 6.3 | 68.8 |
| 7.1 | 62.4 | 7.2 | 92.2 | 7.3 | 136.3 |
| 8.1 | 55.9 | 8.2 | 89.7 | 8.3 | 78.9 |
| 9.1 | 108.3 | 9.2 | 78.9 | 9.3 | 68.6 |
| 10.1 | 71.7 | 10.2 | 90.5 | 10.3 | 77.5 |
| 11.1 | 49.8 | 11.2 | 47.9 | 11.3 | 63.7 |
| 12.1 | 148.7 | 12.2 | 97.7 | 12.3 | 76.9 |
| 13.1 | 110.3 | 13.2 | 110.5 | 13.3 | 88.9 |
| 14.1 | 141.8 | 14.2 | 61.3 | 14.3 | 107.5 |
| 15.1 | 42 | 15.2 | 43 | 15.3 | 56.6 |
| 16.1 | 117.5 | 16.2 | 165.7 | 16.3 | 113.2 |
| 17.1 | 117 | 17.2 | 90.8 | 17.3 | 146 |
| 18.1 | * | 18.2 | 81.2 | 18.3 | 75.7 |
| 19.1 | 131.1 | 19.2 | 118.1 | 19.3 | 92.7 |
| 20.1 | 72.5 | 20.2 | 67.6 | 20.3 | 81.7 |
| 21.1 | 80.5 | 21.2 | 17.9 | 21.3 | 56.5 |
| 22.1 | 276.3 | 22.2 | 150.7 | 22.3 | 159.4 |
| 23.1 | 98.7 | 23.2 | 89.4 | 23.3 | 150.6 |
| 24.1 | 66.4 | 24.2 | 103 | 24.3 | 67.7 |

RNA extractions 2,4,5 November 2009
Rtempus RNA extraction kit product no 4378996 Lot 0908015

* no RNA detected by nanodrop

Appendix IX Endogenous control selection

| Sample Name | Endogenous control | Ct | Ct Mean | Endogenous control | Ct | Ct Mean | Endoge control |
|---|---|---|---|---|---|---|---|
| Sample 1 | Gap | 17.7246 | 17.6405 | Cyc | 17.3807 | 17.2848 | B act |
| Sample 1 | Gap | 17.5564 | 17.6405 | Cyc | 17.1889 | 17.2848 | B act |
| Sample 2 | Gap | 17.2832 | 17.1934 | Cyc | 16.9376 | 16.8539 | B act |
| Sample 2 | Gap | 17.1035 | 17.1934 | Cyc | 16.7703 | 16.8539 | B act |
| Sample 3 | Gap | 17.4102 | 17.2633 | Cyc | 17.2845 | 17.2254 | B act |
| Sample 3 | Gap | 17.1164 | 17.2633 | Cyc | 17.1662 | 17.2254 | B act |
| Sample 4 | Gap | 17.7276 | 17.6723 | Cyc | 17.3871 | 17.4822 | B act |
| Sample 4 | Gap | 17.6169 | 17.6723 | Cyc | 17.5773 | 17.4822 | B act |
| Sample 5 | Gap | 17.2123 | 17.1391 | Cyc | 16.8673 | 16.7985 | B act |
| Sample 5 | Gap | 17.0658 | 17.1391 | Cyc | 16.7298 | 16.7985 | B act |
| Sample 6 | Gap | 17.5796 | 17.5704 | Cyc | 17.4456 | 17.4235 | B act |
| Sample 6 | Gap | 17.5613 | 17.5704 | Cyc | 17.4013 | 17.4235 | B act |
| | mean | 17.41315 | | mean | 17.17805 | | mean |
| | SD | 0.236185 | | SD | 0.273671 | | SD |

Appendix X Dilution template data

| | Dilution | CT |
|---|---|---|
| GAP DH | 1 | 17.86455 |
| | 10 | 20.68525 |
| | 100 | 24.2107 |
| | 1000 | 27.2651 |
| IL-1ra | 1 | 20.04115 |
| | 10 | 23.57535 |
| | 100 | 25.9145 |
| | 1000 | 29.6282 |
