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Development of an ELISA to Detect Folate Receptor alpha (FRα) Autoantibodies, and their Association with Recurrent Miscarriage.

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Declaration

I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma from any university and that to the best of my knowledge and belief, does not contain any material previously publish or written by another person except where due reference is made in the text.

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List of Abbreviations

5MTHF	5 Methyltetrahydrofolate
aCL	anti-Cardiolipin
ACOG	Australian collage of gynaecologists
aPE	anti-phosphatidylethanolamine
APL	Unit for IgA antiphospholipid antibodies
aPLs	antiphospholipid antibodies
APS	Antiphospholipid syndrome
Ax	anti-annexin V
B2GP	Beta 2 – glycoprotein
BMI	Body mass index
BSA	Bovine serum albumin
CFD	Cerebral folate deficiency
CLP	Cleft lip palate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme linked immunosorbent assay
FBP	Fetal bovine serum
FCS	Fetal calf serum
frU	Unit for FRα autoantibodies

FRα	Folate receptor alpha
FRβ	Folate receptor beta
FVL	Factor V Leiden
GPI	Glycosylphosphatidylinositol
GPL	Unit for IgG antiphospholipid antibodies
IVF	In Vitro fertilization
LA	Lupus anticoagulant
LPD	Luteal phase defect
MPL	Unit for IgM antiphospholipid antibodies
mRNA	messenger ribonucleic acid
MTHFR	Methylene-tetrahydrofolate reductase
N	Number
NTD	Neural tube defect
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween20
PCFT	Proton coupled folate transporter
PCOS	Poly cystic ovary syndrome
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase Chain reaction - restriction fragment length polymorphism
PMSF	Phenylmethylsuphonyl fluoride
PT	Prothrombin
RCOG	Royal collage of gynaecologists
RFC	Reduced folate carrier
RIA	Radioimmunoassay
RM	recurrent miscarriage

RNA	ribonucleic acid
RT	reverse transcriptase
SD	standard deviation
SDS	sodium dodecyl sulfate
SST	Serum separating tubes
TBS	Tris buffered saline
TBST	Tris buffered saline Tween20
TF	tissue factor
tHyc	total homocysteine
TISS	Thrombosis in stroke cohort
TM	Thrombomodulin
t-PA	tissue type-plasminogen activator

Chapter 1:
General Introduction

1 Chapter 1: Introduction

1.1 Introduction

Pregnancy can be a joyous moment in a couple's life; yet on the other hand it can also bring much heartache to couples that experience any form of pregnancy complication. A significant proportion of pregnancies don't make full term, in addition, 1% of couples may experience recurrent miscarriage (RM) which can be devastating to the couple (Bansal *et al.* 2011). RM is a multi-factorial disease with approximately 50% of cases classified as having no known underlying cause (Chen *et al.* 2010). Furthermore reliable and clinically useful information to the various treatments available is lacking (Allison *et al.* 2009). There are only a few scientifically established causes for RM, including antiphospholipid syndrome (APS), chromosomal rearrangements and uterine abnormalities; other factors are less defined, for instance hormonal/nutritional deficiencies, autoimmune disease, and disorders of the coagulation system (Obayashi *et al.* 2010, Ticconi *et al.* 2010).

A cause of RM that has been extensively studied in recent years is folate deficiency and hyperhomocysteinemia, with significant data that supports clinical observations (Kumar *et al.* 2003, Nelen *et al.* 1998, Ren *et al.* 2006, Wouters *et al.* 1993). Folate deficiency is associated with many pregnancy complications including pre-eclampsia, reduced foetal growth, sub fertility, neural tube defects (NTD) and miscarriage (ACOG 2002, Antony 2007,

Bisseling *et al.* 2004, Ebisch *et al.* 2007). Additionally, literature strongly emphasises that folate supplementation before and during a pregnancy to be clinically important to the success of a pregnancy (MRC Vitamin Study Research Group 1991). It has been established that folate deficiency can be isolated to the fetal tissues, even in the presence of normal maternal levels; therefore a factor blocking folate transport into the placenta must be present (Olney *et al.* 2002, Rothenberg *et al.* 2004, Steegers-Theunissen *et al.* 2000). There may be several reasons for insufficient folate transport through the placenta, including genetic mutations with folate metabolic enzymes or folate transport proteins, although there is a lack of strong conclusive evidence for these hypotheses (Barber *et al.* 1998, Heil *et al.* 1999, Shaw *et al.* 1998). In 2004 it was demonstrated that autoantibodies directed against the FRα, a folate transport receptor expressed on the apical membrane of trophoblast cells, were present in human sera and able to cause blocking of folate transport in the placenta (Rothenberg *et al.* 2004). Studies to date have examined several folate deficient related diseases including NTD, cleft lip/palate, sub fertility and cerebral folate deficiency in association with FRα autoantibodies with increasing evidence supporting their pathogenesis (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Billie *et al.* 2010, Bliek *et al.* 2006, Boyles *et al.* 2011, Cabrera *et al.* 2008, Molloy Anne M. *et al.* 2009, Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004). With a strong link between autoimmunity such as APS and RM, it is proposed that FRα autoantibodies may also be a factor in increasing the risk of RM, by reducing embryonic folate levels and increasing homocysteine levels (Boyles *et al.* 2011, Vinatier *et al.* 2001). This

review will give an overview of the known causes of RM, the importance of folate during a pregnancy, and folate transport mechanisms within the placenta. Lastly this review will aim to demonstrate the consequence of FRα autoantibodies in relation to pregnancy outcomes, particularly in the setting of RM.

1.2 Recurrent Miscarriage

Approximately 50% of women affected by RM have no known underlying cause, highlighting the lack of understanding for this syndrome (Chen *et al.* 2010, Christiansen *et al.* 2008). Miscarriage is the loss of a clinically recognised pregnancy that weighs less than 500g and happens before 20 weeks gestation; which can occur in 10-25% of women trying to conceive (Branch *et al.* 2010, Christiansen *et al.* 2008, Kavalier 2005, Vinatier *et al.* 2001). RM is defined as the loss of three or more consecutive pregnancies with the same partner, occurring in up to 1% of couples trying to conceive; however the risk is higher in couples without children with an incidence of 2-5% (Bansal *et al.* 2011, Chen *et al.* 2010, Lund *et al.* 2010, Ren *et al.* 2006, Vinatier *et al.* 2001). A majority of women have RMs before 10 weeks with a smaller number after this date, however almost all RMs occur before 15-16 weeks (Branch *et al.* 2010, Vinatier *et al.* 2001). Much debate has centred on an actual definition of RM and many papers have different criteria; with a number of studies characterising RM as 2 or more cases of pregnancy loss, if this is the case the rate of RM would be as high as 5% (Branch *et al.* 2010, Kaandorp *et al.* 2010, Rai *et al.* 2006).

There are many factors that contribute to reproduction and a successful birth during the key phases of implantation, trophoblast invasion and placental development; therefore, the factors that may cause a miscarriage vary greatly. Significant evidence suggests that RM is a heterogeneous condition, with only a couple of established causes identified as causative to date; these include parental karyotype abnormalities, APS, and uterine abnormalities (Obayashi *et al.* 2010). Other, less characterised causes exist, but require further investigation (Ticconi *et al.* 2010). Importantly 40-50% of women diagnosed with RM have no underlying cause and are therefore classified as unexplained RM (Chen *et al.* 2010, Kaandorp *et al.* 2010, Obayashi *et al.* 2010). However, to help physicians and patients better understand RM, there are two main guidelines that can be followed, one produced by the Royal College of Obstetricians and Gynaecologists (RCOG) and the other produced by the American College of Obstetricians and Gynaecologists (ACOG 2002, Royal College of Obstetricians and Gynacologists 2011). A summary of both guidelines outlining possible causes and recommended investigations and treatments are in Table 1.1.

1.2.1 Associated Risks

1.2.1.1 Environmental factors

There are numerous epidemiological factors that might affect the rate of RM. These can include age, weight, smoking, consumption of caffeine or alcohol, and the number of previous miscarriages (Carter *et al.* 2011, George *et al.* 2006, Rasch 2003, Vasudevan *et al.* 2010). It is hard to determine if such factors do play a role in RM with some only playing a minor

role, however much research has centred around these factors to determine if they are true risks for pregnancy complications (Ford *et al.* 2009, George *et al.* 2006, Rasch 2003, Vasudevan *et al.* 2010). One study performed in 2006 analysed a range of these factors to determine causality with RM. The study mainly focused on the associated risks of increased caffeine levels, cotinine levels (from smoking), age, and low folate levels. Both the mean caffeine levels (311mg/d) and mean cotinine levels (43ng/d) were significantly higher in women with RM than controls (George *et al.* 2006). In addition, they demonstrated that RM was associated with an increase in the previous number of RM, myoma uteri, and women without partners.

Obesity during a pregnancy has long been a concern especially in recent years with the population growing more obese, and that it can affect the pregnancy at several different stages (Lo *et al.* 2012). A large meta-analysis carried out in 2006 which included 16 studies between 1964 and 2006, demonstrated a strong association between obesity and RM (Metwally *et al.* 2008). A large study in 2012 demonstrated that obese women with unexplained RM have a 73% increased chance of having another miscarriage in subsequent pregnancies over normal controls (Lo *et al.* 2012). It is not well understood how obesity may affect pregnancy, however obesity is associated with increased levels of oxidative stress and an increased risk of gestational diabetes, which have both shown to be a factor in RM (Gutaj *et al.* 2013). Lastly moderate alcohol consumption of 5 or more units of alcohol per week has been demonstrated to increase risk of spontaneous abortions (Rasch 2003). This is supported in several other studies

demonstrating that what women consume and their lifestyle during a pregnancy can strongly affect the outcome, and are important factors that can be prevented with simple lifestyle changes.

Maternal age has a strong correlation with occurrence of RM with several studies indicating women younger than 24 or older than 35 have a threefold higher rate of RM than women between the ages of 25-34, and women >35 suffering from RM have double the risk of another miscarriage in future pregnancies compared to women <35 years old (Andersen *et al.* 2000, George *et al.* 2006, Lo *et al.* 2012). Maternal age is related to a declining number and quality of oocytes, which can also add to other factors including genetic abnormalities, and hormonal levels (Branch *et al.* 2010). Both these factors increase with age, and a strong correlation has been illustrated in both RM and other pregnancy related complications such as NTD (Andersen *et al.* 2000, George *et al.* 2006). Factors such as age when conceiving are uncontrollable risk factors for RM and women experiencing age associated RM will not benefit from such a diagnosis; nevertheless, such knowledge will allow future women to take into account the risk associated with pregnancy at a later stage in life.

Table 1.1 Outline of both ACOG and RCOG recurrent miscarriage Guidelines

	ACOG	**RCOG**
Year Updated	February 2001	April 2011
Definition	Recurrent miscarriage is defined as two or three or more consecutive pregnancy losses, before 15 weeks.	Recurrent miscarriage, defined as the loss of three or more consecutive pregnancies, from conception to 24 weeks.
Risk factors supported by guidelines	• Antiphospholipid Syndrome • Genetic Factors: parental chromosomal rearrangements, molecular genetic abnormalities, embryonic chromosomal abnormalities • congenital uterine malformations • Endocrine Factors: diabetes mellitus, polycystic ovary syndrome • Immune factors: Antinuclear antibodies • Inherited and acquired thrombophilias	• Epidemiological: including age, number of previous miscarriages, smoking, and caffeine and alcohol consumption. • Antiphospholipid Syndrome • Genetic Factors: parental chromosomal rearrangements, embryonic chromosomal abnormalities • congenital uterine malformations, • Endocrine Factors: diabetes mellitus, thyroid disease, polycystic ovary syndrome • Inherited and acquired thrombophilias
Factors with low significance/ lack evidence	• Epidemiological: conflicting evidence for smoking, alcohol, caffeine use • Endocrine Factors: thyroid disease, Luteal Phase defect • Immune factors: Human leukocyte antigen incompatibility, altered natural killer cells and cytokine imbalance (slight possibility) • Infective agents	• Anatomical factors: cervical weakness • Immune factors: Human leukocyte antigen incompatibility, altered natural killer cells and cytokine imbalance (slight possibility) • Infective agents
Recommended investigations	• Antiphospholipid antibodies • Karyotyping: products of conception and parental peripheral blood • Anatomical: Pelvic ultrasound and hysteroscopy	• Antiphospholipid antibodies • Karyotyping: products of conception and parental peripheral blood • Anatomical: Pelvic ultrasound and hysteroscopy • Thrombophilias: second trimester only of inherited thrombophilias.
Recommended Treatments	• Antiphospholipid syndrome: aspirin and heparin • Genetic Counselling • Anatomical: uterine septum resection • Continual care and counselling by physician	• Antiphospholipid syndrome: aspirin and heparin • Genetic Counselling • Thrombophilias: anticoagulants • Continual care and counselling by physician

1.2.1.2 Genetic

Genetic factors are a well-established cause of RM which can be separated into two main factors, parental and embryonic (Branch *et al.* 2010, Kavalier 2005). Parental balanced chromosomal rearrangements can occur in 2-5% of couples that are affected by RM, with either the paternal or maternal side having the abnormality, however it is more common in women (Kavalier 2005). The two main types of chromosomal rearrangements are reciprocal and Robertsonian translocations (Allison *et al.* 2009, Sugiura-Ogasawara *et al.* 2008, Sugiura-Ogasawara *et al.* 2009). Sugiura-Ogasawara *et al* (2008) performed a large study analysing parental chromosomal rearrangements in 2,382 couples experiencing RM. The study identified 5.4% of couples with a chromosomal rearrangement in either the paternal (34%) or maternal (67%) lineage, with 55% identified as having reciprocal translocations and 10% with Robertsonian translocations (Sugiura-Ogasawara *et al.* 2008). In addition to these two common chromosomal abnormalities; chromosomal inversions, insertions and mosaicisms have also been shown to cause a slight increased risk of RM (Kouru *et al.* 2010, Sugiura-Ogasawara *et al.* 2008)

Embryonic chromosomal abnormality, including aneuploidy, is associated with a large number of miscarriages occurring before 10 weeks gestation. Embryonic aneuploidy is present in up to 50% of aborted fetuses demonstrated by abnormal embryonic karyotype analysis (Ogasawara *et al.* 2000, Sugiura-Ogasawara *et al.* 2009). Even though there is a high rate of embryonic chromosomal abnormality in miscarriages, it is less understood the extent to which embryonic chromosomal abnormality causes RM; as it

has been demonstrated that embryonic aneuploidy occurs at a higher rate in sporadic miscarriages compared to RM (Sullivan *et al.* 2004). Nevertheless, embryonic chromosomal abnormalities tend to occur in older couples experiencing RM, where younger couples are more affected by paternal chromosomal abnormalities (Christiansen *et al.* 2008).

1.2.1.3 Anatomic

Anatomic aetiologies of RM can include uterine anomalies (arcuate/septate), inter-uterine adhesions, uterine fibroids and polyps, however like most factors the overall significance remains unclear (Ford *et al.* 2009, Royal College of Obstetricians and Gynacologists 2011). Vascular insufficiency of the endometrium is thought to be the cause in septate uterus conditions, which may cause inadequate placentation (Branch *et al.* 2010). Such an issue has been demonstrated in 10-25% of women affected by RM compared to only 5% in controls (Branch *et al.* 2010). An important factor to note, is that uterine anomalies tend to be associated with second trimester pregnancy loss, but depends on the type of uterine malformation; women suffering from arcuate uteri (concave fundus of uterus) are more likely to have pregnancy loss in 2nd trimester, whereas septate uteri (uterine cavity portioning) are more prevalent in 1st trimester miscarriage (Woelfer *et al.* 2001). The presence of intrauterine adhesions may also impinge on successful placentation and result in early pregnancy loss (Ford *et al.* 2009). In addition, intramural and sub-mucosal fibroids are also associated with RM as demonstrated in George *et al* 2006. It is therefore recommended that

screening of women for uterine anomalies is performed as there are several factors associated with miscarriage.

1.2.1.4 Immunogenic

Autoimmunity is the second most important factor causing RM in women, after genetic abnormalities, and may be present in up to 30% of women affected by RM (Ticconi *et al.* 2010). There are several different autoimmune disorders associated with RM, including APS, organ specific autoimmunity (subclinical thyroid autoimmunity), systematic autoimmunity (systematic lupus erythematosus, anti-nuclear and anti-ribonucleoprotien), anti-endothelial and anti-trophoblast antibodies (Bansal *et al.* 2011, Branch *et al.* 2010, Jaslow *et al.* 2009, Ornoy *et al.* 2004, Perricone *et al.* 2008, Ticconi *et al.* 2010). Autoimmune disease in pregnancy is complicated and not fully understood; however adverse effects that may cause miscarriage include thrombosis, inflammation, or cytotoxicity and cell death (Di Simone *et al.* 2010, Ticconi *et al.* 2010). It is suggested that autoimmune diseases may act in combination to cause miscarriage, and if a patient is diagnosed with one autoimmune disease it is likely that another autoimmune disease is present. This was demonstrated by Ticconi *et al* (2011) which found that a high proportion (53.84%) of women with anti-nuclear antibodies also had anti-thyroid antibodies. On top of this finding other antibodies were found to be present in women with anti-nuclear antibodies including anti-phospholipid antibodies, anti-mitochondrial antibodies or a combination of multiple autoantibodies (Ticconi *et al.* 2010). Equally, current evidence suggests the presence of any autoimmune disease, even at a minor level, decreases the

survival of the embryo and it may be important to screen patients for a range of autoimmunity conditions (Bansal *et al.* 2011)

1.2.1.5 Antiphospholipid Syndrome (APS)

APS is a major risk factor for RM and has been demonstrated to be causative for approximately 15% of all RM cases (Rai *et al.* 1997, Rai, Regan*, et al.* 1995, Vinatier *et al.* 2001). APS is defined by the persistent presence of a heterogeneous group of antiphospholipid antibodies (aPLs) that have various recognition substrates including phospholipids, phospholipid-binding proteins or both (Di Simone *et al.* 2010, Gris *et al.* 2003). These antibodies are associated with hypercoagulability which leads to adverse pregnancy outcome and/or vascular thrombosis (Gris *et al.* 2003). The clinical manifestations for APS include; 1) Vascular thrombosis in any tissue or organ 2) Pregnancy morbidity including a) three or more consecutive miscarriages before 10 weeks gestation, b) One or more morphologically normal fetal losses after 10 weeks, and c) One or more preterm births before 34th week due to placental disease (Miyakis *et al.* 2006).

New international criteria have been developed in 2006 to diagnose APS requiring at least one clinical (as above) and one laboratory criteria (Matsubayashi 2009, Miyakis *et al.* 2006). Laboratory criteria include elevated levels of any/all antibodies, including 1) lupus anticoagulant (LA) 2) anticardiolipin (aCL), 3) Anti-β_2 glycoprotein (β2GP) (IgM/IgG) antibodies, twice or more over a 12 week period (Miyakis *et al.* 2006, Obayashi *et al.* 2010). Originally, LA antibodies and aCL antibodies were the two main

autoantibodies assessed with APS in association to RM (Miyakis *et al.* 2006), however with further research into the syndrome, β2GP antibodies have also been included (Allison *et al.* 2009, Miyakis *et al.* 2006, Tincani *et al.* 2003). Much study has been performed on these three autoantibodies which have the highest incidence of thrombotic complication in patients with RM.

There are other aPLs associated with increased risk of RM, though they are not clinically assessed in women with RM (Pauer *et al.* 2003). These antibodies include antibodies directed against phospholipids like anti-phosphatidylethanolamine (aPE), phospholipid-binding proteins including anti-annexin V (ax), or against glycoproteins such as tissue factor (TF), thrombomodulin (TM), and tissue type-plasminogen activator (t-PA) (Di Simone *et al.* 2010, Obayashi *et al.* 2010, Pauer *et al.* 2003, Sater *et al.* 2011). Pauer *et al* , 2003 demonstrated that the presence of 7 antibodies, including LA, aCL, aPE, β2GP, ax, t-PA, leads to an increased associated risk of miscarriage in women; with all but TM and β2GP having an independent risk (Pauer *et al.* 2003). Although these antibodies are not clinically tested many studies demonstrate that they play a role within APS manifestations whether independently or collectively with LA, aCL or β2GP autoantibodies.

The main pathological role APS has in causing RM is by placental thrombosis, however it is not fully elucidated how; it is suggested that these antibodies inhibit the phospholipid-dependent coagulation cascade which may cause reduced fibrinolytic activity or venous and arterial thrombosis (Di Simone *et al.* 2010, Gris *et al.* 2003, Ivanov *et al.* , Martinez-Zamora *et al.* 2010, Sater *et*

al. 2011). However, not all RM associated with APS is caused by placental thrombosis, and like other autoimmune disorders other factors may also play a part. These can include, inhibition of trophoblastic function, differentiation, and invasion which is important to successful implantation (Chen *et al.* 2010, Di Simone *et al.* 2010, Ivanov *et al.*), or activation of complement pathways on the stromal decidual cells at the maternal – fetal interface, resulting in inflammatory disease (Martinez de la Torre *et al.* 2007, Ticconi *et al.* 2010). A recent study by Simone *et al* (2010), demonstrated that polyclonal β2GPI antibodies involved in APS decrease endometrial endothelial cell angiogenesis, reducing formation of new capillary structures. It is also important to note that moderate hyperhomocysteinemia, which is also a risk factor for thrombosis, is associated with the presence of APS in RM patients and influences the thrombotic tendency of patients with primary and secondary APS (Gris *et al,* 2003). Overall these pathological factors can work together to increase the risk of RM of women with both APS and hyperhomocysteinemia (Gris *et al,* 2003).

1.2.1.6 Coagulation Factors

As the placenta replaces the yolk sac between 8-10 weeks gestation a complex vascular system is required to maintain successful pregnancy, with chorionic villous vascularisation beginning as early as the fourth week (Gris *et al.* 2003, Lisman *et al.* 2007, te Velde *et al.* 1997). Consequently hypercoagulability can play a detrimental role in fetal development as a result from thrombosis of the uteroplacental vasculature system (Chen *et al.* 2010, Gris *et al.* 2003, Kupferminc *et al.* 1999, Lund *et al.* 2010, Martinez-Zamora *et al.* 2010, Rai *et al.* 2001). There are other factors that may be

responsible for hypercoagulability during a pregnancy causing RM apart from APS, including acquired and inherited thrombophilias (Martinez-Zamora *et al.* 2010, Parand *et al.* 2013, Quere *et al.* 1998). These include factor V G1691A Leiden (FVL) mutation, prothrombin (PT) G20210A mutation, protein C (activated protein C resistance or deficiency), protein S deficiency, Factor XII deficiency, antithrombin III, high levels of factor XI and hyperhomocysteinemia (Allison *et al.* 2009, Branch *et al.* 2010, Foka *et al.* 2000, Glueck *et al.* , Kupferminc *et al.* 1999, Lund *et al.* 2010, Nahas *et al.* 2016, Ogasawara *et al.* 2001, Parand *et al.* 2013, Pauer *et al.* 2003). These factors all play an important role within the coagulation cascade and can cause an increased risk of thrombosis of the venous and spiral arteries of the uterus and placenta, and impaired placental development which may lead to fetal loss (Chen *et al.* 2010, Ford *et al.* 2009, Li *et al.* 2002, Meegdes *et al.* 1988). The FVL and PT mutations have been extensively studied with some studies demonstrating a strong association with RM while, like many other factors that are associated with RM, a few studies report no association and suggest the need for further research (Chen *et al.* 2010, Farahmand *et al.* 2016, Foka *et al.* 2000, Gao *et al.* 2015, Goncalves *et al.* 2016, Lund *et al.* 2010, Rai *et al.* 2001, Sergi *et al.* 2015, Skeith *et al.* 2016). Even though one factor may not be able to cause of RM by itself, a combination of thrombophilic states appears to increase the risk; in addition, many secondary risk factors, such as smoking and obesity increase the risk. (Farahmand *et al.* 2016, Kutteh *et al.* 2006, Preston *et al.* 1996)

1.2.1.7 Endocrinology

Several endocrine aetiologies are associated with an increased risk of RM and may be a cause of up to 20% of RM cases (Li *et al.* 2000). Such risks include diabetes mellitus, thyroid disease, polycystic ovarian syndrome (PCOS), luteal phase defect (LPD) and hyperprolactinemia (Ford *et al.* 2009). Most of these factors only play a role in RM if they are ill-managed. For example, there is only a risk factor with diabetes if glucose levels are not maintained, or for thyroid disease if thyroid hormone replacement treatment is not carried out properly (Dal Lago *et al.* 2011, Ford *et al.* 2009, Gutaj *et al.* 2013). Polycystic ovarian syndrome has been linked with RM in several studies with the prevalence ranging between 4.8-82%, and may cause RM by increased levels of luteinising hormones or elevated testosterone levels (Cocksedge *et al.* 2008, Li *et al.* 2000). While LPD is an inadequate production of progesterone by the corpus letuem, in addition, endometrial maturation is insufficient and therefore causes insufficient placentation (Ford *et al.* 2009). All three, LPD, PCOS, and insulin resistance, have been demonstrated together in patients with RM and may in fact be associated together as a risk factor for RM (Cocksedge *et al.* 2008)

1.2.1.8 Homocysteine and Folate Deficiency

An important factor that has also been identified with RM is homocysteine and folate levels during a pregnancy (Eskes 1997, Kotsopoulos *et al.* , Kumar *et al.* 2003, Nelen *et al.* 1998, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., den Heijer M., & Eskes T. K. A. B. 2000, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., Den Heijer M., Thomas C. M. G., *et al.* 2000, Nelen *et al.* 1997, Steegers-

Theunissen *et al.* 1992, Wouters *et al.* 1993). There is evidence demonstrating that low folate levels may increase the risk of RM (George *et al.* 2002, Kumar *et al.* 2003, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., den Heijer M., & Eskes T. K. A. B. 2000, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., Den Heijer M., Thomas C. M. G., *et al.* 2000, Ronnenberg *et al.* 2007) with a study performed by Kumar *et al* (2003) demonstrating an increase of 16.8% of women with RM having folate deficiency compared to controls; while another study demonstrated 9% of women with RM as folate deficient (Wouters *et al.* 1993). Mild hyperhomocysteinemia caused by disruption in the homocysteine-methionine pathway has been reported as a risk factor for several pregnancy complications including, occlusive arterial and thrombotic disease, NTD, placental pathology, and pre-eclampsia (Eskes 1997, Kumar *et al.* 2003, Nelen *et al.* 1998, Quere *et al.* 1998). There are several studies now demonstrating hyperhomocysteinemia, which is an increase level of homocysteine in the blood above 15μmol/L, increasing the risk of RM (Kumar *et al.* 2003, Nelen *et al.* 1998, Steegers-Theunissen *et al.* 1992, Wouters *et al.* 1993). An early study demonstrated that women with RM had a significant increase in mean fasting homocysteine (tHyc) levels compared to controls, with hyperhomocysteinemia (tHyc > 51μmol/L) being diagnosed in 21% of women with RM (Wouters *et al.* 1993), while another study demonstrated 27% of women with RM to have hyperhomocysteinemia (Nelen W. L. D. M., Blom H. J., Steegers E. A. P., den Heijer M., & Eskes T. K. A. B. 2000). Studies to date demonstrate the importance of both folate and homocysteine status in RM aeitiology.

How low levels of folate or high levels of homocysteine cause RM has not been fully elucidated, however there are many possibilities including structural, neurological, and placental thrombosis (Eskes 1997, Kumar *et al.* 2003). Increased levels of homocysteine in general have been associated with hypercoagulability, in which it has been demonstrated to cause endothelial cell injury, damage to decidual and chorionic vessels, premature arteriosclerosis and thromboembolism, and premature vascular disease (Varga *et al.* 2005, Wald *et al.* 2002, Wouters *et al.* 1993). During the early stages of the pregnancy an increased homocysteine level may cause improper chorionic villous vascularisation, or later in the pregnancy small blood clots occurring within the placental blood vessels blocking fetal blood circulation (Nelen W. L. *et al.* 2000, te Velde *et al.* 1997). It has also been proposed that an increase in homocysteine and a reduction in folate can cause significant DNA damage via decrease in methylation or an imbalance in pyrimidine/purine biosynthesis (Shelnutt *et al.* 2004, Sinclair *et al.* 2007, Wiwanitkit 2005, Zetterberg 2004) .

Factors affecting homocysteine levels can be complex, and may result from either genetic defects within enzymes in the homocysteine pathway or from deficiencies within cofactors of such pathways (Crider *et al.* 2011, Kumar *et al.* 2003, Nelen *et al.* 1998, Ren *et al.* 2006). Folate deficiency is an important predecessor of hyperhomocysteinemia, as it is the main co-factor in converting homocysteine to methionine, and if folate is insufficient homocysteine can build up (Blom *et al.* 2010). This was supported by Kumar *et al* (2003) demonstrating, that when folate levels decreased in RM patients

the level of tHYc increased. There are other deficiencies that can cause hyperhomocysteinemia including, vitamin B12 and vitamin B6 which with folate are cofactors in the conversion of homocysteine to methionine (Blom *et al.* 2010).

The genetic mutation within the 5, 10-Methylenetetrahydrofolate Reductase (MTHFR) enzyme, which is responsible for the synthesis of 5-methylenetetrahydrofolate (5-MTHF), plays an important role in the increase of homocysteine levels (Crider *et al.* 2011, Shaw *et al.* 1998, Zetterberg 2004). The most common mutation within this enzyme is the c.665C>T mutation, affecting the thermolability of the enzyme and therefore reducing its function, resulting in increased homocysteine (Crider *et al.* 2011, Kumar *et al.* 2003, Quere *et al.* 1998, van der Put *et al.* 1995, Zetterberg 2004). The heterozygous mutation CT can cause reduced enzyme activity by 30% and cause mild hyperhomocysteinemia, where the homozygous mutation TT reduces activity by 70% and has been associated with moderate to high hyperhomocysteinemia (Crider *et al.* 2011, Gava *et al.* 2011, Nelen *et al.* 1998, Unfried *et al.* 2002, Zetterberg 2004). There is inconsistent data to demonstrate if this homozygous mutation, is associated with increased risk of RM (Nair *et al.* 2012, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., den Heijer M., & Eskes T. K. A. B. 2000, Nelen *et al.* 1997, Wang *et al.* 2004). Nelen *et al* (1997) demonstrated that patients with the homozygous mutation have a two-three fold increased risk of RM, with a more recent study demonstrating a 6-fold increase in homozygous patients (Nair *et al.* 2012). In addition, a meta-analysis of 16 individual studies performed by Chen *et al* (2016)

identified a significant association between the MTHFR c.665C>T mutation and RM (Chen *et al.* 2016). However, not all studies support the finding of the c.665C>T mutation as a risk factor for hyperhomocysteinemia related RM with two meta-analyses were performed in 2005 (Wiwanitkit 2005) and in 2006 (Ren *et al.* 2006) demonstrating conflicting data between all studies and an overall analysis of no association between MTHFR and RM. (Carp *et al.* 2002, Pauer *et al.* 2003, Ren *et al.* 2006, Vettriselvi *et al.* 2008, Wiwanitkit 2005). This demonstrates that there is still inconsistent evidence that the MTHFR mutation to be a known cause of RM, however it should not be ruled out that hyperhomocystenmia to be associated with RM, and further research should be carried out to identify if there is another factor causing hyperhomocysteinemia, and further RM.

1.2.1.9 Viral/Infections

Viral and bacterial infections have been demonstrated to cause minimal risk of RM. In most cases viral/bacterial infections cause sporadic, not recurrent miscarriages (Branch *et al.* 2010). For a viral/bacterial infection to cause RM the infection must be prolonged and be present without detection throughout all affected pregnancies. Viral and bacterial cultures that may cause miscarriage include, Chlamydia, mycoplasma and ureaplasma (Allison *et al.* 2009). However, there is little evidence supporting such infections with RM.

1.2.2 Diagnostic testing

In many cases, evaluation does not reveal a cause, and in some cases it may find an unrelated cause consequently resulting in the patient being unnecessarily treated which may cause distress when another miscarriage occurs (Branch *et al.* 2010). For this reason, there are guidelines that should be followed when evaluating women with RM, including ACOG and RCOG. The recommended screening processes before the next pregnancy are for antiphospholipid antibodies, parental chromosomal abnormalities, and endocrine factors including LPD, thrombophilias, and uterine anomalies (ACOG 2002, Royal College of Obstetricians and Gynacologists 2011). Other factors that may play a role in RM are not often recommended as there are still inconclusive results in their association with RM. It is also recommended that couples should regularly see their family physicians, and in addition, be assessed for epidemiological factors such as smoking, weight, age and caffeine intake that may play a role in RM , that can easily be prevented (ACOG 2002, Royal College of Obstetricians and Gynacologists 2011).

Guidelines into APS recommend testing for only LA, aCL and β2GP antibodies with two positive results within 12 weeks of medium or high titres (40g/L), but not any other antibodies, as research does not yet support any association to RM (Miyakis *et al.* 2006). Karyotyping is recommended for both products of conception and parental peripheral blood of both partners; however these tests may only identify chromosomal rearrangements and not abnormalities such as mosaicism, or single point genetic mutations and therefore should be taken into consideration (ACOG

2002, Allison *et al.* 2009, Branch *et al.* 2010, Royal College of Obstetricians and Gynacologists 2011). It should also be noted that karyotyping is an expensive diagnostic test, and as such, it has been suggested that routine karyotyping is not justified due to low numbers of cases with such traits (Barber *et al.* 2010). Anatomical factors should be first tested by pelvic ultrasound, and if suspected, further by hysteroscopy, laparoscopy and 3D-ultrasound (Jaslow *et al.* 2009). With a large influence of coagulation on RM , it is also suggested to test for other factors such as the FVL mutation and mutations within the MTHFR, PT genes, and also look for deficiencies in protein S or activated protein C resistance (Ford *et al.* 2009). Keeping in mind that these tests should be performed first, there are still 50% or more women that experience unexplained RM, and therefore the importance of experimental evaluation is critical to women that have no obvious underlying cause (ACOG 2002, Royal College of Obstetricians and Gynacologists 2011).

1.2.3 Prevention and treatment

The prognosis for women with RM is favourable, with persistence and care during the pregnancy. It has been demonstrated in some studies that overall care, with regular obstetric appointments and reassurance increases the chances of a successful pregnancy (ACOG 2002, Royal College of Obstetricians and Gynacologists 2011). However there are a lot of cases that have factors, such as APS and deficiencies, that require treatment for a successful pregnancy (Matsubayashi 2009). Even though treatment is required, treatment may not always be available, with more than 50% of

cases having an unknown cause and even with known causes there is not always a treatment available that is sufficient enough to reduce the risk of RM (Sugiura-Ogasawara *et al.* 2009).

There are not many treatments available to reduce the risk of RM, however ones that are currently used for RM include anticoagulants and vitamin supplements; but the degree of benefit depends on the patient. As hyperhomocysteinemia may play a role in increasing the risk of RM, several papers have demonstrated that supplements containing 0.5mg folic acid can greatly reduce the tHyc levels in women with RM (Crider *et al.* 2011, Nelen *et al.* 1998). In addition, Quere *et al* (1998) demonstrated that women with the c.667C>T MTHFR mutation undergoing folic acid and pyridoxine supplementation had an increase in live birth rate. Anticoagulants include low-dose aspirin and heparin (low molecular weight or unfractionated), either alone or in combination, that are used to treat APS and other thrombophilias (Kaandorp *et al.* 2010, Laskin *et al.* 1997, Rai *et al.* 1997, Reznikoff-Etievant *et al.* 1999, Tempfer *et al.* 2006, Tincani *et al.* 2003). There are still some discrepancies between the treatments with aspirin and heparin demonstrating that aspirin alone is equally effective, or that the type of heparin administered can differentially effect outcomes (Bansal *et al.* 2011). Aspirin works by its inhibition of platelet activation and reduced thrombus formation, where heparin works by interfering with the autoantibodies directed at the heparin-binding epidermal growth factor (EGF) by saturation, which is important in blastocyst implantation (Bansal *et al.* 2011).

Moreover the role of both these treatments is still controversial like most treatments for RM (Rai *et al.* 2006).

A majority of treatments proposed for RM are just that, proposed, and show little evidence for success. Treatments for uterine abnormalities include hysteroscopic resection or myomectomy which have been shown to slightly increase the success rate of live birth; unfortunately they also have a high risk of complications and therefore outweigh the benefits (Ford *et al.* 2009, Grimbizis *et al.* 2001). Treatments for genetic abnormalities are limited to methods of pre-implantation IVF karyotyping which enables selection of an embryo without genetic defects (Sugiura-Ogasawara *et al.* 2008). However the treatment does not correlate with an increase in live birth rates, as single nucleotide or small deletions/insertions genetic mutations are not characterised by karyotyping (Kouru *et al.* 2010, Sugiura-Ogasawara *et al.* 2008). Lastly pre-implantation IVF karyotyping is an expensive treatment that may not be affordable for some couples (Barber *et al.* 2010). As with the previous two mentioned treatments, leukocyte immunization for immunologic intervention has demonstrated no improvement in pregnancies going to term, and in some studies demonstrated an adverse effect on pregnancy (Ata *et al.* 2011, Branch *et al.* 2010).

1.2.3.1 Treatment for Unexplained Recurrent Miscarriage

There are not many treatments available for unexplained miscarriage, largely attributable to an absence of a definitive underlying cause. Although aspirin and heparin are used to treat people with RM and the

presence of APS, studies have also looked at the treatment of unexplained RM with both aspirin and heparin, based on presumed pathogenesis similarities (Reznikoff-Etievant *et al.* 1999). The Initial study in 1999 demonstrated the treatment of unexplained RM with aspirin and prednisone to show the same success rates for live birth as women with RM and APS (Reznikoff-Etievant *et al.* 1999). However, since this first study multiple studies have disproved this theory. Kaandrop *et al* (2010) has since completed the ALIFE study which was a randomized trial containing 299 women, and demonstrated that neither aspirin with nadroparin nor aspirin alone increased the success rate in women with unexplained RM. Another large cohort study, the SPIN study (2010) recruited 294 pregnant women and demonstrated that treatment with enoxaparin and low-dose aspirin proved to be no more successful in women than intensive surveillance alone (Clark *et al.* 2010). Three further published studies all support_ a conclusion that treatment with aspirin and heparin in unexplained RM is ineffective for increasing the chance of live birth and should not be prescribed to treat women (Visser *et al.* 2011) (Pasquier *et al.* 2015, Schleussner *et al.* 2015).

Tempfer *et al* (2006) described a study looking at a multifactorial treatment for patients with unexplained RM. The study comprised of 104 pregnant women. 52 women fell pregnant on a combination of treatments, which included, Prednisone (20mg/d) and progesterone (20mg/d) for the 1st 12 weeks, aspirin (100mg/d) for 38 weeks and folate (5mg) every second day for the whole pregnancy; which were compared to 52 pregnant women not taking treatment (Tempfer *et al.* 2006). Overall there was a 42% increase in

live birth rates in the treated women compared to the untreated women, demonstrating that one treatment or a combination of treatments helped the women carry to term. The authors suggested that factors such as a high dose of folate in the1st trimester and pre-pregnancy treatment, and treatment over the full length may play an important factor in women having a live birth (De-Regil *et al.* 2010, Tempfer *et al.* 2006). It is also important to note that the folate supplementation levels of treated women was 5mg every second day which is far higher than the recommended levels of treatment for women with NTD which is 400-800µg/day (De-Regil *et al.* 2010, MRC Vitamin Study Research Group 1991, Tempfer *et al.* 2006). This suggests a greater requirement for folic acid supplementation in women with RM compared to NTD (De-Regil *et al.* 2010).

1.3 Folate: Essential for Human Physiology

To better understand how folate may affect pregnancy outcome for example in RM, the physiology and mode of transport through the placenta must be understood. Folate is an important compound in many biochemical reactions within the body, including maintenance of the nervous system, DNA replication and repair, cell division, apoptosis, and for normal development from embryogenesis right through adult life (Kim 1999, Mattson *et al.* 2003, van der Linden *et al.* 2006, Wollack *et al.* 2008). Folate is a water-soluble compound from the B vitamin group that includes isoforms folic acid, folinic acid, and 5-methyltetrahydrofolate (5-methylTHF) (Kelemen 2006). Folic acid is the synthetic compound which is monoglutamated and more stable than naturally found folates and therefore used in supplements (Eskes

1997, Laanpere *et al.* 2010, Ramaekers, Blau, *et al.* 2007). 5-methylTHF is the only reduced form of folate found in plasma and has a range of functions within the body via metabolism (Matsue *et al.* 1992). It is also the only folate compound that is able to cross the blood-brain barrier and is transported from maternal circulation to the fetus (Ramaekers, Sequeira, *et al.* 2007). Cells that require a large amount of folate are generally undergoing rapid cell division and growth, although apoptotic cells also require folate for example in embryogenesis (Blount *et al.* 1997, Shelnutt *et al.* 2004, Xiao *et al.* 2005).

Folate plays a major role in the folate-homocysteine metabolic pathway, with 5-MTHF being at the critical junction of the cycle (Blom *et al.* 2010). This pathway is responsible for both synthesis and metabolism of folate and homocysteine, and is the major pathway for nucleotide and amino acid synthesis and methylation reactions. Figure 1.1 illustrates the overall pathway (Mason *et al.* 2000, Mattson *et al.* 2003, van der Linden *et al.* 2006). Folate is brought into the cell as 5-MTHF and reacts with homocysteine to produce methionine and tetrahydrofolate (Dickson *et al.* 2005, Mason *et al.* 2000). The production of methionine is the first step in the methylation cycle, furthermore it is then converted to s-adenosyl methionine (SAM); which is responsible for the methylation of substrates including DNA, RNA, proteins, lipids and amino acids via one carbon transfer reactions (Mason *et al.* 2000, Mattson *et al.* 2003, Shelnutt *et al.* 2004). SAM methylates up to 4% of cytosine residues within DNA to produce 5-methylcytosine, which is constant throughout all cells, maintaining DNA stability and regulating gene

expression (Mason *et al.* 2000). RNA stability is also maintained via methylation of specific bases by SAM, and by the process of the 5' methyl capping of the RNA transcript (James *et al.* 1993, Mattson *et al.* 2003). Post-methylation SAM is converted to S-adenosylhomocysteine, ultimately producing homocysteine, which is also produced by the de-methylation of methionine (Collaboration 1998, Forges *et al.* 2007).