| TGF beta | 1 | 22.19335 |
| | 10 | 25.438 |
| | 100 | 28.4327 |
| | 1000 | 31.87545 |

Appendix XI

| | GAPDH | | IL-1ra | | | | | TGF-beta | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study # | CT | Mean CT | CT | mean CT | Δ CT | ΔΔ CT | Fold change | CT | mean CT | Δ CT | ΔΔ CT | Fold change |
| 1.1 | 17.7246 | 17.6405 | 19.7419 | 19.6967 | 2.0562 | 0 | 1 | 21.904 | 21.85215 | 4.21165 | 0 | 1 |
| 1.1 | 17.5564 | | 19.6515 | | | | | 21.8003 | | | | |
| 1.2 | 17.2832 | 17.19335 | 19.4989 | 19.47965 | 2.2863 | 0.2301 | 0.852576 | 21.6609 | 21.69655 | 4.5032 | 0.29155 | 0.81702 |
| 1.2 | 17.1035 | | 19.4604 | | | | | 21.7322 | | | | |
| 1.3 | 17.4102 | 17.2633 | 19.787 | 19.75695 | 2.49365 | 0.43745 | 0.738439 | 21.7554 | 21.7338 | 4.4705 | 0.25885 | 0.83575 |
| 1.3 | 17.1164 | | 19.7269 | | | | | 21.7122 | | | | |
| 2.1 | 17.7276 | 17.67225 | 19.8532 | 19.856 | 2.18375 | 0 | 1 | 21.977 | 21.92755 | 4.2553 | 0 | 1 |
| 2.1 | 17.6169 | | 19.8588 | | | | | 21.8781 | | | | |
| 2.2 | 17.2123 | 17.13905 | 19.3165 | 19.3852 | 2.24615 | 0.0624 | 0.95767 | 21.5691 | 21.58365 | 4.4446 | 0.1893 | 0.87703 |
| 2.2 | 17.0658 | | 19.4539 | | | | | 21.5982 | | | | |
| 2.3 | 17.5796 | 17.57045 | 19.8827 | 19.9754 | 2.40495 | 0.2212 | 0.857852 | 22.116 | 22.11125 | 4.5408 | 0.2855 | 0.82045 |
| 2.3 | 17.5613 | | 20.0681 | | | | | 22.1065 | | | | |
| 3.1 | 18.199 | 17.9316 | 20.7737 | 20.5459 | 2.6143 | 0 | 1 | 22.4495 | 22.34365 | 4.41205 | 0 | 1 |
| 3.1 | 17.6642 | | 20.3181 | | | | | 22.2378 | | | | |
| 3.2 | 17.5718 | 17.52545 | 19.8562 | 19.8265 | 2.30105 | -0.31325 | 1.242504 | 21.7703 | 21.8294 | 4.30395 | -0.1081 | 1.07780 |
| 3.2 | 17.4791 | | 19.7968 | | | | | 21.8885 | | | | |
| 3.3 | 16.9665 | 16.91115 | 19.6154 | 19.54525 | 2.6341 | 0.0198 | 0.986369 | 21.1418 | 20.95225 | 4.0411 | -0.37095 | 1.29320 |
| 3.3 | 16.8558 | | 19.4751 | | | | | 20.7627 | | | | |
| 4.1 | 16.8343 | 17.09025 | 19.592 | 19.54075 | 2.4505 | 0 | 1 | 21.7589 | 21.79135 | 4.7011 | 0 | 1 |
| 4.1 | 17.3462 | | 19.4895 | | | | | 21.8238 | | | | |
| 4.2 | 17.3459 | 17.31165 | 19.9894 | 20.07035 | 2.7587 | 0.3082 | 0.807649 | 21.9926 | 21.9441 | 4.63245 | -0.06865 | 1.04873 |
| 4.2 | 17.2774 | | 20.1513 | | | | | 21.8956 | | | | |
| 4.3 | 17.7188 | 17.95275 | 20.4731 | 20.6083 | 2.65555 | 0.20505 | 0.867509 | 22.5777 | 22.6583 | 4.70555 | 0.00445 | 0.9969 |
| 4.3 | 18.1867 | | 20.7435 | | | | | 22.7389 | | | | |

| | GAPDH | | IL-1ra | | | | | TGF-β | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study # | CT | Mean CT | CT | mean CT | Δ CT | ΔΔ CT | Fold change | CT | mean CT | Δ CT | ΔΔ CT | Fold change |
| 5.1 | 16.7523 | 16.5766 | 19.5005 | 19.06895 | 2.49235 | 0 | 1 | 21.6779 | 21.67135 | 5.09475 | 0 | 1 |
| 5.1 | 16.4009 | | 18.6374 | | | | | 21.6648 | | | | |
| 5.2 | 16.3949 | 16.32965 | 18.7957 | 18.54775 | 2.2181 | -0.27425 | 1.209365 | 20.4284 | 20.39185 | 4.0622 | -1.03255 | 2.04563 |
| 5.2 | 16.2644 | | 18.2998 | | | | | 20.3553 | | | | |
| 5.3 | 16.8411 | 16.4956 | 19.5854 | 19.2675 | 2.7719 | 0.27955 | 0.823848 | 21.8068 | 21.36525 | 4.86965 | -0.2251 | 1.16885 |
| 5.3 | 16.1501 | | 18.9496 | | | | | 20.9237 | | | | |
| 6.1 | 16.922 | 16.63335 | 18.9998 | 19.0451 | 2.41175 | 0 | 1 | 21.6835 | 21.61155 | 4.9782 | 0 | 1 |
| 6.1 | 16.3447 | | 19.0904 | | | | | 21.5396 | | | | |
| 6.2 | 18.5659 | 18.58545 | 21.4447 | 21.33285 | 2.7474 | 0.33565 | 0.792427 | 23.5968 | 23.42075 | 4.8353 | -0.1429 | 1.10412 |
| 6.2 | 18.605 | | 21.221 | | | | | 23.2447 | | | | |
| 6.3 | 18.3761 | 18.6442 | 19.5447 | 19.56175 | 0.91755 | -1.4942 | 2.817079 | 21.8898 | 21.80995 | 3.16575 | -1.81245 | 3.51238 |
| 6.3 | 18.9123 | | 19.5788 | | | | | 21.7301 | | | | |
| 7.1 | 18.624 | 18.45405 | 19.5638 | 19.5697 | 1.11565 | 0 | 1 | 21.6099 | 21.4428 | 2.98875 | 0 | 1 |
| 7.1 | 18.2841 | | 19.5756 | | | | | 21.2757 | | | | |
| 7.2 | 17.3018 | 17.2427 | 19.2272 | 19.26745 | 2.02475 | 0.9091 | 0.532517 | 21.0575 | 21.1063 | 3.8636 | 0.87485 | 0.54531 |
| 7.2 | 17.1836 | | 19.3077 | | | | | 21.1551 | | | | |
| 7.3 | 16.8125 | 16.77945 | 18.8501 | 18.745 | 1.96555 | 0.8499 | 0.554823 | 20.8902 | 20.89995 | 4.1205 | 1.13175 | 0.45636 |
| 7.3 | 16.7464 | | 18.6399 | | | | | 20.9097 | | | | |
| 8.1 | 17.3882 | 17.13275 | 20.2083 | 20.0194 | 2.88665 | 0 | 1 | 21.5455 | 21.6363 | 4.50355 | 0 | 1 |
| 8.1 | 16.8773 | | 19.8305 | | | | | 21.7271 | | | | |
| 8.2 | 18.0809 | 18.011 | 21.3888 | 21.0455 | 3.0345 | 0.14785 | 0.902595 | 22.7876 | 22.66895 | 4.65795 | 0.1544 | 0.89850 |
| 8.2 | 17.9411 | | 20.7022 | | | | | 22.5503 | | | | |
| 8.3 | 16.0418 | 16.02 | 18.6382 | 18.6128 | 2.5928 | -0.29385 | 1.225907 | 20.5654 | 20.5941 | 4.5741 | 0.07055 | 0.95227 |