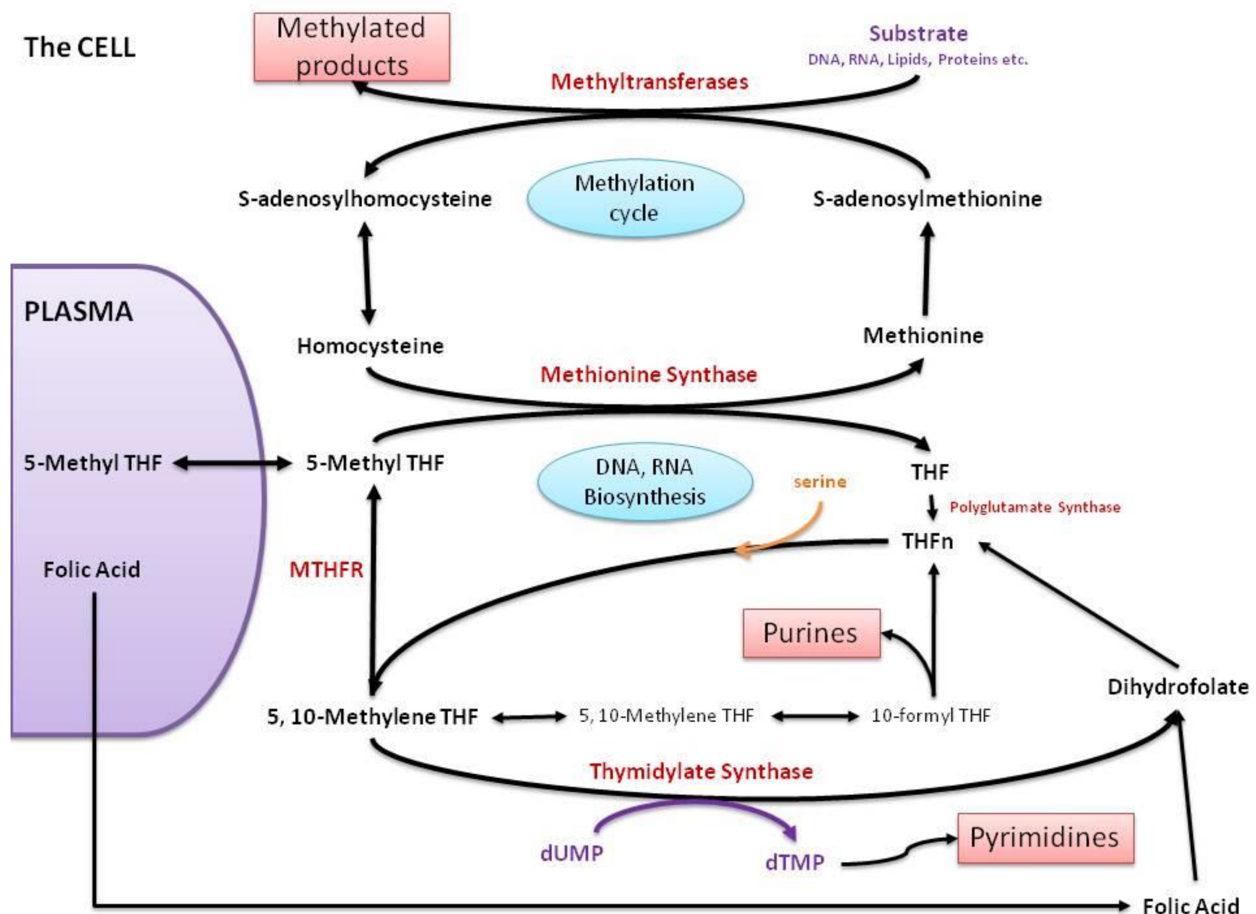


Figure 1.1 Simplified diagram of the Folate Cycle. 5-methylTHF is an important co-factor in the methylation cycle, methylating specific bases within DNA, RNA, and proteins, as well as a requirement for pyrimidines and purine production.

The synthesis of tetrahydrofolate from homocysteine/5-MTHF metabolism is the first important step in the DNA/RNA biosynthesis pathway, and requires a large amount of folate to maintain a balance (Blount *et al.* 1997, James *et al.* 1993, Mason *et al.* 2000). In this cycle pyrimidines are formed when the enzyme thymidylate (dTMP) synthase catalyses a reaction between 5,10 methyleneTHF and dUMP to produce dTMP (Blount *et al.* 1997, Choi *et al.* 1998). Purines are produced when 5,10 MethyleneTHF is converted to 10-formyltetrahydrofolate further resulting in the production of dGTP and dATP (James *et al.* 1993). Overall folate plays a vital role in the production of both

pyrimidines and purines which is an important process in DNA synthesis and repair with every cell (Blount *et al.* 1997, Mason *et al.* 2000)

1.3.1 Folate Transport in the Placenta

The placenta is a protective barrier allowing exchange of nutrients from the maternal blood supply to the fetus (Henderson *et al.* 1995, Yasuda, Hasui, Kobayashi*, et al.* 2008, Yasuda, Hasui, Yamamoto*, et al.* 2008). Approximately 600μg/d of folate is required for development of the embryo, therefore adequate uptake of folate from the maternal circulation is critical and a specialised mechanism is required to transport the folate across the placenta (Antony 2007, Henderson *et al.* 1995). There are three mechanisms that can transport folate in and out of a cell, the reduced folate carrier, the proton coupled folate transporter and folate receptors (Figure 1.2) (Carter *et al.* 2011, Yasuda, Hasui, Yamamoto*, et al.* 2008, Zhao *et al.* 2009). All three, have specific tissue distribution and are membrane specific (apical/basolateral membranes) depending on their function and affinity to transport folate (Henderson *et al.* 1995, Opladen *et al.* 2007).

In the placenta the initial uptake of folate is facilitated by the folate receptor alpha (FRα) present on the microvillous membrane of the syncytiotrophoblast cells, the absorptive cells of the placenta (Henderson *et al.* 1995, Prasad *et al.* 1998). FRα comes into direct contact with the maternal blood supply, where 5-methylTHF binds to FRα with high affinity ($K_d \approx$ 1-3nM) and is internalised via endocytosis (Henderson *et al.* 1995, Kranz *et al.* 1995). The uptake of folate causes an increase in 5-methylTHF concentration in the

intravillious blood three times that of the maternal blood (Henderson *et al.* 1995, Prasad *et al.* 1998). The second step of folate transport within the placenta occurs via the reduced folate carrier (RFC) (Prasad *et al.* 1998). The RFC is present on the basolateral membrane of the syncytiotrophoblast and is an ion channel that moves folate from high concentration to a low folate concentration, this allows efflux of 5-MTHF from the intravillous blood to the fetal blood system (Prasad *et al.* 1995, Yasuda, Hasui, Yamamoto, *et al.* 2008). The advantage of initial transport via FRα from maternal to intravillous blood is that FRα has a greater affinity to bind folate than the RFC and is therefore better suited for the high folate requirements of the developing embryo (Prasad *et al.* 1995, Yasuda, Hasui, Kobayashi, *et al.* 2008, Yasuda, Hasui, Yamamoto, *et al.* 2008, Zhao *et al.* 2009). In addition, it is able to transport folate against a concentration gradient when maternal blood 5-MTHF concentrations are low (Henderson *et al.* 1995).

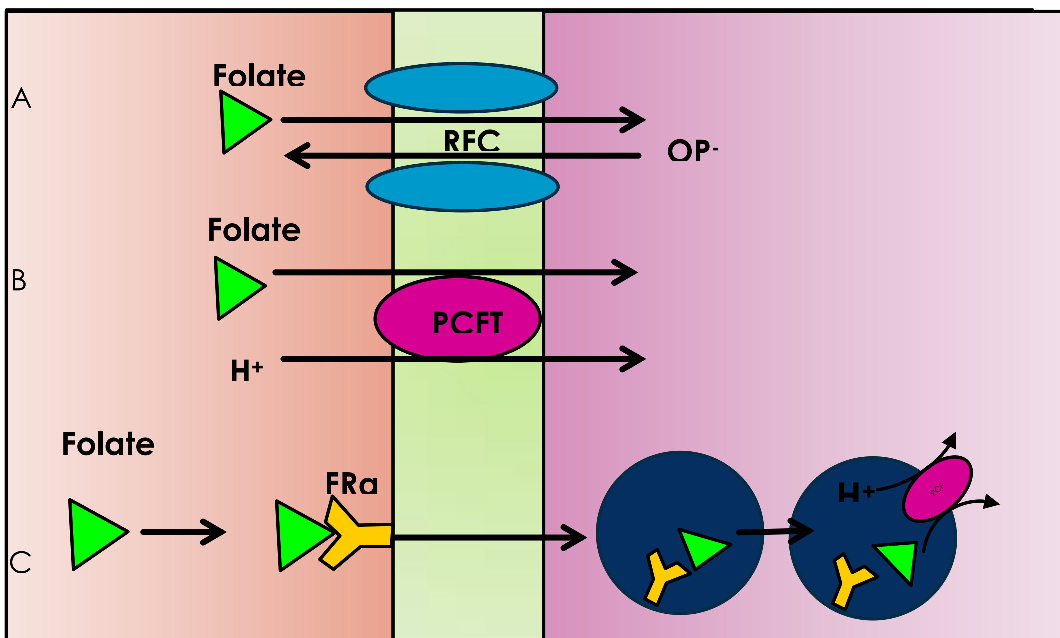


Figure 1.2 The three folate transport mechanisms. A. The reduced folate carrier (RFC) B. Protein Coupled Folate transporter (PCFT), C. Folate Receptor Alpha

1.3.1.1 Reduced Folate Carrier (RFC)

The RFC is an integral membrane bound glycoprotein that plays a significant role in placental-fetal transport of folate during pregnancy (Henderson *et al.* 1995). The RFC transports folate through the cell membrane via a bidirectional anion exchange pump mechanism (Bisseling *et al.* 2004, Prasad *et al.* 1998, Prasad *et al.* 1995, Yangfeng *et al.* 1995, Yasuda, Hasui, Kobayashi*, et al.* 2008). Therefore, folate can be transported in or out of a cell via the RFC, depending on the cell requirements. Distribution of the RFC is ubiquitous throughout cell tissues, and is the predominant transporter of folate for tissues with low folate requirements (Bisseling *et al.* 2004, Yasuda, Hasui, Kobayashi*, et al.* 2008). A defining property of the RFC compared to the FRα is its lower affinity for folate and the concentration at which it functions ($K_d \approx$ 1-100μM) (Kranz *et al.* 1995). RFC has a higher binding affinity for 5-methylTHF than folic acid, but cannot transport folate when circulating folate concentrations are low, and unlike FRα, cannot transport folate against a concentration gradient (Henderson *et al.* 1995, Yasuda, Hasui, Kobayashi*, et al.* 2008)

1.3.1.2 Folate Receptor Alpha (FRα)

FRα is a 38 kDa glycosylphosphatidylinositol (GPI) linked glycoprotein bound to the cell membrane of specific cells (Galmozzi *et al.* 2001, Ratnam *et al.* 1989, Yasuda, Hasui, Kobayashi*, et al.* 2008). FRα is one of three isoforms of the folate receptor protein, the others being FRβ and FRγ which are encoded by a group of genes located on chromosome 11q13 (Barber *et al.* 1998). The three genes encoding FRα, FRβ and FRγ are FOLR1, FOLR2, and

FOLR3, respectively (Elnakat *et al.* 2004, Elnakat *et al.* 2006, Kelemen 2006). The FOLR1 gene consists of seven exons, six introns and two TATA-less promoters (Tran *et al.* 2005, Zheng *et al.* 2003). Exons 5 and 6 are predicted to be responsible for a key part of the binding pocket of the protein; however it has no known crystal structure as yet (Barber *et al.* 1998). The addition of a GPI tail post translation on FRα and FRβ functions as a signal to transport the protein to the membrane surface of the cell (Kinoshita *et al.* 2008, Luhrs *et al.* 1989, Mauritz *et al.* 2008, Smart *et al.* 1996, Varma *et al.* 1998). It also mediates clustering to caveolae or lipid rafts within the cell membrane instead of clathrin coated pits which are mediated via another cytoplasmic domain (Anderson *et al.* 1992, Ritter *et al.* 1995). FRγ differs from FRα and FRβ as the protein does not undergo post translational addition of a GPI tail, instead acting as a secretory protein circulating throughout the body (Kelemen 2006, Mauritz *et al.* 2008).

There are several differences between FRα and the FRβ which may explain the higher rate of FRα expression in tissues throughout the body. The amino acid homology between the two isoforms is only 71% (Barber *et al.* 1998, Elnakat *et al.* 2004, Kelemen 2006). In addition, the C-terminal end also varies with the FRα having 25 residues and the FRβ having only 19 residues (Elnakat *et al.* 2004). Furthermore, another variation is the affinity of the receptors to bind folate which plays an important role in the uptake of folate within the cells, even in comparison with the RFC. FRα has a much higher affinity for both 5-methylTHF and folic acid, with a K_d= ~1-3nmol/L and a K_d= ~0.4nmol/L, respectively, and is the only isoform near saturation at normal

folate concentration (Bisseling *et al.* 2004, Doucette *et al.* 2001, Kranz *et al.* 1995). Whereas FRβ has an affinity to bind 5-methylTHF with a K_d equal to 0.3-4.0μmol/L and folic acid with a K_d of only 100-200μmol/L (Doucette *et al.* 2001, Jhaveri *et al.* 2004). FRα is capable of binding and transporting folate in nanomolar concentrations of extracellular folate, whereas the RFC and FRα require much greater extracellular folate concentrations and may explain the difference in distribution and high expression of the FRα in folate demanding tissues compared to FRβ and RFC (Antony *et al.* 1981, Henriques *et al.* 1996, Opladen *et al.* 2007).

Several studies have researched the distribution of the FRα. Distribution of FRα is highly tissue specific, is limited throughout the body and, in general, is only found in tissues that require a large influx of folate (Weitman, Weinberg*, et al.* 1992). The major tissues that the FRα is expressed in high concentrations are the placenta, kidney, and choroid plexus (Birn *et al.* 2005, Pinard *et al.* 1996, Ross *et al.* 1994), however a lower expression has been demonstrated in over ten different tissues. In addition, the FRα can be highly expressed in specific cancer cells which has been the focus of much research, particularly as a means of drug delivery to tumours (Basal *et al.* 2009, Bueno *et al.* 2001, Dainty *et al.* 2007, Ebel *et al.* 2007, Elnakat *et al.* 2004, Evans *et al.* 2001, Gates *et al.* 1996, Kalli *et al.* 2008, Knutson *et al.* 2006, Lu *et al.* 2003, Muller *et al.* 2008, Parker *et al.* 2005, Ross *et al.* 1994, Yang R. *et al.* 2007).

FRα is generally expressed on the epithelial cells on the apical membrane found in the placenta, kidney and choroid plexus (Elnakat *et al.* 2004, Muller

et al. 2008, Yasuda, Hasui, Yamamoto*, et al.* 2008). Expression of the receptor on the apical membrane allows the receptor to be in direct contact with circulating 5-methylTHF in serum, allowing transport of folate into specific tissues via FRα (Muller *et al.* 2008, Wu *et al.* 1999). This is demonstrated in the choroid plexus which requires sustained folate transport across the blood brain barrier (Holm *et al.* 1991, Weitman, Lark*, et al.* 1992, Weitman, Weinberg*, et al.* 1992). In the kidney, high expression of the FRα is also found in the apical membrane of the proximal tubules, allowing folate to be recaptured from urine that is being excreted (McMartin *et al.* 1992, Muller *et al.* 2008, Sandoval *et al.* 2004). Therefore FRα plays an important role in folate transport for a selected range of tissues that require large quantities of folate.

1.3.1.2.1 Mechanisms of FRα

FRα transports folate via receptor mediated endocytosis, however the whole process is complicated and not well understood (Anderson *et al.* 1992, Mauritz *et al.* 2008, Yang J. *et al.* 2007). FRα is a specific membrane bound receptor that utilizes an endosome to concentrate and internalize folate (Anderson *et al.* 1992, Jhaveri *et al.* 2004, Ritter *et al.* 1995, Yang J. *et al.* 2007, Zhao *et al.* 2009). The GPI tail plays a major role in the determination of the FRα location, as it mediates clustering to the caveolae, or lipid raft, while other transmembrane proteins are attached to the clathrin coated pits by their cytoplasmic domain (Anderson *et al.* 2002, D'Alincourt Salazar *et al.* 2007, Kinoshita *et al.* 2008, Miotti *et al.* 2000, Pike 2003, Ritter *et al.* 1995). Caveolae are only expressed in a few cell types whereas lipid rafts are

uniformly expressed in all cell types (Anderson *et al.* 1992). This mechanism of endocytosis using the caveolae and lipid rafts is also known as potocytosis (Anderson *et al.* 1992, Mauritz *et al.* 2008, Yang J. *et al.* 2007) .

Figure 1.3 demonstrates the basic mechanism of endocytosis that FRα utilizes to import folate into the cytoplasm of the cell (Kamen *et al.* 2004, Lacey *et al.* 1989, Rijnboutt *et al.* 1996, Ritter *et al.* 1995). The first step of this process is binding of folate to FRα (Zhao *et al.* 2009). A fluid filled endosome, also known as a GPI-anchored protein enriched endocytic compartment, is then formed by invagination of the lipid raft or caveolae membrane, which is then kept in close proximity to the cell membrane in the cytoplasm (Anderson *et al.* 1992, Kamen *et al.* 1991, Rijnboutt *et al.* 1996, Ritter *et al.* 1995, Rothberg *et al.* 1990, Sabharanjak *et al.* 2004). Once the endosome has formed within the cell, the folate must be released into the cytoplasm (Zhao *et al.* 2009). An increase in acidity occurs via influx of protons into the endosome during transit within the cytoplasm (Kamen *et al.* 1991, Yang J. *et al.* 2007, Zhao *et al.* 2009). This drop in pH to 6.0~6.5 is required for the dissociation of the folate from the FRα in the endosome (Kamen *et al.* 2004, Wibowo *et al.* 2013, Yang J. *et al.* 2007, Zhao *et al.* 2009)

Once the folate has been released from the receptor it is then free to move into the cytoplasm via a folate transporter. Studies have demonstrated that an anion channel may be responsible for the export of folate from the endosome to the cytoplasm (Matsue *et al.* 1992, Ritter *et al.* 1995, Yang J. *et al.* 2007). A study carried out by Zhao *et al* (2009) provided evidence that

the proton-coupled folate transporter (PCFT) plays a role in this transport and is present within the endosomal perinuclear compartment (Wollack *et al.* 2008, Zhao *et al.* 2009). The last step in endocytosis is the recycling of the FRα to the cell membrane, allowing more folate to be sequestered by the cell and allowing a high intake of folate required by the placenta during embryo development (Zhao *et al.* 2009). A more recent study identifying the crystal structure of the FRα, identified three conformational phases the receptor changes to depending on the step of folic acid binding/FRα cycling. Phase I is in neutral pH and the deep binding pocket is open, phase II folic acid binds and the binding pocket forming a FRα-folate complex and change in binding pocket, and then when the pH shifts to 6.5 the binding pocket closes releasing the folic acid bound (Wibowo *et al.* 2013); supporting what was demonstrated in earlier papers.

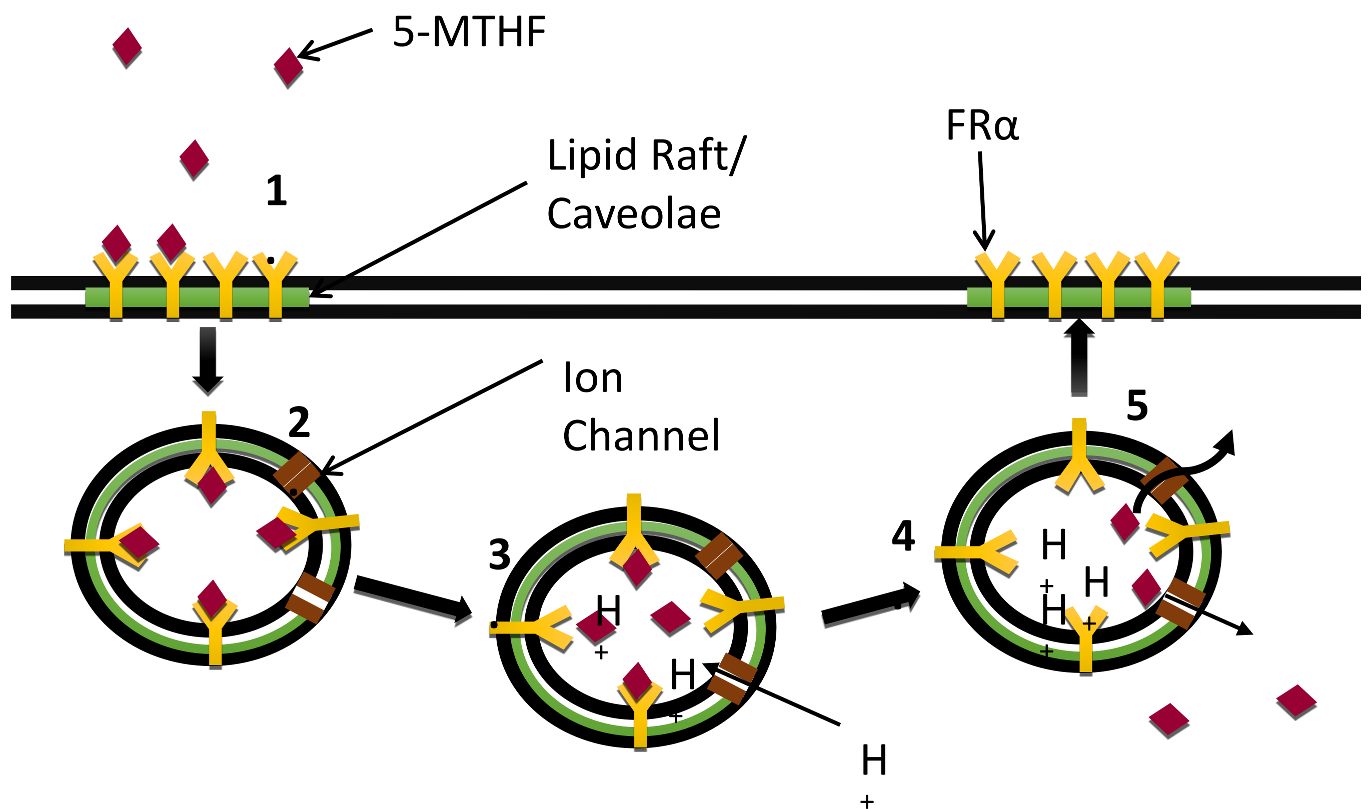


Figure 1.3 FRα mediated endocytosis of 5-methyltetrahydrofolate into the cell. 5-methylTHF binds to the FRα clustered in the lipid raft/caveolae (1) and is transported into the cell via formation of an endosome (2). Folate is released from the receptors after an increase in acidity (3) and transported into the cytoplasm via an ion channel (4). Endosomes then re-fuse with the cell membrane recycling the FRα to the cell surface (5).

1.3.2 Folate Deficiency and Pregnancy

Folate deficiency is defined as plasma folate level of <3ng/mL or an erythrocyte folate level of <140ng/mL and can have a significant effect during fetal development (Blount *et al.* 1997). Folate, which is required 5-10 fold greater during gestation, has been well documented to have a significant effect on embryogenesis, since folate is required for DNA, RNA and protein synthesis (Antony 2007, Chmurzynska , Finnell *et al.* 2004). As early as 1948, folate deficiency has been associated with adverse effects during gestation and congenital abnormalities which include low birth weights, sub fertility, spontaneous abortion, NTD's, and other pregnancy complications (Antony 2007, Bisseling *et al.* 2004, Ebisch *et al.* 2007, Hogan *et al.* 1950, O'Dell *et al.* 1948, Smithells *et al.* 1976, Tamura *et al.* 2006). In 1976 Smithells *et al* discovered that there was an association between low folate levels and an increase risk of NTD's, which subsequent studies have supported (Cabrera *et al.* 2004, Kirke *et al.* 1993, Zhao *et al.* 2006). Three major consequences can occur when folate is deficient within the body leading to such disorders above, including reduced methylation, an imbalance of the nucleotide pool and an increase in homocysteine levels; ultimately leading to a decrease in cell proliferation and increase in apoptosis (Blount *et al.* 1997, Choi *et al.* 1998, Li *et al.* 2006, Mattson *et al.* 2003, Xiao *et al.* 2005).