| 8.3 | 15.9982 | | 18.5874 | | | | | 20.6228 | | | | |
| 9.1 | 17.2035 | 17.01735 | 18.9983 | 18.87075 | 1.8534 | 0 | 1 | 21.4367 | 21.16305 | 4.1457 | 0 | 1 |
| 9.1 | 16.8312 | | 18.7432 | | | | | 20.8894 | | | | |
| 9.2 | 17.5738 | 17.10615 | 19.8445 | 19.82415 | 2.718 | 0.8646 | 0.549199 | 22.4435 | 21.76885 | 4.6627 | 0.517 | 0.69882 |
| 9.2 | 16.6385 | | 19.8038 | | | | | 21.0942 | | | | |
| 9.3 | 17.7053 | 17.0485 | 19.2161 | 19.0591 | 2.0106 | 0.1572 | 0.896764 | 21.407 | 20.9593 | 3.9108 | -0.2349 | 1.17682 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9.3 | 16.3917 | | 18.9021 | | | | | 20.5116 | | | | | |
| | GAPDH | | | | IL-1ra | | | | | TGF-β | | | |
| Study # | CT | Mean CT | CT | mean CT | Δ CT | ΔΔ CT | Fold change | CT | mean CT | Δ CT | ΔΔ CT | Fold change |
| 10.1 | 16.8752 | 16.8834 | 18.7871 | 18.88385 | 2.00045 | 0 | 1 | 21.3159 | 20.87595 | 3.99255 | 0 | 1 |
| 10.1 | 16.8916 | | 18.9806 | | | | | 20.436 | | | | |
| 10.2 | 17.0767 | 16.7965 | 19.1485 | 19.0397 | 2.2432 | 0.24275 | 0.845133 | 21.5227 | 21.0868 | 4.2903 | 0.29775 | 0.81352 |
| 10.2 | 16.5163 | | 18.9309 | | | | | 20.6509 | | | | |
| 10.3 | 18.1074 | 17.7111 | 20.5383 | 20.61525 | 2.90415 | 0.9037 | 0.534514 | 22.7601 | 22.5346 | 4.8235 | 0.83095 | 0.56215 |
| 10.3 | 17.3148 | | 20.6922 | | | | | 22.3091 | | | | |
| 11.1 | 19.0293 | 18.4211 | 21.8694 | 21.5687 | 3.1476 | 0 | 1 | 23.4888 | 23.2728 | 4.8517 | 0 | 1 |
| 11.1 | 17.8129 | | 21.268 | | | | | 23.0568 | | | | |
| 11.2 | 18.2695 | 17.76065 | 21.3151 | 21.28235 | 3.5217 | 0.3741 | 0.771587 | 22.5111 | 22.3388 | 4.57815 | -0.27355 | 1.20877 |
| 11.2 | 17.2518 | | 21.2496 | | | | | 22.1665 | | | | |
| 11.3 | 18.2507 | 17.5301 | 19.9839 | 19.919 | 2.3889 | -0.7587 | 1.691965 | 22.1076 | 21.93085 | 4.40075 | -0.45095 | 1.3669 |
| 11.3 | 16.8095 | | 19.8541 | | | | | 21.7541 | | | | |
| 12.1 | 17.1986 | 17.0866 | 20.6481 | 20.5041 | 3.4175 | 0 | 1 | 22.5954 | 22.27325 | 5.18665 | 0 | 1 |
| 12.1 | 16.9746 | | 20.3601 | | | | | 21.9511 | | | | |
| 12.2 | 18.9264 | 18.63165 | 22.0108 | 21.59965 | 2.968 | -0.4495 | 1.365567 | 23.7315 | 23.5526 | 4.92095 | -0.2657 | 1.20221 |
| 12.2 | 18.3369 | | 21.1885 | | | | | 23.3737 | | | | |
| 12.3 | 19.0778 | 18.70795 | 21.1256 | 21.26515 | 2.5572 | -0.8603 | 1.815416 | 23.6731 | 23.17935 | 4.4714 | -0.71525 | 1.64176 |