One of the most important factors affected by folate deficiency is pyrimidine and purine biosynthesis within cells (Blount *et al.* 1997, Choi *et al.* 1998). When folate is deficient it causes a reduction in its pre-cursor 5-10

methyleneTHF and as a result synthesis of thymidylate is decreased, further leading to an increased cellular dUMP/dTMP ratio (Blount *et al.* 1997, Choi *et al.* 1998, O'Neill 1998). This imbalance in nucleotides causes uracil (dUMP) to be mis-incorporated into the DNA (Blount *et al.* 1997). Uracil is excised from the DNA through DNA repair, resulting in single strand breaks, however with extremely depleted folate levels the chance of two closely spaced uracil bases on opposing DNA strands is increased resulting in double stranded DNA breaks. In addition, a reduction of thymidylate slows DNA synthesis reducing cell proliferation and increases apoptosis (Laanpere *et al.* 2010). As a result DNA synthesis and repair can be impaired inducing DNA strand breaks, chromosomal anomalies, and mutagenesis (Choi *et al.* 1998). Mis-incorporation of uracil and ultimately genetic instability is greater in cells undergoing rapid DNA synthesis, therefore rapidly dividing cells, such as during embryogenesis, are most affected (Blount *et al.* 1997, Shelnutt *et al.* 2004, Xiao *et al.* 2005).

Another outcome of folate deficiency is the decrease in the generation of SAM which as a result reduces the level of methylation within the cell (Mattson *et al.* 2003, Shelnutt *et al.* 2004). A decrease in DNA methylation can cause DNA instability, a reduction in replication, gene transcriptional activation and DNA strand breakage and mutations which can further trigger apoptosis (Mattson *et al.* 2003, Shelnutt *et al.* 2004). In addition, the reduction of SAM can affect methylation of proteins, phospholipids and neurotransmitters, and importantly histones in DNA (Forges *et al.* 2007, Mattson *et al.* 2003). Therefore, sufficient levels of folate are required to

maintain DNA, RNA and protein stability; otherwise if folate is deficient there may be an increase in genetic mutations and cell death.

Folate deficiency has another consequence which is increased levels of homocysteine within the cell (Blom *et al.* 2010, Coppola *et al.* 2000). Homocysteine, which is a sulphur containing amino acid, has many cytotoxic effects within tissues (Blount *et al.* 1997, Zetterberg 2004). Studies have demonstrated a strong inverse association between serum folate levels and homocysteine; when folate decreased tHyc levels increased (Collaboration 1998, Ray *et al.* 1999, Solanky *et al.* 2010). Homocysteine is metabolized by two pathways forming either methionine or cysteine, with both pathways being dependent of folate, therefore if folate is deficient then minimal homocysteine is converted resulting in the accumulation of homocysteine in the cells (Collaboration 1998, Laanpere *et al.* 2010, Tsitsiou *et al.* 2011). The accumulation of homocysteine can have many impacts ultimately leading to DNA strand breaks and hypomethylation, oxidative stress and trophoblast apoptosis. A major factor that is caused by increased levels of homocysteine vascular thrombosis which is demonstrated in many pregnancy complications, including RM (Coppola *et al.* 2000, D'Angelo *et al.* 1997, Di Simone *et al.* 2003, Durand *et al.* 2001, Kuch *et al.* 2001). Elevated homocysteine levels have been shown to cause thrombosis by; toxic accumulation in endothelial cells causing dysfunction of vascular endothelium; ability to inhibit platelet aggregation, deregulation; increase of clotting factors and tissue factors; and the inhibition of the anticoagulant

protein thrombomodulin (Alsulaimani *et al.* 2013, Ashfield-Watt *et al.* 2001, Goldstein 2000, He *et al.* 2004, Lonn *et al.* 2006)

As described earlier folate has to pass through the placenta to enter fetal circulation and therefore it is important to note in any pregnancy complication related to folate, that folate deficiency may occur only in fetal tissues while the maternal folate levels remain normal (MRC Vitamin Study Research Group 1991, Olney *et al.* 2002, Steegers-Theunissen *et al.* 2000). A large amount of work has gone into describing this in relation to NTD however it may also be a factor in RM, which may also be associated to folate deficiency (Berry *et al.* 1999, Botto *et al.* 2005, Czeizel 2009, Czeizel *et al.* 1992, Czeizel *et al.*). Over the last few decades studies into maternal nutrition, NTD's and pregnancy related complications have discovered that use of vitamin supplementation can greatly reduce the risk of having an affected pregnancy; in NTD it has been demonstrated to reduce the risk in up to 70% of cases (Kirke *et al.* 1993, MRC Vitamin Study Research Group 1991, Olney *et al.* 2002, Steegers-Theunissen *et al.* 2000). However what was not expected, was that a large proportion of these women did not have maternal folate deficiency, yet folate supplementation greatly increased their chance of an uncomplicated pregnancy (Czeizel 2009, Czeizel *et al.* 1992, MRC Vitamin Study Research Group 1991). Today it is recommended that folic acid supplementation of 0.4mg/day is required in women trying to conceive to reduce such risk as NTD, subfertility and RM (Bower *et al.* 2009, Steegers-Theunissen *et al.* 2000, Werler *et al.* 1993). The mechanism of folate supplementation reducing the risk in women that do not have maternal

folate deficiency is not well understood, though it is suggested that it may overcome blocking of folate-transport across the maternal-fetal blood barrier (Werler *et al.* 1993).

1.3.3 Factors Leading to Fetal Folate Deficiency

Factors that may be responsible for causing folate deficiency within the embryonic tissues, and not maternally, are defects within folate transport proteins or folate metabolism enzymes and blocking of folate transport (Da Costa *et al.* 2003, Steinfeld *et al.* 2009). It has been demonstrated in mouse models that FOLR1 nullizygous knock out causes embryo death, and therefore it is proposed that the role of FRα in folate transport as a mechanism in folate deficiency may cause RM (Piedrahita 1999, Tang *et al.* 2003, Taparia *et al.* 2007). Studies investigating genetic mutations within folate metabolism enzymes show conflicting results. There have only been a couple of studies into FRα mutations and pregnancy, with De Marco *et al* (2000), demonstrating 4 out of 50 patients that had NTD to have a unique mutation in exon 7 and the 3'UTR, however there is no strong evidence associating genetic mutations of the FRα with defective folate transport in the placenta (Barber *et al.* 1998, De Marco *et al.* 2000, Heil *et al.* 1999, Holmes *et al.* 1999, Nilsson *et al.* 2004, van der Linden *et al.* 2006, van der Put *et al.* 1995). As a result, the role of these mutations in the aeitiology of folate-preventable pregnancy complications has not been established.

There is a high prevalence of autoantibodies that are responsible for many autoimmune diseases associated with increased risk of pregnancy complication. The first known human autoimmune disease was discovered in 1904 and demonstrated that antibodies can attack self-antigens (Schwartz 2005). An autoimmune disease is classified as an illness that involves the immune system producing antibodies to mistakenly attack the organs and tissues it is supposed to protect (Ornoy *et al.* 2004). Each autoantibody binds to a specific antigen; however they can be directed against a range of antigens including DNA, RNA, membrane proteins, phospholipids, nuclear proteins, thymoglobulins, annexin and matrix proteins (Da Costa *et al.* 2003, Gris *et al.* 2003). The aetiology of an autoimmune disease may be due to a complex group of autoantibodies (working together), and not just a single autoantibody, for example in APS (Gris *et al.* 2003). Since the first discovery there have been over 80 autoimmune diseases reported, with approximately 75% of these occurring during child bearing age (Ornoy *et al.* 2004). It has been demonstrated that a significant number of pregnancy related complications for example NTD's, RM and infertility are associated with autoimmune disease, and that autoantibodies can have a dose dependent increase on the risk of congenital defects and impaired embryo development (Matsubayashi 2009, Ornoy *et al.* 2004, Ticconi *et al.* 2010, Tincani *et al.* 2003). As there are still numerous unrecognised factors that may cause fetal folate deficiency, and it is well known that immunological responses can have a substantive impact on embryonic development, an immunological factor affecting folate transport was investigated (Barrow *et al.* 1971, Brent 1967).

1.4 The Presence of FRα Autoantibodies

In 2003 it was first proposed that one such mechanism may be the presence of autoantibodies directed against the FRα circulating in the maternal blood blocking cellular folate uptake in the placenta (Da Costa *et al.* 2003, Rothenberg *et al.* 2004). This study investigated the effect of FRα antiserum and embryogenesis in a rat model. It was confirmed that antiserum containing both FRα and FRβ when injected, caused a dose dependent effect on embryogenesis (Da Costa *et al.* 2003). Large doses (>0.3mL) of antiserum caused 100% embryo resorption, while lower doses showed 50% mortality of the embryo, and an increase of NTD affected offspring in the 50% that were born (Da Costa *et al.* 2003). Since this original study, a large population based study demonstrated that 7.2% of the population has FRα autoantibodies present, and several further studies have demonstrated the presence of FRα autoantibodies capable of blocking folate transport from maternal to fetal blood and an association between increased risk of NTD's, sub fertility, and neurodegenerative disorders such as cerebral folate deficiency (Berrocal-Zaragoza M. I. *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Frye *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers, Blau*, et al.* 2007, Ramaekers *et al.* 2013, Ramaekers *et al.* 2005, Ramaekers, Sequeira*, et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004).

The first autoantibody directed against FRα detected in human serum was reported by Rothenberg *et al* (2004). This study showed an association between the presence of FRα-blocking autoantibodies in maternal serum and an increased risk of the offspring being born with NTD's (Rothenberg *et*

al. 2004). The study examined sera from 12 women who previously had, or currently had, a pregnancy affected by NTD's. Out of the twelve case women tested, nine showed the presence of FRα autoantibodies in their serum, compared to two out of twenty controls that had never had a pregnancy affected by NTD's (Rothenberg *et al.* 2004). The population of this study was small, however it did suggest a possible explanation for the low folate concentration in the developing embryo compared to the maternal folate concentration and blocking of folate transport (Bliek *et al.* 2006, Cabrera *et al.* 2008, Molloy Anne M. *et al.* 2009, Rothenberg *et al.* 2004). Other limitations of this study included a lack of longitudinal quantitation of autoantibody titres in case women and, therefore called for further research to be performed (Rothenberg *et al.* 2004).

Three other studies have further investigated the presence of FRα autoantibodies and NTD, two which support the first study and one that does not support any association (Boyles *et al.* 2011, Cabrera *et al.* 2008, Molloy Anne M. *et al.* 2009). The second study was performed by Cabrera *et al* (2008). This study examined a slightly larger cohort than Rothenberg *et al* (2004), specifically 29 women with pregnancies affected by NTD's and 76 control samples of women with normal pregnancies (Cabrera *et al.* 2008). The serum collected was between weeks 15 and 18 of the pregnancy in both case and control subjects. The study demonstrated that the mean concentration of FRα autoantibodies and mean concentration of folic acid being blocked was significantly higher in case mothers compared to control subjects (Cabrera *et al.* 2008). In comparison to the pilot study which

illustrated only 10% of control subjects with the FRα autoantibodies, this research showed a continuous distribution of autoantibodies in both case and control subjects (Rothenberg *et al.* 2004). However, a significant difference between case and controls was shown, supporting the hypothesis that high titres of FRα autoantibodies present within maternal serum, blocked folate transport and ultimately increased the risk of NTD's in expecting mothers (Cabrera *et al.* 2008).

Molloy *et al* (2009) demonstrated a lack of association between folate receptor autoantibodies and NTD's in two studies performed in Ireland. The cohort was larger; one arm consisting of 103 mothers with a history of pregnancies complicated by NTD using frozen plasma and arm two comprising 38 women with a history of pregnancies complicated by NTD using fresh serum samples (Molloy Anne M. *et al.* 2009). The study did demonstrate the presence of FRα autoantibodies and their capability of blocking folic acid binding. However, a modest but non-significant association was seen, between the presence and titre of blocking FRα autoantibodies and the increased risk of NTD, as 17% of cases compared to 13% of controls contained FRα autoantibodies. No association was seen between binding antibodies and NTD with 29% of cases compared to 32% of controls (Molloy Anne M. *et al.* 2009).

Before 2011 there had only been two studies carried out investigating the association between the presence of FRα autoantibodies and oral clefts, including cleft lip and palate (CLP), which is a birth related defect also

associated with fetal folate deficiency (Billie *et al.* 2010, Bliek *et al.* 2006). The initial study was a small clinical trial analysing 11 cases and 10 controls (Bliek *et al.* 2006). A total of 9 out of 11 cases contained FRα autoantibodies at significant levels, compared to 3 out of 10 controls (Bliek *et al.* 2006). The last study to be carried out examined 185 cases of cleft lip in Denmark, that found there was no significant difference of mean FRα autoantibody levels between cases and control (Billie *et al.* 2010). However, in Denmark at the time of study, there was little folate supplementation leading to overall low folate status which could affect the overall association (Billie *et al.* 2010).

The most recent study looking at the presence of FRα autoantibodies in relation to both NTD or CLP was in 2011 (Boyles *et al.* 2011). The study supported previous findings as it identified an increased risk of NTD in women with high FRα autoantibodies at the time of pregnancy (adjusted odds ratio =1.4 95% confidence interval 1.0-1.8), demonstrating a higher level of inhibition of folic acid binding (Boyles *et al.* 2011, Cabrera *et al.* 2008, Rothenberg *et al.* 2004). However, the study showed no evidence of any association between the presence of FRα autoantibodies and cleft palate (adjusted odds ratio=0.7 95% confidence interval 0.8-1.4), supporting the findings of Billie *et al* (2010) (Boyles *et al.* 2011). The studies for both NTD and CLP had variations in sample size, period of sample collection and analysis techniques, which need to be taken in to account before making a strong conclusion for either agreeing or disagreeing that FRα autoantibodies increase the risk of these pregnancy related complications (Billie *et al.* 2010,

Bliek *et al.* 2006, Boyles *et al.* 2011, Cabrera *et al.* 2008, Molloy Anne M. *et al.* 2009, Rothenberg *et al.* 2004).

To date only one study has investigated whether an association exists between FRα autoantibodies and an increased risk of sub-fertility (Berrocal-Zaragoza Maria Isabel *et al.* 2009). Berrocal-Zargoza *et al* (2009) proposed that such an association did exist as previous studies demonstrated autoantibodies responsible for low folate status can result in increased risk of NTD's. The study examined 17 cases of sub-fertility collecting a total of 83 blood samples from women at different time points throughout their menstrual cycle (Berrocal-Zaragoza Maria Isabel *et al.* 2009). The results showed an association between the presence of autoantibodies and sub-fertility, with 18 out of 83 case samples testing positive to FRα autoantibodies, in comparison to only 1 out of 104 control samples (Berrocal-Zaragoza Maria Isabel *et al.* 2009). The authors suggested that autoantibodies directed against FRα may represent a 12-fold increased risk of sub-fertility and underlying cause for multiple miscarriages, which to date has not been further investigated (Berrocal-Zaragoza Maria Isabel *et al.* 2009).

The main research focus, apart from NTD's, has been cerebral folate deficiency and related neurological effects (Frye *et al.* 2013, Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2013, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008, Schwartz 2005). Cerebral folate deficiency is not a pregnancy complication, but studies carried out investigating the presence of FRα autoantibodies and

reduced folate concentrations, can greatly improve the knowledge of how these FRα autoantibodies can cause different pregnancy complications (Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008, Schwartz 2005). The mechanisms of fetal folate deficiency and cerebral folate deficiency are very similar; folate in both cerebral spinal fluid and fetal blood supply is required to be transported over a barrier such as the placenta or choroid plexus (Ramaekers *et al.* 2005, Rothenberg *et al.* 2004). There are seven studies to date that demonstrate a high significance in the association of such FRα autoantibodies and increased risk of cerebral folate deficiency and related disorders such as Rett syndrome and autism, study results demonstrated in table 1.2 (Frye *et al.* 2013, Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2013, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008). Firstly, it was established that as the FRα autoantibody titre increased in patients, the level of folate available in the cerebral spinal fluid decreased (Ramaekers *et al.* 2005). All studies showed a significant association between FRα autoantibodies and cerebral folate deficiency with the first carried out in 2005 demonstrating 25 out of 28 cases to have FRα autoantibodies present, while 28 controls did not (Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008). It was also shown that the presence of the FRα autoantibodies increased over time and with this there was an increase in disease markers i.e. aggressiveness in autism (Ramaekers, Blau, *et al.* 2007). These studies are important as they show how the presence of the FRα autoantibodies could potentially reduce the

amount of folate transport not only through the blood brain barrier but in the placenta as the main mechanism is the FRα.

Table 1.2 Study results for FRα autoantibodies in CFD related diseases

Study	Cases/controls	Result
Ramaekers et al. 2005	28 CFD cases 28 controls	• 25 of 28 cases positive for high affinity FRα blocking autoantibodies • 0 of 28 controls positive for high affinity FRα blocking autoantibodies
Ramaekers et al. 2007 (a)	33 Rett cases 28 controls	• 8 of 33 cases positive of blocking FRα autoantibodies • 0 of 28 controls positive for high affinity FRα blocking autoantibodies • A mean 1.25 pmol FR blocked/ml serum (0.29-2.04 range)
Ramaekers et al. 2007 (b)	25 Low functioning autism cases	• 19 of 25 cases positive for bloc king FRα autoantibodies -a mean 1.09 pmol FR blocked/ml serum (0.1-4.19 range)
Ramaekers et al. 2008	12 FRα CFD patients	• This study illustrated that reducing milk in 12 CFD patients with FRα autoantibodies to reduce mean antibody titres from 2.08pmol of FRα blocked to 0.35pmol. • Re-exposure to milk in 9 patients increased FRα autoantibody titres to 6.53pmol
Ramaekers et al. 2013	75 Infantile autism cases 30 controls	• 35 of 75 cases positive for high affinity FRα blocking autoantibodies • 1 of 30 controls positive for high affinity FRα blocking autoantibodies • a mean 0.21 pmol FR blocked/ml serum (0.1-4.19
Frye et al. 2013	93 ASD cases	• 70 of the 93 cases (75.3%) positive for FRα autoantibodies. • 16 cases of high FRα autoantibodies correlated with low cerebrospinal fluid 5-MTHF concentrations

1.4.1 The Genesis of FRα Autoantibodies

One question that has been raised in many reports investigating the presence of these FRα autoantibodies is why are they there, or how have they developed Several aetiologies have been proposed, and a few studies have investigated such reasons (Ramaekers *et al.* 2008). The first theory that was suggested was that the presence of approximately 5-8mg/L folate binding proteins (FBP) in milk could cause an immune response (Ramaekers *et al.* 2008, Schwartz 2005). The FBP found in cow's milk shares 90% homology to the human FRα and therefore antibodies directed against the FBP could cross react with the FRα (Berrocal-Zaragoza M. I. *et al.* 2009, Ramaekers *et al.* 2008, Schwartz 2005). Ramaekers *et al* (2008) demonstrated this in cerebral folate deficiency, when milk was removed from the diet, the levels of FRα autoantibodies was significantly decreased after 3-13 months (2.08pmol to 0.35pmol) (Ramaekers *et al.* 2008). Whereas in control patients with FRα autoantibodies, but still containing milk in their diet, there was no reduction in autoantibody titre after 12-24 months; in fact a significant increase in antibody titre was observed over time (Ramaekers *et al.* 2008). It was also demonstrated that subsequent reintroduction of milk into the diet increased antibody titres threefold in cases that had milk removed from the diet (Ramaekers *et al.* 2008). A further study carried out in 2009 investigated milk consumption and the presence of FRα autoantibodies without any association to a disorder (Berrocal-Zaragoza M. I. *et al.* 2009). This study revealed that the risk of having FRα autoantibodies increased with increasing quintile of milk intake (Berrocal-Zaragoza M. I. *et al.* 2009).

Like most immune disorders the mechanisms of how the immune system changes to produce pathogenic, autoantibody producing B cells and T cells which turn against itself are hard to determine (Berrocal-Zaragoza M. I. *et al.* 2009, Ramaekers *et al.* 2008, Schwartz 2005). In the case of milk and FBP this can only occur when a breakdown in the gastrointestinal tract happens exposing the antigen to the immune system (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Ramaekers *et al.* 2008). The immune response will only occur if the person has some other factor affecting the gastrointestinal tract immune response, like with most children in the early weeks of life their immune system may be weak (Berrocal-Zaragoza M. I. *et al.* 2009). Further supporting the postulation of FBP as the causative agent, the majority of the FRα autoantibodies identified in CFD/autism are of subclass IgG4, which suggests long term antigen exposure, supporting the presence of FRα autoantibodies due to an immune response against a substance that people drink in everyday lives (Ramaekers *et al.* 2008).

There are several other factors that have been proposed, mostly theories that remain untested. Previous induced/spontaneous abortions due to other factors may lead to the placenta breaking down or that placental FRα is subject to degradation and therefore the FRα is released into the circulating blood supply becoming a target antigen that is incorrectly detected by the immune system (Molloy Anne M. *et al.* 2009, Opladen *et al.* 2007, Rothenberg *et al.* 2004). This may be a trigger for antibody production as it has been demonstrated that women with previous pregnancy loss have an increased risk of miscarriage in future pregnancies, in addition to NTD's

(Andersen *et al.* 2000). It has also been suggested that the presence of antiphospholipids increase after either induced/spontaneous abortion, also giving rise to further RM if they were present in the original pregnancy loss (Gris *et al.* 2003). Another theory that has been proposed, is the formation of FRα autoantibodies due to N-homocysteinylation of the FRα as a product of elevated homocysteine levels, caused by folate deficiency (Berrocal-Zaragoza M. I. *et al.* 2009, Cabrera *et al.* 2008). This N-homocysteinylation occurs to the lysine residues of the FRα in the presence of homocysteine thiolacetone creating a new auto-antigen for the immune system to recognise (Taparia *et al.* 2007). However, it may not be a significant factor in the production of FRα autoantibodies in pregnancy related complications, as most women don't have folate deficiency or increased homocysteine levels in the maternal blood circulation (Berrocal-Zaragoza M. I. *et al.* 2009).

1.4.2 The Biological Role of FRα Autoantibodies on Fetal Development

FRα autoantibodies may be present in a patient's serum, but do they affect embryonic development? And if so, 'how' is an important question that needs investigating. The crystal structure for the FRα has only recently been described demonstrating a complex deep binding pocket of the receptor where folate binds to, which can change confirmation in different pH and when folic acid binds (Chen *et al.* 2013, Della-Longa *et al.* 2013, Wibowo *et al.* 2013). There are several important amino acids involved in folic acid binding that have been identified which are Y76, F78, D97, Y101, W118, R119, G153, W154, W156 and W187, illustrating they expand over numerous regions

of the protein (Wibowo *et al.* 2013). Nonetheless, a large amount of work has explored the pathogenesis of these FRα autoantibodies present in patient serum, investigating two mechanisms that may cause pregnancy complication and ultimately RM (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Ebel *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Rothenberg *et al.* 2004, Schwartz 2005). Firstly, two types of FRα autoantibodies can be present in patient serum; blocking and binding (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Cabrera *et al.* 2008). Studies to date have all analysed for the presence of blocking antibodies, measuring the amount of folate blocked, and demonstrated that all patients with FRα autoantibodies contain blocking autoantibodies of some capability (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Ramaekers, Blau, *et al.* 2007, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004). Binding FRα autoantibodies are categorised by binding to an epitope that does not affect the binding of folic acid, therefore not impairing the overall transport (Cabrera *et al.* 2008). This was demonstrated in the study carried out by Cabrera *et al* (2008) which identified patient serum containing FRα autoantibodies that exhibited a low level of folic acid blocking. This observation has further been confirmed in cancer patients where it was demonstrated that patient serum reacted to an average of 3 epitopes of the FRα analysed (Knutson *et al.* 2006). These studies in combination support the hypothesis that binding and blocking autoantibodies are raised against a range of different epitopes, with some patients having a multi epitope immune response against the FRα.

The presence of blocking FRα autoantibodies, bind to the FRα protein on the cell membrane at its folate binding site, impairing 5-MTHF binding and then further being transported through the cells (Molloy Anne M. *et al.* 2009). The main consequence of this folate blocking is folate deficiency within the fetal tissues (Cabrera *et al.* 2008). FRα autoantibodies have a higher affinity of binding to the FRα than folate with a mean affinity of 5.54×10^{10} L/mol; it was also established that the mean affinity of FRα autoantibodies capable of blocking folate transport in KB cells is 2.2×10^{10} L/mol (Ramaekers *et al.* 2005, Rothenberg *et al.* 2004). Folate deficiency itself can affect pregnancy outcomes as it affects the methylation of proteins and DNA, as well as causes an imbalance in nucleotide biosynthesis (Mason *et al.* 2000, Mattson *et al.* 2003). However, the effect folate deficiency can also consequently cause is an increase in homocysteine levels within the embryonic tissues (Di Simone *et al.* 2003). High levels of homocysteine (tHyc), as described earlier, are toxic to cells and can cause a range of problems including reduced biological methylation, DNA synthesis, and cell division together with vascular dysfunction including atherosclerosis and placental endovascular (Coppola *et al.* 2000, Molloy A. M. *et al.* 2009, Molloy *et al.* 2002, van der Put *et al.* 2001, Vollset *et al.* 2000, Zhao *et al.* 2006). Both the decrease of available folate and increase in homocysteine within the embryonic tissues has been associated with increasing levels of FRα autoantibodies present within patient sera (Bliek *et al.* 2006, Boyles *et al.* 2011, Ramaekers, Blau, *et al.* 2007, Rothenberg *et al.* 2004). In addition the increase/decrease of these factors has been proven to cause a significant effect on pregnancy outcome, including increasing the associated risk of RM (George *et al.* 2002,

Steegers-Theunissen *et al.* 1992, Steegers-Theunissen *et al.* 2000, Wouters *et al.* 1993).

Similar to the type of autoantibody present in a specific patient (blocking/binding) the level of FRα autoantibody present can affect the overall involvement they have within the pregnancy. It has been proposed that a significant level of FRα autoantibodies is required to have complete impact on folate transport via the folate receptor (Da Costa *et al.* 2003, Ramaekers, Sequeira*, et al.* 2007). A study looking in to the presence of FRα autoantibodies in Rett syndrome demonstrated that out of the 8 patients that had FRα blocking autoantibodies only 6 had low folate levels in their CSF; while the two without cerebral folate deficiency were the only two with low FRα autoantibody titres (Ramaekers, Sequeira*, et al.* 2007). Thus, pointing out the fact that a positive/negative result cannot be used, and analysis of the exact level of FRα blocking autoantibodies needs to be determined.

The second mechanism proposed is the role of FRα autoantibody binding causing an antibody dependent cytotoxicity, and/or activation of complement, causing cell death and possibly RM (Da Costa *et al.* 2003, Ebel *et al.* 2007, Ticconi *et al.* 2010). It is well known that autoantibodies can cause this effect on cells and can cause placental injury and fetal death (Ticconi *et al.* 2010). A study carried out by da Costa *et al.* (2003) found that when high concentrations of FRα antiserum and a pharmacologic dose of dexamethasone where administered prior to the administration of FRα positive sera prevented 97% of embryonic resorption in rats, indicating an immune-mediated cytolytic reaction rather than a folate deficient response

within the embryo. Previous studies evaluating monoclonal antibodies (MOv18, MOv19, MORAb-003) directed against the FRα also demonstrated an antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity when binding to the FRα (Coney *et al.* 1991, Ebel *et al.* 2007). Mechanisms suggested include the involvement of signalling pathways with lyn kinase in addition to the α and β subunit of the G protein (Ebel *et al.* 2007, Miotti *et al.* 2000); a result of induction of cell lysis. However, at lower doses of FRα antiserum, folinic acid was able to overcome the effects of FRα blocking, suggesting that both folate deficiency and antibody cytotoxicity may be a risk factor for pregnancy complications (Da Costa *et al.* 2003). The studies demonstrate the overall function of the FRα in cell proliferation and suggest that the FRα upon autoantibody binding may undergo a functional change affecting both folate transport and cell signalling (Boyles *et al.* 2011). Once again, the levels of autoantibodies present may play a significant role in the biological affect they have at a cellular level, and consequently the overall affect in pregnancy. For example, the consequence may be NTD's at lower levels or RM at higher levels of FRα autoantibodies (Da Costa *et al.* 2003).

The presence of FRα autoantibodies may only be clinically significant in the limited number of organs/tissues where FRα is expressed highly, due to an elevated level of folate required (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Ramaekers *et al.* 2005, Schwartz 2005). Two examples are in embryonic tissue where a high concentration of folate is required for cell division or in the CSF where a large amount of folate is required for brain development (Antony 2007, Pitkin 2007, Ramaekers *et al.* 2005). It is also important to note that FRα

autoantibodies may have a limited effect on other tissues that do express the FRα such as the kidney proximal tubules and lungs as the FRα is expressed on the luminal membrane which does not come in contact with circulating antibodies (Elnakat *et al.* 2004, Holm *et al.* 1991, Wu *et al.* 1999). The placenta is one of the tissues expressing high levels of FRα demonstrating the potential effect that the FRα autoantibodies may have on embryonic tissues; resulting in pregnancy complications when no other clinical manifestations are present.

1.4.3 Potential Treatment

Treatment for FRα autoantibodies causing folate deficiency in specific tissues must either overcome the binding of the autoantibodies to the receptor or find another transport mechanism to bypass the FRα. To do this it must have an extremely high binding affinity to the receptor and also be in such a high concentration that it can be transported via the RFC or by passive diffusion. The obvious treatment to overcome FRα is by folic acid supplementation, as previous studies have demonstrated its positive affects in fetal folate deficiency related NTD (Bower *et al.* 2009, MRC Vitamin Study Research Group 1991). Rothenberg et al. (2004) suggested this as it can be transported via the RFC, but the dose that would be required would be much higher than is what is currently recommended for pregnancy. However, this may not work for all pathologies caused by the presence of FRα autoantibodies for example cerebral folate deficiency or if these autoantibodies do in fact cause RM.

Another treatment that has been proposed is folinic acid; a 5-formyl derivative of tetrahydrofolic acid and is readily converted to other reduced forms of folate. The first study carried out looking at FRα antisera in rats, demonstrated that folinic acid was able to overcome embryo loss caused by the antisera. It is thought that folinic acid can overcome the blocking of folate by several pathways, as it is converted to the active form 5MTHF. The high level of 5MTHF can then be transported via the RFC, overcome the binding of the autoantibodies to the FRα or be transported by passive diffusion. The current treatment of FRα autoantibodies in children with CFD is a high dose of folinic acid. Initial treatment is 1mg/Kg/day of folinic acid and may be increased to 2-3mg/Kg/day if required. A study in 2007 demonstrated that 100% of patients treated with this high dose of folinic acid, had their 5MTHF cerebral spinal fluid level normalize after treatment; with the 2 youngest patients having full recovery and were cured of autism. Studies to date demonstrate that both folic acid and folinic acid can overcome FRα autoantibodies however the dose required is dependent on the binding affinity of the FRα autoantibodies present or high enough to be transported via the RFC or passive diffusion.

1.4.4 Potential Role of FRα Autoantibodies in Recurrent Miscarriage

The importance of these anti-FRα autoantibodies in pregnant women is evident. To date no studies have examined the involvement of these autoantibodies in RM, yet there might be a strong association between the two as folate deficiency is a risk factor for RM. It has been proven that fetal

folate deficiency can occur when maternal folate levels are normal (MRC Vitamin Study Research Group 1991, Olney *et al.* 2002). Based on this it is hypothesised that a factor affecting folate transport across the maternal-fetal barrier is responsible. With little evidence supporting genetic mutations within folate transport mechanisms another cause is likely responsible (Barber *et al.* 1998, Heil *et al.* 1999, Nilsson *et al.* 2004, van der Linden *et al.* 2006). Studies have demonstrated that these autoantibodies directed against the FRα can cause a similar pathological effect on pregnancies as the well-established APS, which include hyperhomocysteinemia related thrombosis and complement and cell mediated cytotoxicity, and in addition can also cause folate deficiency affecting DNA/RNA and protein stability (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Cabrera *et al.* 2008, Da Costa *et al.* 2003, Ebel *et al.* 2007, Rothenberg *et al.* 2004, Vinatier *et al.* 2001). Therefore, the possibility that these FRα autoantibodies can increase the risk of RM is plausible.

1.5 Statement of Aims

1. Develop and validate an ELISA assay specific for the detection of FRα autoantibodies in patient serum and plasma.

2. Assess the risk associated with FRα autoantibody prevalence in the setting of recurrent miscarriage

3. Identify the prevalence and risk of FRα autoantibodies in stroke- a disease also associated with folate deficiency and hyperhomocysteinemia

4. Determine the inhibitory effect of anti-FRα antibodies on cell proliferation using an *in vitro* model

Chapter 2: Materials

2 Chapter 2: Materials

2.1 Chemical Reagents

2.1.1 General

Item	Company
agarose powder	Amresco, USA
Dipotassium phosphate	BDH Chemicals, England
Disodium Phosphate	BDH Chemicals, England
Folic acid	Sigma Aldrich, USA
Glacial Acetic Acid	Ajax Finechem, Australia
Glycine	Amresco, USA
hydrochloric acid (concentrated)	Ajax Finechem, Australia
isopropanol	BDH Chemicals, England
methanol	Biolab, Australia
Potassium Dihydrogen Orthophosphate	BDH Chemicals, England
sodium azide	BDH Chemicals, England
Sodium Bicarbonate	Sigma Chemical Co. USA
Sodium Carbonate	Sigma Chemical Co. USA
Sodium Chloride	Rowe Scientific, Australia
Sodium Hydroxide pellets	BDH Chemicals, England
sulphuric acid	Ajax Finechem, Australia
Thiazol Blue Tetrazolium Bromide (MTT)	Sigma Aldrich, USA
Tris	Amresco, USA
Tween20	Sigma Chemical Co. USA

2.1.2 Cell Culture

Item	Company
0.05% trypsin/EDTA	Gibco® Life Technologies, Australia
0.25% Trypsin EDTA	Gibco® Life Technologies, Australia
Dimethyl Sulphoxide	Sigma, MO, USA
Dulbecco's Modified Eagle Medium (DMEM), high glucose, with L-glutamine	Gibco® Life Technologies, Australia
Fetal Bovine Serum	HyClone, GE Healthcare, Australia
non-essential amino acids	Gibco® Life Technologies, Australia
Penicillin-Streptomycin	Gibco® Life Technologies, Australia
RPMI 1640 medium with L-glutamine	Gibco® Life Technologies, Australia
RPMI 1640 medium without Folic acid	Gibco® Life Technologies, Australia
Sodium Pyruvate (100 mM)	Gibco® Life Technologies, Australia

2.2 Western Blotting

Item	Company
Hybond™-c extra, Supported nitrocellulose membrane	GE Healthcare, UK
4x NuPAGE® LDS Sample buffer	Life Technologies, Australia
Acqua stain	Acqua science, UK
BenchMark Dual Prestain protein Ladder	Bio-Rad, USA
Biorad PowerPac 300	Bio-Rad, USA
CL-Xposure X-ray Film	Thermo Scientific, USA
NuPAGE® Antioxidant	Life Technologies, Australia
NuPAGE® MES SDS Running Buffer (20X)	Life Technologies, Australia
NuPAGE® MOPS SDS Running Buffer for large MW protein separation	Life Technologies, Australia
NuPAGE® Sample Reducing Agent (10X)	Life Technologies, Australia
Pierce SuperSignal™ West Pico Chemiluminescent substrate	Pierce, Thermo Scientific, USA
Precise protein ladder	Life Technologies, Australia
SDS-PAGE using NuPAGE® Novex® 4-12% Bis-Tris Gel	Life Technologies, Australia
Tween20	Sigma Chemical Co. USA
Whatman 3MM Paper	Whatman International, Australia

2.3 PCR reagents

Item	Company
acetylated BSA	Promega, USA
antisense primer	Invitrogen™, USA
Buffer B	Promega, USA
HinfI restriction enzyme	Promega, USA
$MgCl_2$	Invitrogen™, USA
PCR buffer	Invitrogen™, USA
Platinum® Taq DNA Polymerase	Invitrogen™, USA
QIAquick PCR Purification Kit	Qiagen, Australia
TaqMan Genotyping master mix	Applied Biosystems, USA
TaqMan® SNP genotyping Kit	Applied Biosystems, USA
water for injection	Pfizer, USA

2.4 ELISA

Item	Company
6X His tag® peptide	Abcam, USA
Peirce™ TMB substrate	Pierce, Thermo Scientific, USA
Bovine Serum Albumin (BSA)	Sigma Aldrich, USA
Gelatin from cold water fish skin	Sigma Aldrich, USA
Protein-Free (PBS) Blocking Buffer	Pierce, Thermo Scientific, USA

2.5 Primary Antibodies

Item	Company
Leaf™ Purified Mouse IgG1, k Isotype Control	Biolegend, USA
mouse monoclonal anti FRα MOv18	Enzo Life Sciences, USA
PE labelled anti mouse IgG antibody	Dako, Australia

2.6 Secondary Antibodies

Item	Company
antibody anti-mouse IgG-HRP	Rockland, USA
anti-chicken IgY HRP conjugated antibody	Santa Cruz Biotechnology, Inc. USA
anti-FRα F5753 mouse monoclonal antibody	US Biological, USA
anti-human IgG,IgM,IgG HRP	Santa Cruz Biotechnology, Inc. USA
FITC labelled anti Human IgG/IgA/IgM antibody	Rockland, USA

2.7 Blood Collection

Item	Company
5ml SST™ tube	Beckon, Dickinson Company, USA
BD Vacutainer Citrate Blood collection tube	Beckon, Dickinson Company, USA
BD Vacutainer Plastic Lithium Heparin tubes	Beckon, Dickinson Company, USA
BD Vacutainer SST 11 advance tubes	Beckon, Dickinson Company, USA
in BD Vacutainer EDTA tubes	Beckon, Dickinson Company, USA

2.8 Chicken IgY production

Item	Company
Freund's Complete Adjuvant	Sigma Aldrich, USA
H_2O for irrigation	Baxter, USA
Phosphate buffered saline pH 7.2	Gibco® Life Technologies, Australia
Pierce® Chicken IgY Purification Kit	Thermo Fisher Scientific Inc, USA

2.9 Kits

Item	Company
Anti-beta-2-Glycoprotein I IgA	Orgentec, UK
Anti-beta-2-Glycoprotein I IgG/IgM	Orgentec, UK
Anti-Cardiolipin IgG/IgM is an ELISA	Orgentec, UK
EZ1 DNA Blood 350µl Kit	Qiagen, Australia
QIAquick PCR Purification Kit	Qiagen, Australia

2.10 Laboratory Consumables

Item	Company
0.2 mL thin-walled PCR tubes	Scientific Specialities Inc, USA
15 ml Falcon tube	Falcon, BD, USA
2 mL screw-cap cryogenic vials	(Nalgene, Nunc, NY, USA)
50 ml Falcon tube	Falcon, BD, USA
Clear flat-bottom 96 well polystyrene ELISA plates	Greiner, Sigma Aldrich, USA
Ni-NTA HisSorb plate	Qiagen, Australia
U-shaped Nunc Thermo Immunol 2HB Polystyrene Plates	Greiner, Sigma Aldrich, USA
0.5ml tubes	Eppendorf, Germany
1.5ml tubes	Eppendorf, Germany
Glass Cover Slips	Menzel-Glaser, Germany
T75 Tissue Culture Flask	Sarstedt, USA
T25 Tissue Culture Flask	Sarstedt, USA

2.11 Equiptment

Item	Company
AGFA CP-100 film developer	AGFA Gaevert N.V., Belgium
Asys UVM 340 microplate reader	Biochom, UK
BD FACSCanto II Flow cytometer	BD Biosceciences Australia, Australia
BioRad mini Trans-Blot cassette and cell apparatus	Biorad, Australia
EZ1 advanced instrument	Qiagen, Australia
FluoStar Optima spectrophotometer	BMG Labtech, USA
NanoDrop 1000	Thermo Fischer Scientific Inc., USA
Verti® thermocycler	Thermo Fisher Scientific, USA
Xcell Surelock® Mini cell	Life Technologies, Australia
CO2 Incubator	Sanyo Electric Co, Japan
Haemocytometer	Hawksley, UK
pH Cube pHMeter	TPS, Australia

2.12 Computer Programs

Item	Company
Chromas Lite 2.0	Chromas, USA
Endnote XVI	ISI ResearchSoft, USA
GraphPad Prism 7	GraphPad Prism Software Inc, USA
Microsoft Office	Microsoft Corp, USA
NanoDrop 1000 software	Thermo Scientific, USA
Quantity One	Biorad, Australia

Chapter 3:
FRα ELISA Development

3 Chapter 3: FRα ELISA Development

3.1 Introduction

Autoimmunity in recurrent miscarriage has been well studied, with ~15% of all cases associated with anti-phospholipid syndrome (Bansal *et al.* 2011, Kuon *et al.* 2015). Human FRα autoantibodies were first discovered in 2004 in patients with neural tube defects, however in 2003 it was demonstrated that antibodies against the folate receptor alpha in rats caused resorption of the embryo leading to failed pregnancy (Da Costa *et al.* 2003, Rothenberg *et al.* 2004). Since these first two discoveries there have been multiple studies analysing FRα autoantibodies in patients with folate deficient diseases including neural tube defects, cerebral folate deficiency including Rett syndrome and autism, subfertility and more recently cardiovascular disease; with a majority demonstrating that the autoantibodies are capable of blocking folate transport via the FRα (Berrocal-Zaragoza M. I. *et al.* 2009, Billie *et al.* 2010, Bliek *et al.* 2006, Boyles *et al.* 2011, Cabrera *et al.* 2008, Frye *et al.* 2014, Frye *et al.* 2013, Lewandowski *et al.* 2013, Molloy Anne M. *et al.* 2009, Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004, Vo *et al.* 2015). Various methods have been used to detect FRα autoantibodies, with no standard method having been developed, nor is there currently a commercial kit available for the detection of FRα autoantibodies highlighting the need for a reliable assay that is quick, easy and effective (Sequeira *et al.* 2013).

3.1.1 Current Methods of Detection

Initial studies detected folate receptor autoantibodies using methods based on radio-immunoassay (Bliek *et al.* 2006, Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Rothenberg *et al.* 2004). Radioimmunoassay (RIA) has been used to detect both binding and blocking autoantibodies, with a slight variation to the method; patient serum is charcoal stripped to remove free folate, and either incubated with FRα bound to [H^3] folic acid for binding assays or pre-incubated with the membranes of the FRa expressing KB cell line, then [H^3] folic acid is added to the autoantibody-FRa complex to test for blocking antibodies. (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Cabrera *et al.* 2008, Ramaekers *et al.* 2005, Rothenberg *et al.* 2004). The level of radioactivity was proportional to the amount of binding autoantibodies, and inversely proportional to blocking autoantibodies (Rothenberg *et al.* 2004). This method although well-developed has many disadvantages in a routine lab, particularly for large-scale cohort analysis. Disadvantages include the risks associated with handling radioactive antigens or antibodies by laboratory staff, radioactive waste and the need for purpose built buildings and laboratories designed for radioactive work. In addition to health concerns, there are also costs for special equipment and handing procedures (Lequin 2005). Therefore, a method that does not require radioactivity would be better suited for the detection of FRα autoantibodies in large-scale cohorts.

The first group that looked at a method other than RIA was Cabrera *et al* (2008), who developed an ELISA based method to detect FRα

autoantibodies. The method developed used human FRα and bovine folate binding protein (FBP) printed onto ELISA plates to detect IgG and IgM (3). Two further studies also adopted the ELISA method to analyse both IgG and IgM antibodies against recombinant FRα, and in addition developed an ELISA method for the determination of blocking FRα autoantibodies using Folic acid-HRP (Billie *et al.* 2010, Boyles *et al.* 2011). More recent studies now employ the RIA for the detection of blocking FRα autoantibodies, while analysis is performed on an ELISA to detect binding FRα autoantibodies and their antibody classes, as it is a more rapid method of detection (Frye *et al.* 2014, Frye *et al.* 2013, Lewandowski *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2008, Sequeira *et al.* 2013, Shapira *et al.* 2015). There is significant variation between ELISA methods for FRα autoantibodies, and still no commercial standardised assay has been developed.