| 12.3 | 18.3381 | | 21.4047 | | | | | 22.6856 | | | | |
| 13.1 | 18.4363 | 18.10115 | 20.8768 | 20.28315 | 2.182 | 0 | 1 | 22.2295 | 21.92355 | 3.8224 | 0 | 1 |
| 13.1 | 17.766 | | 19.6895 | | | | | 21.6176 | | | | |
| 13.2 | 15.4145 | 15.9397 | 20.0112 | 19.59865 | 3.65895 | 1.47695 | 0.359247 | 22.1919 | 21.7015 | 5.7618 | 1.9394 | 0.26072 |
| 13.2 | 16.4649 | | 19.1861 | | | | | 21.2111 | | | | |
| 13.3 | 19.4076 | 18.7604 | 21.1242 | 20.7987 | 2.0383 | -0.1437 | 1.104735 | 24.3199 | 23.70455 | 4.94415 | 1.12175 | 0.45953 |
| 13.3 | 18.1132 | | 20.4732 | | | | | 23.0892 | | | | |
| 14.1 | 16.8259 | 16.6481 | 17.7182 | 17.5671 | 0.919 | 0 | 1 | 22.0287 | 21.82305 | 5.17495 | 0 | 1 |
| 14.1 | 16.4703 | | 17.416 | | | | | 21.6174 | | | | |
| 14.2 | 18.8601 | 18.92235 | 19.6329 | 19.22935 | 0.307 | -0.612 | 1.528377 | 23.8989 | 23.9806 | 5.05825 | -0.1167 | 1.08425 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14.2 | 18.9846 | | 18.8258 | | | | | 24.0623 | | | | |
| 14.3 | 18.9217 | 19.02475 | 20.3187 | 20.2851 | 1.26035 | 0.34135 | 0.789302 | | 24.7142 | 5.68945 | 0.5145 | 0.70003 |
| 14.3 | 19.1278 | | 20.2515 | | | | | 24.7142 | | | | |

| | GAPDH | I | | | IL-1ra | | | | | TGF-β | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study # | CT | mean CT | CT | mean CT | Δ CT | ΔΔ CT | Fold change | CT | mean CT | Δ CT | ΔΔ CT | Fold change |
| 15.1 | 17.6741 | 17.5487 | 20.0887 | 19.9753 | 2.4266 | 0 | 1 | 22.2259 | 21.74735 | 4.19865 | 0 | 1 |
| 15.1 | 17.4233 | | 19.8619 | | | | | 21.2688 | | | | |
| 15.2 | 17.8332 | 17.70295 | 20.4688 | 20.6986 | 2.99565 | 0.56905 | 0.674061 | 23.4075 | 23.1263 | 5.42335 | 1.2247 | 0.42788 |
| 15.2 | 17.5727 | | 20.9284 | | | | | 22.8451 | | | | |
| 15.3 | 17.2787 | 17.4212 | 21.811 | 21.71775 | 4.29655 | 1.86995 | 0.273583 | 23.7575 | 23.52905 | 6.10785 | 1.9092 | 0.26624 |
| 15.3 | 17.5637 | | 21.6245 | | | | | 23.3006 | | | | |
| 16.1 | 18.2436 | 18.214 | 20.4422 | 20.7991 | 2.5851 | 0 | 1 | 23.6125 | 23.54445 | 5.33045 | 0 | 1 |
| 16.1 | 18.1844 | | 21.156 | | | | | 23.4764 | | | | |
| 16.2 | 18.4614 | 18.0157 | 21.8337 | 21.73175 | 3.71605 | 1.13095 | 0.456615 | 24.2625 | 24.2841 | 6.2684 | 0.93795 | 0.52197 |
| 16.2 | 17.57 | | 21.6298 | | | | | 24.3057 | | | | |