The current method used in the Coagulation Research laboratory at Royal Perth Hospital utilizes flow cytometry processes without the need for radioisotopes. The method makes use of a transiently transfected Cos-7 cell line via an expression vector containing the FOLR1 open reading frame, producing a cell with FRα expressed on the cell surface. Patient sera is added to the transfected cell; if FRα auto antibodies are present they bind to the FRα expressed on the cell surface. Detection of the bound autoantibodies is confirmed by the addition of FITC-labelled anti Human IgG, IgA, IgM antibodies that bind to the human autoantibody. A positive control consisting of a monoclonal antibody against FRα (MOv18, Alexis Biochemical®) is run simultaneously to quantify transfection efficiency and for

normalization of FRα autoantibodies signal strength. This method is advantageous as it facilitates the detection of autoantibodies that bind to the correct confirmation of the FRα, however it does have limitations. The method is time consuming as the cells must be transiently transfected; which translates to a 72h timeframe for the analysis for one patient sample. In addition to time factors, the method utilises large quantities of antibodies, which means the method is expensive. Therefore, a simpler more rapid test, with high sensitivity, and specificity is required, and this study has chosen to further develop an ELISA to detect FRα autoantibodies.

3.1.2 ELISA's

The ELISA, is a common serological technique utilized to detect either antibodies, or antigens within a sample, is an ideal tool for FRα autoantibody detection (Mendoza *et al.* 1999). It was first invented by two independent laboratories Peter Perlmann and Eva Engvall at Stockholm University in Sweden and by the research group of Anton Schuurs and Bauke van Weemen in The Netherlands simultaneously in 1971 (Engvall *et al.* 1971, Van Weemen *et al.* 1971). Commercialization of the process occurred in the late 70's, leading to the development of fully automated test instruments by the 1980's (Lequin 2005). There are now many variations of ELISA techniques, the most common are 'direct' analysis, 'indirect' analysis, sandwich ELISA, and competitive ELISA's (Figure *3.1*). The ELISA has a range of uses and is utilized in many different industries, from medical laboratories to quality assessment organisations. The advent of ELISA technology has enabled wide screen testing of multiple pathologies and is also used in the fields of, food safety,

environmental monitoring and even toxicological purposes (Bai *et al.* 2006, Lequin 2005, Novack *et al.* 2006)

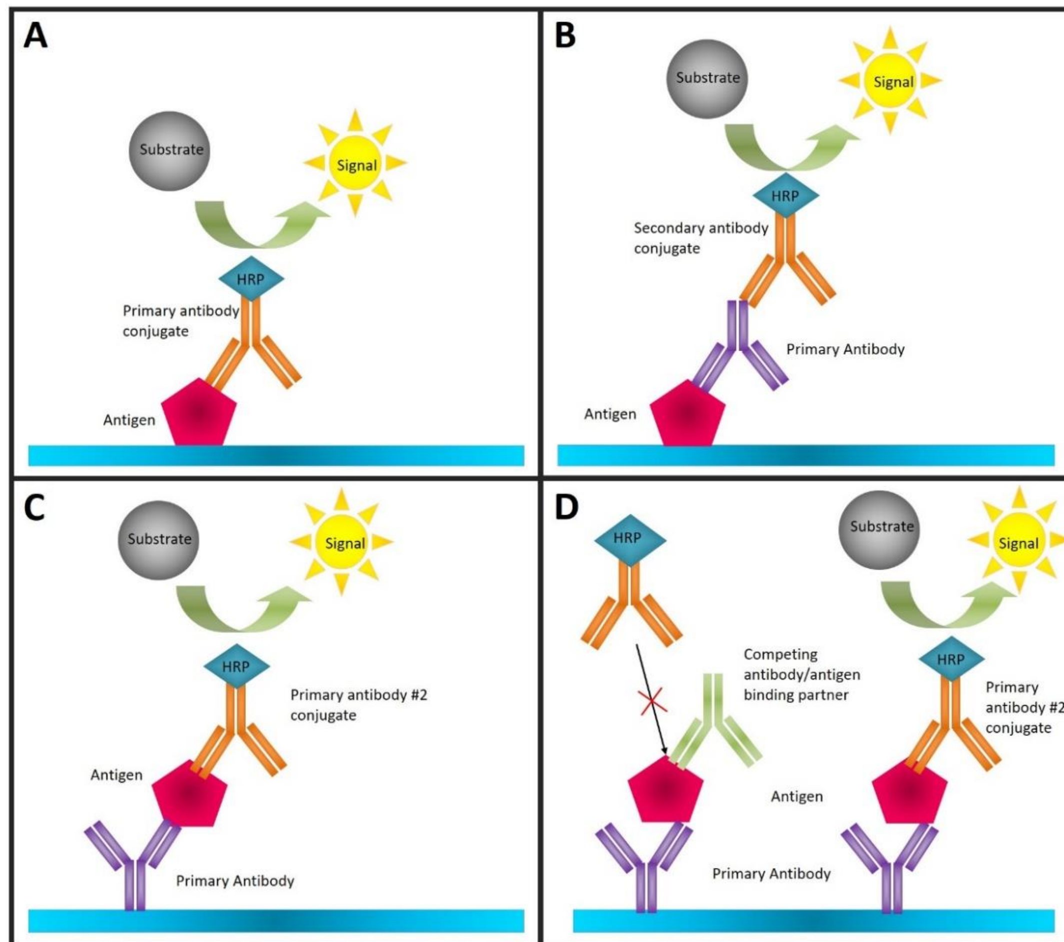


Figure 3.1 Four common ELISA techniques. a) direct ELISA, b) indirect ELISA, c) sandwich ELISA, d) competitive ELISA.

There are four main principle steps in an ELISA with slight variations depending on the analyte. These include coating with either antigen/antibody, blocking, the antibody/antigen reaction, and finally signal development and measurement. The type of ELISA chosen depends on what is required to be detected for example a direct or sandwich ELISA is used for the detection of a specific antigen within a sample, while indirect

ELISA is an assay that can be used to identify the presence of a particular antibody within a sample (Cox KL 2012 (updated 2014)). As this study aims to detect the presence of FRα autoantibodies in sera, the indirect ELISA was chosen to analyse samples. The 'Indirect' ELISA works on the basis that a known antigen is bound to the bottom of an ELISA plate (*Figure 3.2*). Test sample is then added to the ELISA plate. If specific antibodies are present within the sample, they will bind to the cognate antigen. Identification of the bound antibodies is facilitated by an enzyme linked secondary antibody that is specific to the primary bound antibody. The addition of a substrate reacts with the enzyme linked secondary antibody, generating a chromogenic change or fluorescence. A colour change or fluorescence correlates with antibodies present within the sample, however if no change is evident the sample is negative for the antibody of interest. One important use for an indirect ELISA test is the screening of antibody serum concentrations in disease state, which is demonstrated by the first screening test used worldwide for HIV (Lequin 2005)

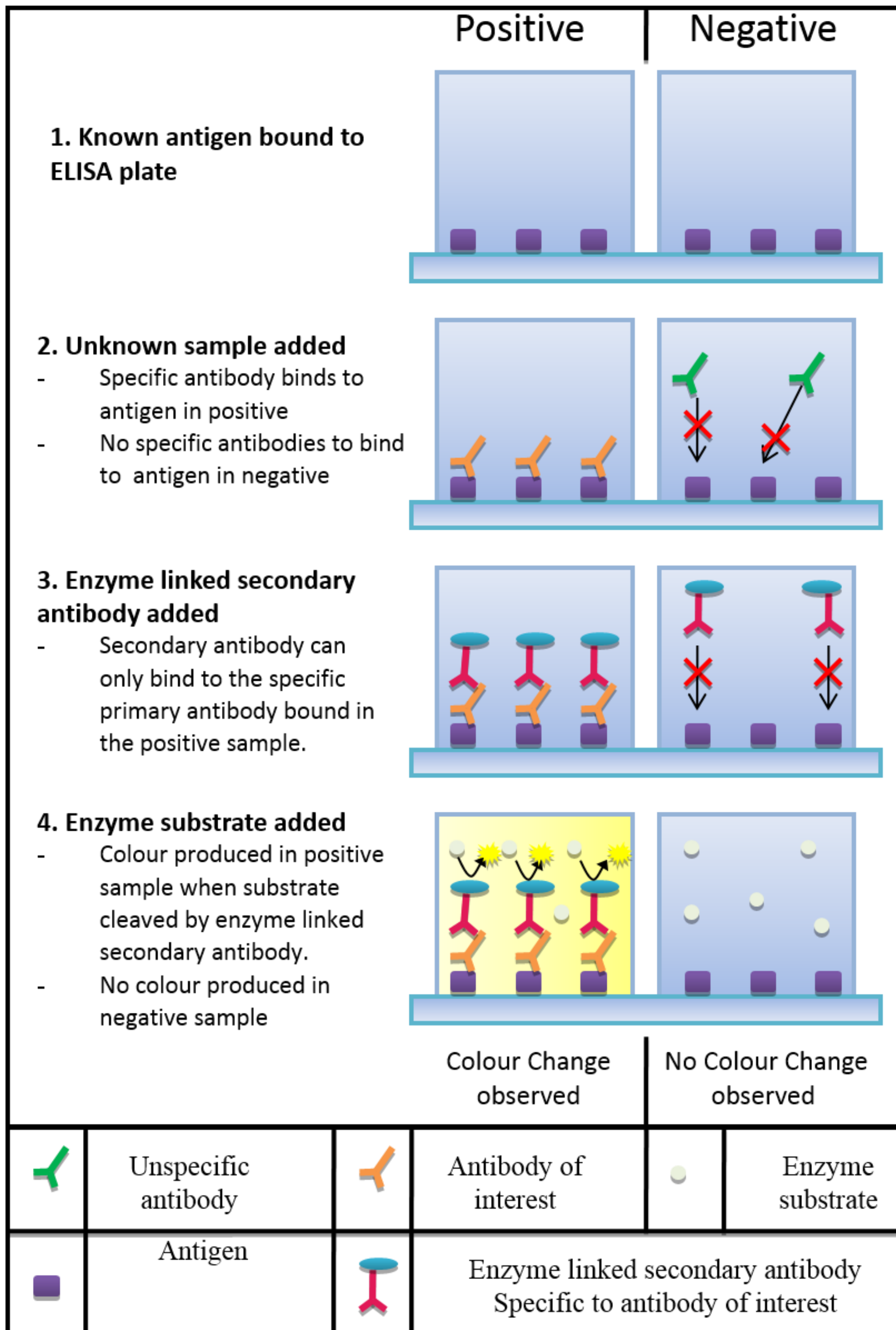


Figure 3.2 Simple depiction of 'Indirect' ELISA analysis of positive and negative samples for detection of a specific antibody of interest. The indirect ELISA test utilizes a known antigen bound to the bottom of a microplate well. An unknown sample is then added, if the specific antibody is present it will bind to the known antigen, where other antibodies present within the sera will be washed out. An enzyme linked secondary antibody is added which binds only to the primary antibody in the positive well. Addition of the enzyme substrate generates a colour change in a positive sample and no colour change in a negative sample.

3.1.3 Development and Optimisation

As described earlier there is currently no standard test or recommendations for the detection of FRα autoantibodies. However, there are general guidelines and optimization steps that can be followed for the development of a valuable ELISA assay. As the ELISA is often based on a large immune complex which has various steps that all need to work together, selection of the right antigen/antibodies are important for it to work successfully; additionally all reagents and protocols should be considered and maintained throughout the assay (Cox KL 2012 (updated 2014))

3.1.3.1 Solid Phase

The solid phase of an ELISA refers to the type of surface that is chosen for the antibody/antigen to be bound to, and can vary in material, shapes, and activation reactions all affecting the binding capacity (Biesiadecki *et al.* 2011, Cox KL 2012 (updated 2014), de Jager *et al.* 2006). The strongest binding capacity of the solid phase is not necessarily the best option for all ELISA's and therefore for each assay the solid phase needs to be selected based on the binding capacity of the protein. The most common plate that is selected for ELISA method is 96 well polystyrene plate, whereby the proteins/antibodies bind via passive absorption due to hydrophobic interactions (de Jager *et al.* 2006). Other common plates that are used are polycarbonate, polypropylene, or nylon, and may also be gamma-irradiated to increase the positive charge (Shekarchi *et al.* 1984). Speciality plates for specific protein interactions include, nickel/copper, streptavidin, protein A and G coated, and maleic-anhydride or maleimide activated

plates; however various sample matrices can react with these plates therefore should be used cautiously to avoid false positives.

3.1.3.2 Protein Coating

Coating buffers and concentration of the antigen/antibody on the plate also play a part for a successful ELISA optimisation. Coating buffers affect the binding capacity of the protein to the plate, and often the pH of these buffers play a major role. The most common coating buffer is carbonate/bicarbonate buffer at pH 9.4, while a phosphate buffered saline, might also work just as effectively. The concentration of the coating protein is often optimal between 1-10μg/ml, however too low a concentration can affect the limit of detection, also there may not be sufficient antibody-antigen binding together; whereas too high a concentration can cause unstable multi-layer formation from protein-protein interactions, saturation or nonspecific interactions within the sample matrix (Gibbs 2001). For the detection of FRα autoantibodies, there has been no one study that has demonstrated the most effective FRα antigen to use or concentration; antigens used include bovine, human placental and recombinant FRα proteins.

3.1.3.3 Blocking

An optimal blocking buffer is necessary in an ELISA protocol to eliminate any non-specific binding of components in the sample matrix, or secondary antibodies, to the plate. The blocking buffer normally contains proteins or other molecules that absorb to the free binding sites on the plate surface

after coating with antigen. A blocking buffer should not cross-react with any other factors in the ELISA such as antibodies or sample matrix. Blocking buffers are generally phosphate buffered saline (PBS) containing proteins such as bovine serum albumin (BSA), non-fat skim milk powder, fetal calf serum (FCS), fish gelatine, casein or a combination of these. There is also commercial protein free blocking buffers available which may be more suitable.

3.1.3.4 Sample Collection and Preparation

Careful consideration must be given when selecting the sample type and sample collection parameters. There are several different sample types that may be tested for autoantibodies in an immunoassay including serum, citrate and EDTA plasma samples; each with different components that can affect the overall functionality of the ELISA. Serum and plasma, are complex substances with high capacity for cross reaction or matrix interference; therefore, an appropriate sample buffer is required to dilute the sample to reduce non-specific binding or cross-reactivity, increase linearity and optimise sensitivity. Generally, the sample buffer, like the blocking buffer, is a mix of PBS supplemented with a competitor protein, but at a lower concentration. Appropriate storage of serum/plasma samples at 2-8°C for no longer than 24 hour and -20° or colder for long term storage is critical for accurate detection of autoantibodies. If incorrectly stored antibody activity can decrease or non-specific binding can occur (Sequeira *et al.* 2013). Heat inactivation of samples, which is often performed, should also be avoided due to inconsistences of antibody levels and false positives (Lopez *et al.* 1998).

3.1.3.5 Wash Steps

Wash steps in an ELISA are required to remove unbound and unwanted components, reducing the background without affecting the key reaction of the ELISA, increasing the efficiency and reproducibility of the assay. There are several important factors in a successful wash step, which include the buffer used (especially the pH of the buffer), incubation time, temperature and the number of wash steps performed. The most common buffer used for this is phosphate buffered saline (PBS), however often it has the surfactant Tween 20 added to improve removal of non-specifically bound material.

3.1.3.6 Temperature and Time

Incubation times and temperatures for coating, blocking, sample addition, wash times and colour development all play an important role in the overall result of the ELISA. Coating generally occurs at 4°C overnight or 37°C for one-four hours. At higher temperatures with shorter incubation times, coating variation between wells can increase. Other incubation steps including blocking, primary and secondary antibody application are performed between 1-2 hours at room temperature or 37°C. Evaporation can cause variation at all steps of the ELISA especially in edge wells increasing solute concentrations, reducing binding capability or denaturing proteins; therefore, plates should always be sealed to reduce this error.

3.1.4 Validation

Finally, there are several critical steps to ensure you get the most accurate and reproducible result in detecting the level of an analyte in a sample in

this case the FRα autoantibody. The inter-assay and intra-assay coefficient of variation (CV) should always be determined, as it demonstrates the level or accuracy and reproducibility of the assay. The CV should not be greater than 10% when run on automated equipment and 15% for a manual ELISA; an antibody concentration that has a CV >20% is considered the limit of detection for the ELISA.

The aim of this chapter is to develop and validate an ELISA specific for the detection of FRα autoantibodies in patient serum as it would have many advantages over previous methods used for analysing FRα autoantibodies including RIA (Lequin 2005). It is highly sensitive and highly specific in detecting both antibodies and antigens and is often referred to as the 'gold standard' in a diagnostic setting (Novack *et al.* 2006). ELISAs are both rapid and allow for screening of multiple samples on a single test plate simultaneously (Mendoza *et al.* 1999). The advantage of producing an ELISA test over RIA is there is no need for the use of radioisotopes, therefore the method is safer for general laboratory use (Lequin 2005). Other advantages include the reduction of consumables, as micro ELISA plates can be used at the same sensitivity as other techniques; for example flow cytometry uses a significant amount of secondary antibodies for analysis of one sample (Bai *et al.* 2006). Ultimately the ELISA methodology will reduce time, costs and hazard, allowing a sensitive and specific test which will allow for rapid screening of case mothers and controls affected with NTD's or multiple miscarriages.

3.2 Methods

3.2.1 General Methods

3.2.1.1 Tissue Culture

All mammalian cell culture was performed in a class II, Laminar flow hood, with aseptic technique performed throughout all methods. All cell lines used are listed in Table 3.1.

Table 3.1 Eukaryotic Cell Lines

Cell type	***Origin***	***Information***	***ATCC N°***
Cos-7	African Green Monkey Kidney	Cell line Negative for FRα	CRL-1651
Cos-7 FRa	FRa Stably transfected African Green Monkey Kidney	Cell line altered to express the FRa	
KB	Human, Nasopharyngeal Carcinoma	A carcinoma cell line which overexpresses the FRα to increase folate uptake within the cell	CCL-17

The Cos-7, Cos-7 FRα and KB cell lines cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®) media and Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco®, Life Technologies Australia Pty Ltd, Australia), respectively, containing 10% heat inactivated Fetal Bovine Serum (HyClone, GE Healthcare, Australia), 1mM sodium

pyruvate (Gibco®, Life Technologies Australia Pty Ltd, Australia), 10µg/ml Penicillin-Streptomycin (Gibco®, Life Technologies Australia Pty Ltd, Australia), and 1x non-essential amino acids (Gibco®, Life Technologies Australia Pty Ltd, Australia). Cells were incubated at 37°C in a 5% CO_2 atmosphere and passaged at 80% confluence.

3.2.1.1.1 Passaging

When cell cultures reached 80-90% confluence the media was removed and washed with 1 x PBS buffer which was then aspirated. 1ml of 0.05% trypsin/EDTA (Gibco®, Life Technologies Australia Pty Ltd, Australia) was added to the flask and incubated for at 37°C 3min, or until cells became non-adherent. Cell culture medium was added to deactivate the trypsin. Cells were centrifuged at 290 x g for 5 mins at 20°C to pellet and the trypsin removed. Fresh cell culture medium was added for seeding at either 1:5 or 1:10 dilutions. Cells were incubated at 37° C with 5% CO_2 until required.

3.2.1.1.2 Cryopreservation

Media was aspirated and the cells washed with 1 x PBS buffer. 1ml of 0.05% trypsin/EDTA (Gibco®, Life Technologies Australia Pty Ltd, Australia) was then added to the flask and incubated at 37°C for 3 minutes, or until cells became non-adherent. Cell culture medium was added to deactivate trypsin. Cells were pelleted by centrifugation at 290 x g for 5 mins at 20°C and re-suspended in 750 µL of 20% FCS in DMEM (Gibco®)/750 µL of 20% dimethyl sulphoxide (DMSO; Sigma, MO, USA) before being transferred to

pre-labelled 2 mL screw-cap cryogenic vials (Nalgene, Nunc, NY, USA) and cooled gradually to -80°C.

Cells to be revived were removed from -80°C and quickly thawed at room temperature and immediately added to 10ml of fresh culture medium depending on the cell line. Cells were incubated at 37°C at 5% CO_2 for several hours to allow for adhesion and then the media was changed to remove DMSO present.

3.2.1.2 SDS Page Gel Electrophoresis

All proteins were separated on a denaturing SDS-PAGE using NuPAGE® Novex® 4-12% Bis-Tris Gel (Life Technologies Australia Pty Ltd, Australia). Identification of protein molecular weight was confirmed using protein ladders (Precise protein ladder; Life Technologies Australia Pty Ltd, Australia; or BenchMark Dual Prestain protein Ladder; Biorad, Australia). A small aliquot of each sample was mixed with an equal volume of 4x NuPAGE® LDS Sample buffer (Life Technologies Australia Pty Ltd, Australia), and incubated for 10 min at 70ºC. Volumes of 5µL-20 µL depending on protein concentration were loaded onto the gel with either NuPAGE® MOPS SDS Running Buffer for large MW protein separation (Life Technologies Australia Pty Ltd, Australia), or NuPAGE® MES SDS Running Buffer (20X)(Life Technologies Australia Pty Ltd, Australia). Electrophoresis was performed at 200V for 45 minutes using Xcell Surelock® Mini cell (Life Technologies Australia Pty Ltd, Australia). If a reducing gel was required, the protein sample was mixed with both 4x NuPAGE® LDS Sample buffer (Life Technologies Australia

Pty Ltd, Australia) and NuPAGE® Sample Reducing Agent (10X) (Life Technologies Australia Pty Ltd, Australia) prior to heating at 70°C for 10 minutes; and then run in running buffer with 500ul of NuPAGE® Antioxidant (Life Technologies Australia Pty Ltd, Australia) added to the inner tank.

3.2.1.3 Coomassie Blue Staining

SDS page gels were immersed in 15mls of Acqua stain (Acqau science, UK) and incubated at room temperature, shaking for 15 minutes. Acqua stain (Acqau science, UK) was removed, and the gel was washed with 1 x PBS.