| 16.3 | 17.4582 | 17.5121 | 21.04 | 21.27 | 3.7579 | 1.1728 | 0.44356 | 23.8839 | 23.7742 | 6.2621 | 0.93165 | 0.52425 |
| 16.3 | 17.566 | | 21.5 | | | | | 23.6645 | | | | |
| 17.1 | 18.8194 | 18.4201 | 21.2876 | 21.03985 | 2.61975 | 0 | 1 | 24.2918 | 23.9701 | 5.55 | 0 | 1 |
| 17.1 | 18.0208 | | 20.7921 | | | | | 23.6484 | | | | |
| 17.2 | 16.9814 | 16.91045 | 19.658 | 19.59385 | 2.6834 | 0.06365 | 0.95684 | 22.3036 | 21.97745 | 5.067 | -0.483 | 1.39764 |
| 17.2 | 16.8395 | | 19.5297 | | | | | 21.6513 | | | | |
| 17.3 | 17.3168 | 17.2489 | 19.3626 | 19.3253 | 2.0764 | -0.54335 | 1.457353 | 22.274 | 21.97045 | 4.72155 | -0.82845 | 1.77577 |
| 17.3 | 17.181 | | 19.288 | | | | | 21.6669 | | | | |
| 18.1 | 15.7966 | 16.1627 | 18.9398 | 19.32425 | 3.16155 | 0 | 1 | 21.0631 | 21.13555 | 4.97285 | 0 | 1 |
| 18.1 | 16.5288 | | 19.7087 | | | | | 21.208 | | | | |
| 18.2 | 16.6071 | 16.8028 | 19.704 | 19.97805 | 3.17525 | 0.0137 | 0.990549 | 21.4714 | 21.45095 | 4.64815 | -0.3247 | 1.25240 |
| 18.2 | 16.9985 | | 20.2521 | | | | | 21.4305 | | | | |
| 18.3 | 17.7924 | 18.56995 | 19.465 | 19.68135 | 1.1114 | -2.05015 | 4.14149 | 21.9354 | 21.6178 | 3.04785 | -1.925 | 3.79736 |
| 18.3 | 19.3475 | | 19.8977 | | | | | 21.3002 | | | | |
| 19.1 | 16.1848 | 16.08695 | 19.5139 | 19.41175 | 3.3248 | 0 | 1 | 21.5637 | 21.24175 | 5.1548 | 0 | 1 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19.1 | 15.9891 | | 19.3096 | | | | | 20.9198 | | | | |
| 19.2 | 16.7414 | 16.29205 | 18.7057 | 19.0439 | 2.75185 | -0.57295 | 1.487562 | 21.4445 | 21.42945 | 5.1374 | -0.0174 | 1.01213 |
| 19.2 | 15.8427 | | 19.3821 | | | | | 21.4144 | | | | |
| 19.3 | 16.0895 | 15.9629 | 19.3874 | 19.3236 | 3.3607 | 0.0359 | 0.975423 | 21.367 | 21.16415 | 5.20125 | 0.04645 | 0.96831 |
| 19.3 | 15.8363 | | 19.2598 | | | | | 20.9613 | | | | |

| Study # | CT | mean CT | CT | mean CT | Δ CT | ΔΔ CT | Fold change | CT | mean CT | Δ CT | ΔΔ CT | Fold change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20.1 | 17.0076 | 16.95695 | 21.4632 | 20.90725 | 3.9503 | 0 | 1 | 23.7543 | 22.9583 | 1.4951 | 0 | 1 |
| 20.1 | 16.9063 | | 20.3513 | | | | | 22.1623 | | | | |
| 20.2 | 18.0418 | 17.80305 | 20.7298 | 20.5934 | 2.79035 | -1.15995 | 2.234497 | 23.2276 | 22.64085 | 1.91105 | 0.41595 | 0.74952 |
| 20.2 | 17.5643 | | 20.457 | | | | | 22.0541 | | | | |