3.2.1.4 Western Blot

3.2.1.4.1 Western Transfer

After electrophoresis, the SDS page gel was removed from the plastic cassette and loaded into a BioRad mini Trans-Blot cassette and cell apparatus (Biorad, Australia) with nitrocellulose membrane in ice cold transfer buffer as per manufacturer's protocol. Proteins were transferred over night at 35V in a cool room.

3.2.1.4.2 Immunoblotting

Following overnight transfer the membrane was removed from the transfer cassette and cut to size and incubated in Tris buffered saline containing 0.05% Tween20 (TBST)/3% skim milk powder for 1 hour. All immunoblotting was carried out at room temperature and on an orbital shaker. The membrane was transferred into primary antibody diluted in TBST/1% skim milk powder for 1 hour. Membranes were then washed 3x in TBST for 10mins, incubated in

secondary antibody diluted in TBST/1% skim milk powder for 1 hour, and further washed 3 x in TBST for 10mins. Membranes were then incubated in enhanced chemiluminescence (ECL) Pierce SuperSignal™ West Pico Chemiluminescent substrate (Pierce, Thermo Scientific, USA) for 5 minutes, excess ECL was removed and the membrane was exposed to X-ray film. X-ray film was developed using an AGFA CP-100 film developer.

3.2.2 ELISA Optimisation

All optimisation and final ELISA protocols were performed with the commercial recombinant FRα protein (Sino Biological, China).

3.2.2.1 Nickel Coated ELISA Plate

A nickel coated Ni-NTA HisSorb plate (Qiagen, Australia) was the first plate to be utilised in the optimization of an ELISA to detect FRα autoantibodies. The general protocol from Qiagen for the Ni-NTA HisSorb plates was used for all optimizations with these plates, which is as follows. Preparation of protein, either recombinant FRα or 6xHIS peptide (control)(Abcam, USA) was prepared at various concentrations in a PBS/BSA buffer. 200µl of the diluted protein solution was added to each well. A negative control of 200ul PBS/BSA only with no protein was also added to additional wells. Protein was allowed to bind for 1 hour at room temperature, with shaking. The solution was then flicked off and washed 4 times for 30 seconds with 300µl of PBST and dried thoroughly between each step. Primary antibody consisting of either anti-FRα antibody or patient serum samples are diluted in PBS/BSA at various concentrations and 200µl are added to wells in triplicate for both FRα

coated and negative control wells; then incubated for 1 hour at room temperature while shaking. After 1 hour, the plates were washed 4 times with 300µl of PBST for 30 seconds each wash step, then dried thoroughly by blotting. 200ul of specific HRP conjugated secondary antibody diluted in PBS/BSA, was then added to the each well and incubated for a further 1 hour at room temperature, while shaking. The wells were then washed again with 300µl of PBST four times for 30 seconds and dried thoroughly, before 100µl of TMB substrate (Peirce, Thermo Scientific, USA) was added to each well an incubated for 5 minutes or until a blue colour reaction was seen. Finally, 100µl of 2M H_2SO_4 was added to each well to stop the colourmetric reaction, and the plate was read at 450nm using the Asys UVM 340 microplate reader (Biochom, UK). Further optimization, using the nickel coated plates, was the addition of a blocking step after the binding of the antigen to the plates. In this step 300µl of the chosen blocking buffer was added to each well and incubated for 1 hour at room temperature, while shaking; and there was no wash step performed between the blocking step and the addition of the primary antibody, the blocking solution was simply flicked off and dried thoroughly.

3.2.2.2 Basic ELISA Protocol

For development of the ELISA with both clear U-shaped Nunc Thermo Immunol 2HB Polystyrene Plates and Clear flat-bottom 96 well polystyrene ELISA plates (Greiner, Sigma Aldrich), an ELISA protocol was adapted from the ELISA technical guide and protocols Tech Tip # 65 (Thermo Scientific) and an in-house ELISA that is used to detect ADAMTS13 autoantibodies. All incubation and wash steps remained the same for all optimizations as

follows. Firstly, antigen was diluted in 0.2M sodium carbonate/bicarbonate coating buffer (pH 9.4) at the desired concentration and 100µl was added to each test well, with negative control wells of coating buffer only, included for each ELISA optimization. The coating antigen was incubated at 4°C overnight. The following day the plates were washed three times with 250µl of the selected wash buffer for 5 minutes with thorough drying between each wash step; this was repeated after primary and secondary antibody incubation. After the first wash step, 250µl of the chosen blocking buffer was then added to each well and incubated for one and a half hours at room temperature while shaking. Blocking buffer was removed and 100µl of primary antibody was added per well, incubated at room temperature for one hour with shaking, and then washed. Serum samples or monoclonal anti-FRα control antibodies were diluted in the chosen dilution buffer and 100µl/well was loaded onto the plate, followed by one hour incubation at room temperature. Primary antibodies were detected by the addition of 100µl/well of corresponding secondary antibody labelled with horse radish peroxidase (HRP) in selected dilution buffer, and further incubated for one hour at room temperature. After the final wash step, 100µl of TMB substrate (Pierce, Thermo Scientific, USA) was added per well and developed while shaking for the desired time; then 100µl/well of 2M H_2SO_4 was added to stop the enzymatic reaction and any further colour development. Intensity of the colour produced was measured using Asys UVM 340 microplate reader (Biochom, UK) and for each sample and condition and the FRα positive wells were compared to the FRα negative wells. The basic ELISA protocol as described above was used for each optimization, however concentrations of antigen, primary and secondary antibodies in addition the composition of

buffers was varied throughout the optimization and will be described in the results section.

3.2.2.3 Validation

Inter-assay, intra-assay, limit of blank and limit of detection analysis was performed for the final optimized anti-FRα autoantibody ELISA. The overall protocol for the ELISA was as followed in section 3.3.5. For the intra-assay coefficient four samples were selected a high positive (FR3), a moderate positive (B2), a weak positive sample (S41), and a negative sample (FR0) for FRα autoantibodies. Five replicates of each serum sample were plated in duplicate on a single FRα-coated ELISA plate. Both limit of blank and limit of detection were calculated with these results. Inter-assay variability was determined using data obtained from the 3 controls FR3, S41, and FR0. Ten individual plates were run separately; each with 2 duplicates of each sample diluted twice, with a total of four wells for each sample. Calculations performed for validation analysis are demonstrated below.

<u>Inter-assay</u>

$$\text{Covariant of variation each sample} = \left(\frac{\text{Standard deviation of mean of all plates}}{\text{Mean of all plates}} \right) \text{X } 100$$

Overall Inter-assay variation = Average of each Covariant of variation

<u>Intra-Assay</u>

$$\text{Covariant of variation each sample} = \left(\frac{\text{Standard deviation of the mean of all sample replicates}}{\text{Mean of all sample replicates}} \right) \text{X } 100$$

Overall inter-assay variation = Average of each covariant of variation

Limit of Blank

$LoB = Mean_{(negative)} + 1.645\ (SD_{negative})$

Limit of Detection

$LoD = LoB + 1.645\ (SD_{weak\ positive})$

3.2.2.4 Standardisation

To reduce plate to plate variation, the FRα autoantibody ELISA was standardised against a known positive and a known negative sample. For each ELISA, the unknown samples were indexed against FR3 control, and FR0 control analysed in triplicate on each plate. This was performed to standardise the absorbance reading variation between each plate. To calculate the index the formula below was used:

$$\frac{OD_x - OD_n}{OD_p - OD_n} \times 100$$

Where OD_x is the optical density of unknown serum sample, OD_n is the optical density of the negative control, and OD_p is the optical density of high positive control.

3.2.2.5 Folate Receptor alpha Autoantibody Positivity

To determine if a sample was positive, a selection of normal serum samples were collected and analysed on the FRα autoantibody ELISA, as per section 3.3.5. Each sample was assayed in triplicate, at a 1:50 dilution. For each ELISA plate the positive FR3 and the negative FR0 sample was run, to standardise the ELISA plate. The positive and negative control FR3 and FR0 respectively was identified using the flow cytometry method outlined in section 3.2.2.6. The positive control was identified by a significant right shift (a distinct population) for FRα positive Cos-7 cells stained with FITC when analysed against FRα negative Cos-7 cells, while negative control was identified when no FITC signal shift was observed.

3.2.2.5.1 Sample Collection

Ethics was approved by Royal Perth Hospital for the collection of a population of normal controls for the determination of a baseline for the FRα autoantibody ELISA. A call for volunteers was advertised in the Haematology Department at Royal Perth Hospital with the aim to collect at least 30 volunteers. Each volunteer was allocated a number for sample de-identification. One 5ml SST™ (Beckon, Dickinson Company, Australia) tube was collected via a trained phlebotomist and samples were sent to the laboratory for analysis.

3.2.2.5.2 Sample Preparation

Each SST™ tube was allowed to sit for 30 minutes before spinning; however, they were spun within 2 hour of collection. The SST tube was spun at 1100xg

for 15 min at room temperature, and the serum collected. Serum was immediately frozen at -80°C until required.

3.2.2.5.3 Statistics

Baseline samples were indexed as described in section 3.2.2.4 and the overall mean and standard deviation for all normal population samples was calculated. A low positive sample was classified as a value between two and five standard deviations above the mean, while a high positive sample was classified as a value greater than 5 standard deviations above the mean.

3.2.2.6 Flow Cytometry Detection of FRα Autoantibodies

The presence of FRα autoantibodies in serum samples were measured using a flow cytometry method that was previously developed in our laboratory (Coagulation Research, Royal Perth Hospital, Perth, WA), with the use with the stably transfected cell line Cos-7 FRα. T25 flasks either containing confluent Cos-7 FRα cells or Cos-7 cells were washed with 4 ml of 1 x PBS, aspirated and 3 ml of chilled flow buffer (pH 7.2) was added to each culture flask. Using a 10 ml pipette cells were removed from the surface of the cell culture flask via vigorous pipetting until liberated and then transferred to a 50 ml Falcon tube (Falcon, BD, USA). A cell count was performed and cell numbers adjusted to 1.9 x 10^6 cells/ml with chilled flow buffer. 1ml of each transfected and non-transfected cell culture was transferred to pre-labelled 1.5 ml Eppendorf® tubes and incubated at 4°C rotating for 1 hour. Samples were centrifuged at 16,000 x g for 1 minute and the supernatant discarded. The pellet was washed twice in 400 µL of chilled flow buffer, centrifuged and

the supernatant removed. The pellet was then resuspended in 100 µL of primary antibody mix (1:3 dilution of patient serum, 1:333 dilution of FRα F5753 antibody (US Biological, USA), in chilled flow buffer. Samples were incubated at 4° C for 45mins, and the 2x 400 µL flow buffer wash steps repeated. Pellets were then resuspended in 100 µL of secondary antibody mix (1:50 PE labelled anti mouse IgG antibody (Dako, Australia); 1:100 FITC labelled anti Human IgG/IgA/IgM antibody (Rockland, USA), in chilled Flow Buffer) and incubated in the dark for 45 mins at 4° C. Cells were washed again 2x 400 µL of chilled flow buffer, resuspended in 450 µL of chilled flow buffer and transferred to flow tubes. Expression levels of the FRα were measured using a BD FACSCanto II Flow cytometer at an excitation of 488nM and emission detected at 530/30nM and 585/42nM for fluorescenin isothiocyanate (FITC) and phycoerythrin (PE), respectively.

3.3 Results

A significant amount of optimization was required for development of an ELISA to detect FRα autoantibodies in serum samples due to the large amount of cross reactivity that was initially observed. Initial testing for FRα autoantibodies was based off protocols described for specific plates being used and an in-house ELISA utilized to detectADAMTS13 autoantibodies. A positive serum sample FR3 and a negative serum sample FR0 was determined using flow cytometry (results not shown) and utilized in all further optimization protocols for ELISA anti-FRα development.

3.3.1 Commercial Recombinant FRα Antigen

A recombinant FRα protein was purchased from Sino-Biological (China) which is expressed as a soluble form in the cell line CHO-K1 (Chinese Hamster Ovary). This recombinant form of the FRα is 220 amino acids compared to the 257aa of the native FRα, as the pro-peptide and signal peptide have been removed, with the addition of a poly-histidine tag on the c-terminus. Confirmation of a pure protein was performed on SDS-PAGE gel and Coomassie blue staining, which demonstrated the protein running at the correct weight of 37kDa. A western blot was also performed and confirmed antigenicity of the recombinant FRα with two different monoclonal anti-FRα antibodies, mouse monoclonal anti FRα MOv18 (ENZO Life Sciences, USA) at 1:5,000 dilution and F5753 (US Biological, USA) at 1:50,000 dilution. The presence of the poly histidine tag was confirmed) by a western blot, using the 'The' His anti-HIS antibody (Genscript, USA) at a 1:10,000 dilution (Results not shown).

3.3.2 FRα antigen Concentration

The first step in the development of the FRα autoantibody ELISA was determinating the optimal concentration of FRα antigen on the ELISA plate. Initial testing was performed with six concentrations of recombinant FRα protein between 1-10μg/ml and detected by the monoclonal mouse anti-FRα antibody MOv18 (Enzo Life Sciences, USA) at a set concentration of 1:2000 with the secondary antibody anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc. USA) being tested at 1:50,000, 1:100,000 and 1:200,000. Figure 3.3 shows that above concentrations of 5μg/ml of FRα absorbance levels plateau for all three concentrations of secondary antibody, this demonstrated that the secondary antibody dilution was the limiting factor in the analysis, and that the concentration of 5μg/ml of recombinant FRα bound to the plate represented the optimal concentration. After the final ELISA protocol was optimised to detect FRα autoantibodies another optimization of FRα concentration was carried out using the known positive anti-FRα, sera FR3, a known negative, sera FR0, and MOv18; at 1, 2.5, 5, 7.5, 10ug/ml, to confirm that the 5μg/ml was the correct concentration to use for the ELISA. As shown in Figure 3.4 5μg/ml of recombinant FRα was the correct concentration to use for a 1:50 dilution sera dilution with a 1:30,000 anti-human IgG,IgM,IgG HRP (Rockland, USA) antibody dilution.

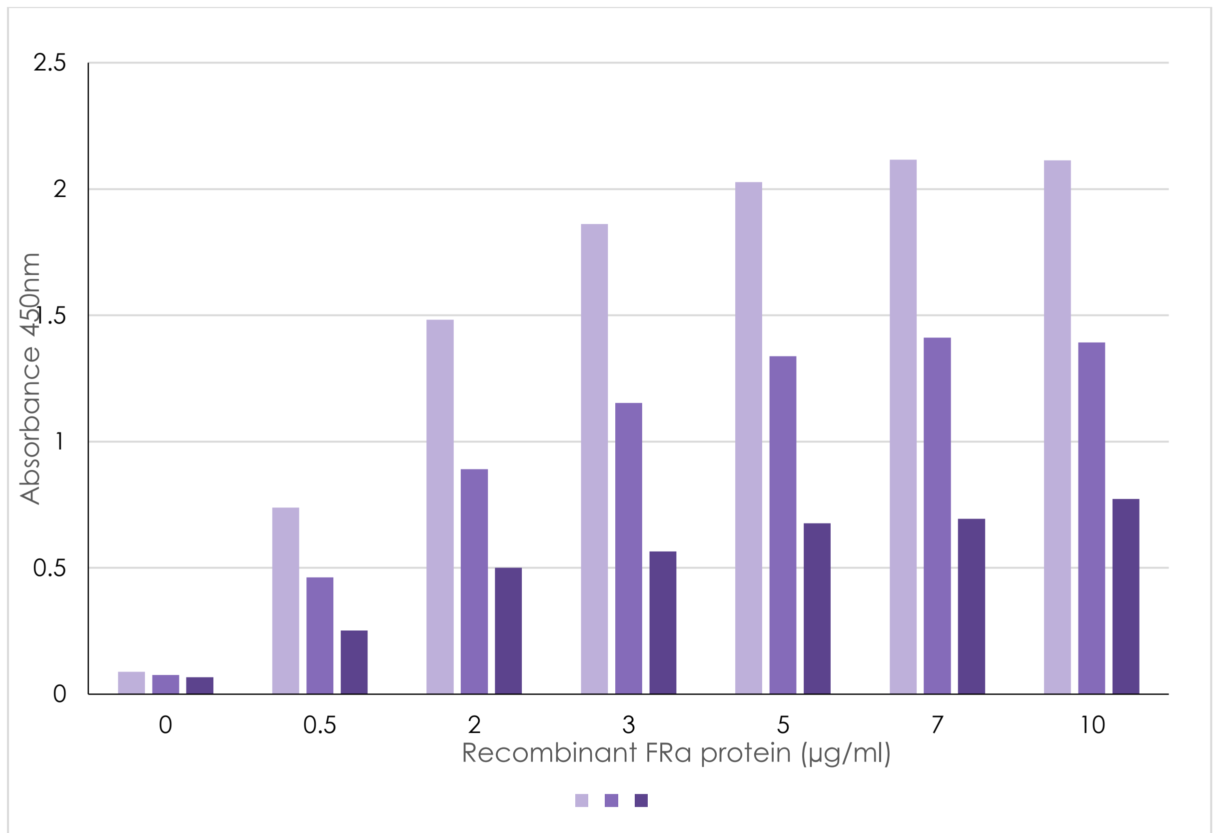


Figure 3.3 Determination of optimal concentration of recombinant FRα protein using monoclonal antibody MOv18 with basic ELISA protocol (section 3.2.2.2). Seven concentrations of recombinant FRα protein were coated in a total volume of 100ul 0.2M Sodium carbonate/bicarbonate coating buffer on clear U-shaped Nunc Thermo Immunol 2HB Polystyrene plates overnight. Primary antibody anti-FRα MOv18 at a concentration of 1:2000, followed by the secondary antibody, anti-mouse IgG HRP at three concentrations 1:50,000, 1:100,000, 1:200,000.

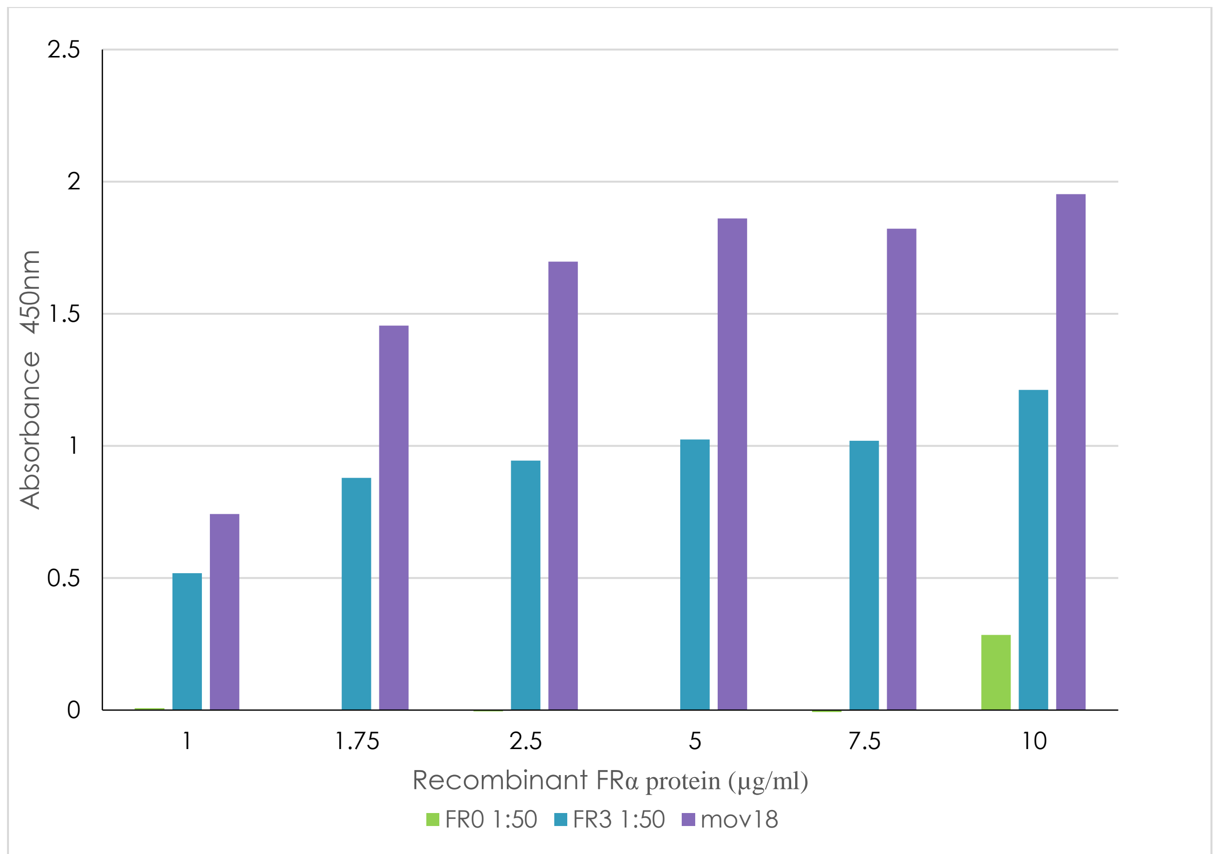


Figure 3.4 Determination of optimal concentration of recombinant FRα protein using a known positive and negative human serum, and the monoclonal antibody MOv18 with the optimised ELISA protocol (section 3.3.5). Six concentrations of recombinant FRα protein was coated in a total volume of 100ul 0.2M Sodium carbonate/bicarbonate coating buffer on clear flat-bottom 96 well polystyrene ELISA plates (Greiner, Sigma Aldrich) overnight. Primary antibody concentrations were 1:2000 for anti-FRα MOv18 and a 1:50 serum dilution for both FR3 and FR0. Primary antibodies were detected by either a 1:15,000 anti-mouse IgG HRP (Santa Cruz Biotechnology, Inc. USA) or a 1:30,000 dilution for anti-Human IgG/M/A HRP antibody (Rockland, USA).