| 20.3 | 17.2414 | 17.03235 | 19.9467 | 19.8908 | 2.85845 | -1.09185 | 2.131472 | 23.7853 | 22.7095 | 5.67715 | 4.18205 | 0.05509 |
| 20.3 | 16.8233 | | 19.8349 | | | | | 21.6337 | | | | |
| 21.1 | 17.0203 | 16.95295 | 19.5415 | 19.3911 | 2.43815 | 0 | 1 | 22.206 | 21.50675 | 4.5538 | 0 | 1 |
| 21.1 | 16.8856 | | 19.2407 | | | | | 20.8075 | | | | |
| 21.2 | 18.6465 | 18.703 | 21.4007 | 21.2819 | 2.5789 | 0.14075 | 0.907047 | 24.8024 | 23.7432 | 5.0402 | 0.4864 | 0.71380 |
| 21.2 | 18.7595 | | 21.1631 | | | | | 22.684 | | | | |
| 21.3 | 16.7992 | 16.83965 | 19.2894 | 19.0158 | 2.17615 | -0.262 | 1.19914 | 21.4978 | 21.5002 | 4.66055 | 0.10675 | 0.92867 |
| 21.3 | 16.8801 | | 18.7422 | | | | | 21.5026 | | | | |
| 22.1 | 15.8497 | 15.6385 | 18.5248 | 18.3503 | 2.7118 | 0 | 1 | 19.6837 | 20.29185 | 4.65335 | 0 | 1 |
| 22.1 | 15.4273 | | 18.1758 | | | | | 20.9 | | | | |
| 22.2 | 15.6649 | 15.75975 | 18.3364 | 18.8721 | 3.11235 | 0.40055 | 0.757569 | 20.1917 | 20.386 | 4.62625 | -0.0271 | 1.01896 |
| 22.2 | 15.8546 | | 19.4078 | | | | | 20.5803 | | | | |
| 22.3 | 16.7238 | 16.8432 | 19.4188 | 19.34975 | 2.50655 | -0.20525 | 1.152886 | 21.4171 | 21.58545 | 4.74225 | 0.0889 | 0.94023 |
| 22.3 | 16.9626 | | 19.2807 | | | | | 21.7538 | | | | |
| 23.1 | 16.4728 | 16.5893 | 19.9844 | 19.39175 | 2.80245 | 0 | 1 | 22.6081 | 21.55315 | 4.96385 | 0 | 1 |
| 23.1 | 16.7058 | | 18.7991 | | | | | 20.4982 | | | | |
| 23.2 | 16.8686 | 16.6795 | 20.7326 | 20.29435 | 3.61485 | 0.8124 | 0.569434 | 22.5854 | 21.8229 | 5.1434 | 0.17955 | 0.88297 |
| 23.2 | 16.4904 | | 19.8561 | | | | | 21.0604 | | | | |
| 23.3 | 15.6531 | 15.5606 | 18.4418 | 18.35305 | 2.79245 | -0.01 | 1.006956 | 20.2967 | 20.27225 | 4.71165 | -0.2522 | 1.19102 |
| 23.3 | 15.4681 | | 18.2643 | | | | | 20.2478 | | | | |
| 24.1 | 16.4616 | 16.51105 | 19.3507 | 19.30945 | 2.7984 | 0 | 1 | 21.7492 | 21.4801 | 4.96905 | 0 | 1 |
| 24.1 | 16.5605 | | 19.2682 | | | | | 21.211 | | | | |
| 24.2 | 17.4202 | 17.01335 | 20.1599 | 20.04615 | 3.0328 | 0.2344 | 0.850038 | 22.058 | 22.0122 | 4.99885 | 0.0298 | 0.97955 |
| 24.2 | 16.6065 | | 19.9324 | | | | | 21.9664 | | | | |
| 24.3 | 15.218 | 15.66365 | 19.5193 | 19.61515 | 3.9515 | 1.1531 | 0.449658 | 22.0865 | 22.1056 | 6.44195 | 1.4729 | 0.36025 |
| 24.3 | 16.1093 | | 19.711 | | | | | 22.1247 | | | | |