3.3.3 Plate Selection

3.3.3.1 Nickel Coated

Initial testing for the FRα autoantibody ELISA was performed on a nickel based plate Ni-NTA HisSorb plate (Qiagen, Australia) that binds Histindine (His) tagged proteins specifically. This plate was utilized as the recombinant FRα protein (Sino-biological) contains a poly-histidine tag on the C-terminal end of the protein. Preliminary testing demonstrated in Figure 3.5 was performed using FRα at 2µg/ml, with 1:50, 1:100, 1:200 dilutions of both FR3 and FR0 serum samples. In addition, a 6x His peptide was used as a negative control to test for background. High absorbance levels were demonstrated in both serum samples at the lowest concentration of 1:200, with FR0 having nearly a two-fold absorbance value over FR3. High background levels were displayed in wells where there was no FRα present and moderate absorbance in wells that contained 6xHis peptide only. There was no signal observed in no serum controls, ruling out cross-reactivity of the secondary anti-human IgG, IgM, IgA with the plate or other buffers. This first trial for detecting FRα autoantibodies failed, demonstrating high cross-reactivity of human sera with the Ni-NTA His-Sorb (Qiagen, Australia) plate.

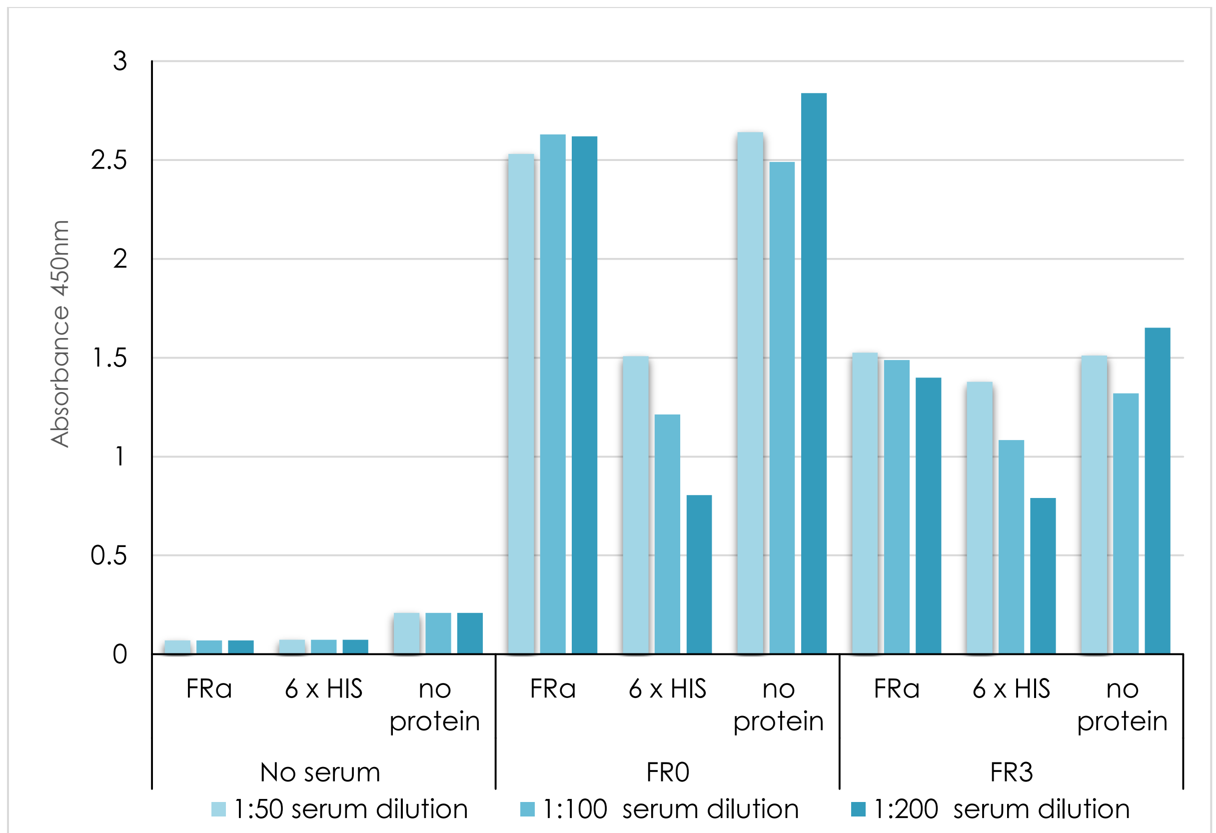


Figure 3.5 Anti-FRα ELISA utilising Ni-NTA HisSorb plates and a positive and negative anti-FRα human serum. Three different coating substrates were used, the FRα recombinant protein and recombinant 6xHIS peptide at 2µg/ml protein and a no protein control. Testing FR3 (positive) serum, FR0 (negative) serum at a 1:200 dilution and no serum at 1:50, 1:100, and 1:200 dilution. Anti-FRα antibodies were detected with a 1:30,000 dilution of anti-Human IgG/M/A HRP labelled secondary antibody.

Ni-NTA His-Sorb (Qiagen, Australia) plates are purchased pre-blocked, however due to the large amount of background and cross-reactivity it was proposed that an additional 1 hour blocking step after FRα binding might be required to reduced non-specific binding. Several different blocking buffers were tested including various concentrations of 6 x His peptide, 1, 3, 5% BSA, 3% non-fat dry skim milk powder in 1x PBS, and a pre-formulated Protein-Free (PBS) Blocking Buffer (Peirce, Thermo-scientific, USA); none of the blocking

buffers tested reduced the non-specific binding (results not shown). In addition to testing different blocking methods, PBS was tested as an alternative wash buffer in replace of PBST, however did not reduce serum-plate cross reactivity.

3.3.3.2 Nunc Thermo Immunol 2HB Polystyrene Plates

As the nickel plates were highly non-specific, further development of the FRα autoantibody ELISA plate was performed on clear U-shaped Nunc Thermo Immunol 2HB Polystyrene Plates; which have a high binding affinity for proteins. Various optimizations were performed utilizing these plates as demonstrated in Table 3.2 which investigated different blocking buffers, different concentrations of serum and secondary anti-human IgG, IgM, IgA HRP (Rockland, USA) antibody levels. Factors that were maintained over all assays were FRα concentration of 5μg/ml, wash buffer 1x PBST, and incubation times and temperatures. Results demonstrated that all blocking buffers used except the Protein-Free (PBS) Blocking Buffer (Peirce, Thermo-scientific, USA) and skim milk powder had either a moderate or low signal to noise ratio for seras FR3 and FR0, with negative FR0 serum reacting with both the non-coated and FRα coated plate. Blocking with skim milk demonstrated low signal to noise ratio for the positive FR3 sample, however no signal was detected for the negative FR0 serum on coated and non-coated wells. There was no signal was seen with Protein-Free (PBS) Blocking Buffer (Peirce, Thermo-scientific, USA) even for positive FR3 serum control in FRα coated wells, while the monoclonal anti-FRα antibody confirmed that FRα was present on the plate. The negative control which had no patient serum

present was in fact negative in all assays using the Nunc Thermo Immunol 2HB Polystyrene plates; suggesting that the presence of patient serum either cross reacted with the blocking buffers used or with the plates due to inherent failure of the blocking buffers.

Table 3.2 Optimisation of blocking and dilution buffers with the Nunc Thermo Immunol 2HB ELISA plates (Greiner, Sigma Aldrich, USA).

blocking buffer	***Concentrations***	***Antibody dilution buffer***	***Positive Serum Result***	***Negative serum***
Skim milk powder PBS	3%	1% Skim milk in PBS	High Background	No signal
Protein-Free (PBS) Blocking Buffer (Peirce, Thermo-scientific)	neat	Protein-Free (PBS) Blocking Buffer (Peirce, Thermo-scientific)	No signal	No signal
BSA in PBS	1%	1% BSA	High Background	High Background and Fra binding
	3%	1% BSA	High Background	High Background and FRα binding
	5%	1% BSA	High Background	High Background and FRα binding
Fish gelatine in PBS	1%	1% fish gelatine	low signal to noise ratio	low signal to noise ratio
	3%	1% fish gelatine	low signal to noise ratio	low signal to noise ratio
Casein in PBS	0.20%	Casein 0.2%	low signal	low signal
	0.30%		low signal	low signal
	0.40%		low signal to noise ratio	low signal to noise ratio
	0.50%		low signal to noise ratio	low signal to noise ratio

3.3.3.3 96 well polystyrene ELISA plates

Clear flat-bottom 96 well polystyrene ELISA plates (Greiner, Sigma Aldrich, USA) were further tested, to determine if there was an increase in the signal to noise ratio that was observed in the Nunc Thermo Immunol 2HB Polystyrene Plates (Greiner, Sigma Aldrich, USA). The first trial that was performed utilizing these plates tested 3% Fish gelatine (Sigma Aldrich, USA) in PBS as the blocking buffer with 1% dilution buffer (serum dilution 1:25 with 1:100,000 anti-human IgG, IgM, IgA HRP antibody) to see if there was still a low signal to noise ratio compared to the Nunc Thermo Immunol 2HB Polystyrene Plates. This assay also demonstrated a slightly increase signal to noise ratio for FR3 and FR0 however the overall signal on this plate was lower compared to the high binding plates (results not shown).

The next blocking buffer tested was fetal calf serum (HyClone, GE Healthcare, Australia) at a concentration of 10%. FR3 and FR0 was used at dilution of 1:25, 1:50 1:100 with anti-human IgG, IgA, IgM at a dilution of 1:15,000, 1: 30,000, 1:50,000, however unlike the previous ELISA assays PBS was used as the wash buffer. Figure 3.6 demonstrates that there was minimal background for both FR3 and FR0, with a strong signal for FR3 in wells containing FRα; however there was still also a strong absorbance level detected for FR0 in wells containing the FRα protein, which was expected to be negative, based on the flow cytometry results.

A further test that yielded a positive result was performed using 3% skim milk powder/PBS as the blocking buffer; while the dilution of the primary and

secondary antibodies remained in 10% FCS/PBS. Antibody dilutions were 1:10 serum/1:100,000 2°Ab, 1:25 with 1:50,000 or 1:100,000 2°Ab, or 1:50 with 1:15,000, 1:30,000 and 1:50,000 2°Ab; which was anti-human IgG IgM IgA-HRP. A high signal to noise ratio was seen for FR3 (2.356/.0431) in the 1:50 serum/1:30,000 2°Ab dilution, which also demonstrated a low FR0 absorbance (Figure 3.7). This was repeated to confirm that further assays obtained the same results, and it was indeed blocking with 3% skim milk powder/PBS with the antibody dilution in 10% FCS/PBS that gave the highest signal to noise ratio. Different variations in blocking and antibody dilution was performed with the skim milk powder and FCS as demonstrated in Figure 3.8, which supports the first assay in the blocking with 3% skim milk powder/PBS, and the serum/2°Ab dilution in 10% FCS/PBS reduced background absorbance, and FR0 was not positive for FRα binding.

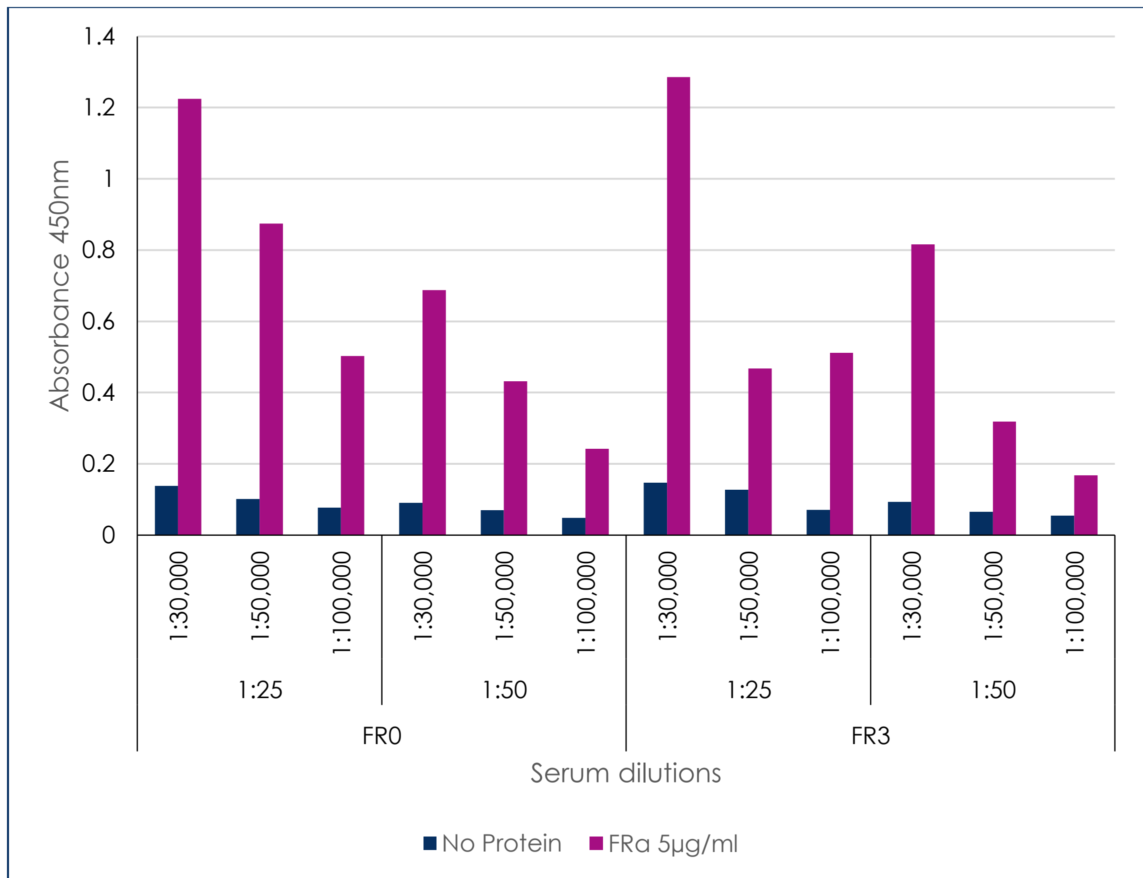


Figure 3.6 Anti-FRα ELISA oprimisation utilising clear flat-bottom 96 well polystyrene ELISA plates with 10% FCS as blocking buffer (Greiner, Sigma Aldrich). Plates coated with 5µg/ml recombinant FRα. Blocked with 10% FCS/PBS. Positive anti-FRα serum FR3, negetive serum FR0 at either 1:25 or 1:50 dilution in 10% FCS/PBS with secondary antibody anti-human IgG/IgM/IgA diluted at 1:30,000, 1:50,000 and 1:100,000 in 10% FCS/PBS.

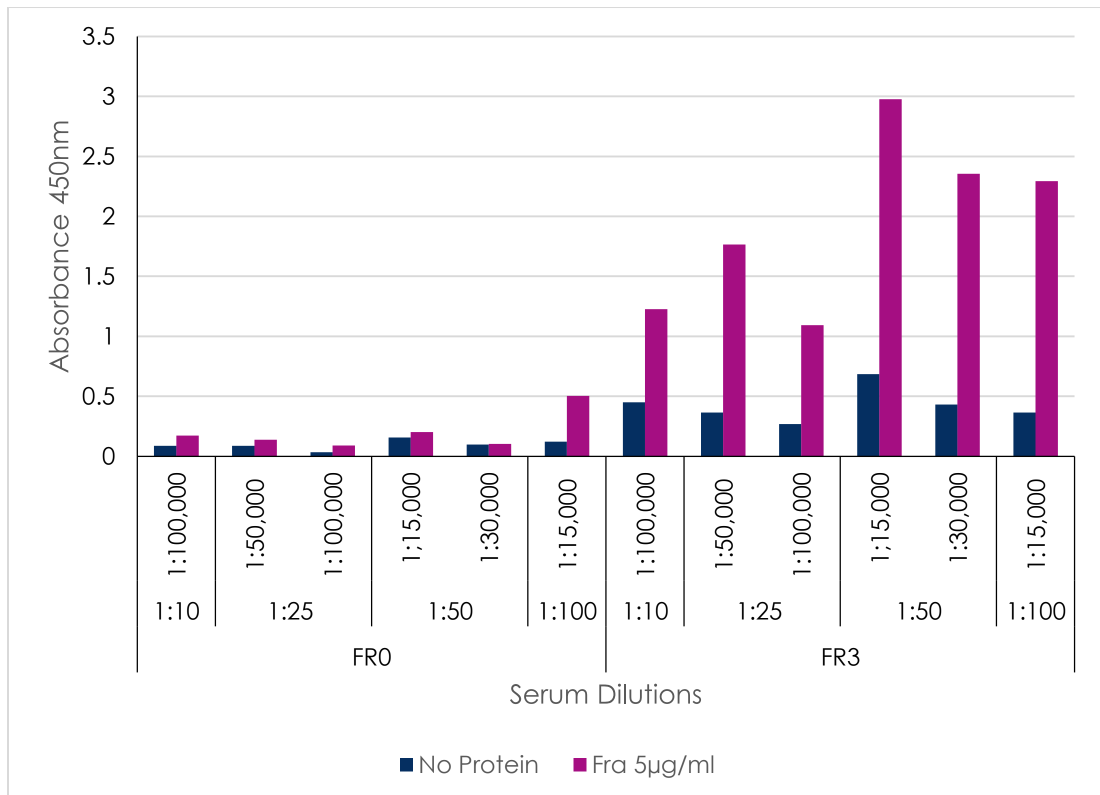


Figure 3.7 Anti-FRα ELISA opitimisation utilising clear flat-bottom 96 well polystyrene ELISA plates with 3% skim milk as the blocking buffer (Greiner, Sigma Aldrich). Plates coated with 5µg/ml recombinant FRα. Blocked with 3% skim milk powder/PBS. Positive anti-FRα serum FR3, negetive serum FR0 in 10% FCS/PBS with secondary antibody anti-human IgG/IgM/IgA diluted in 10% FCS/PBS.

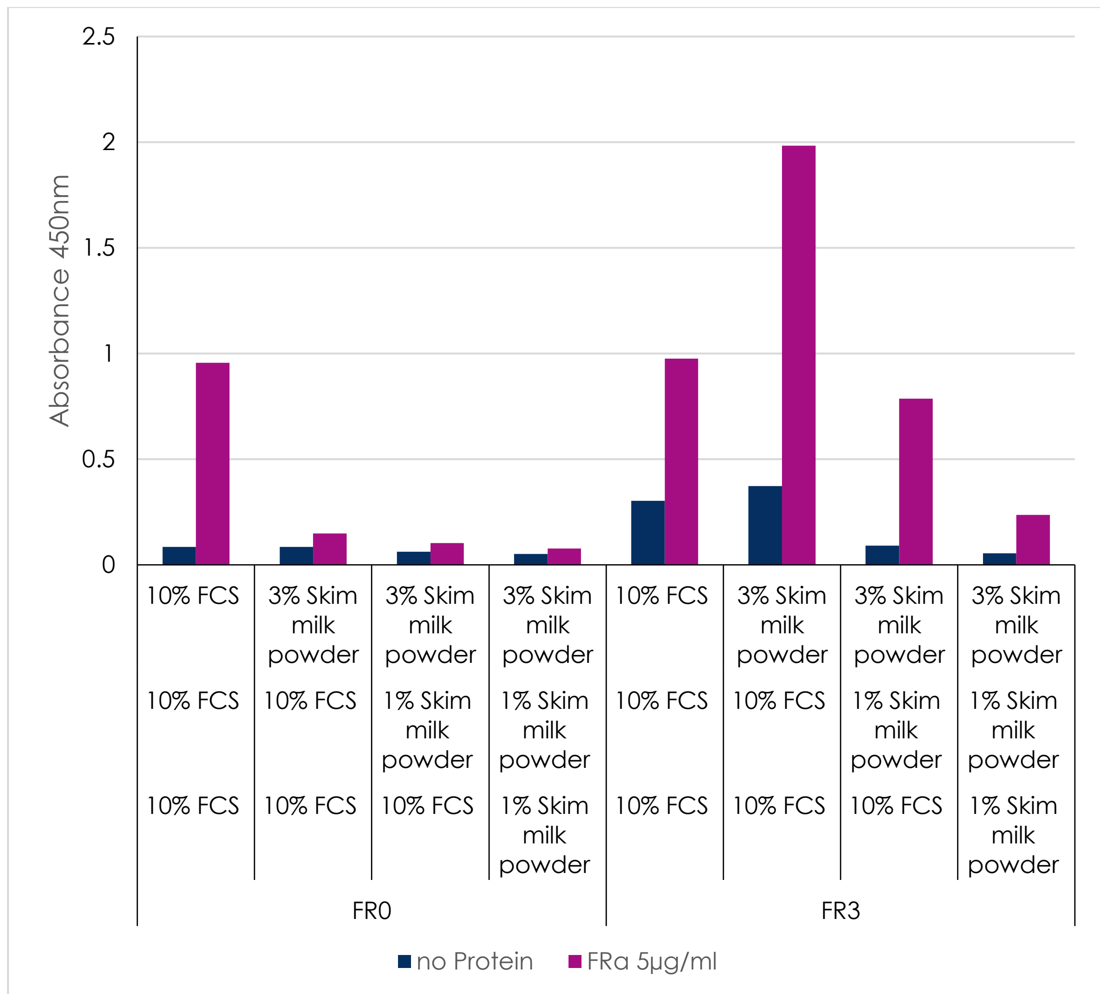


Figure 3.8 Anti-FRα ELISA Blocking and antibody dilution opitimisation utilising clear flat-bottom 96 well polystyrene ELISA plates and combination of 10% FCS and skim milk powder buffer (Greiner, Sigma Aldrich, USA). Plates coated with 5µg/ml recombinant FRα. Various combinations of 10% FCS/BSA and 3 % skim milk/PBS used for blocking and dilution of both serum and secondary antibody. Positive anti-FRα serum FR3 and negetive serum FR0 diluted at 1:50, detected with the secondary antibody anti-human IgG/IgM/IgA at a 1:30,000 dilution.

3.3.4 Wash Steps

Although the combination of blocking with 3% skim milk powder/PBS and diluting both primary and secondary antibodies in 10% FCS/PBS was successful in producing a result that had high signal to noise ratio for the positive control FR3 and no signal for the negative control FR0; it could be improved. Wash buffer used in the initial optimization of the clear flat-bottom 96 well polystyrene ELISA plates (Greiner, Sigma Aldrich) was PBS for 3 x 4 minute washes at three steps in the ELISA: 1) before blocking, 2) after primary antibody incubation 3) after secondary antibody incubation. Further optimization of the anti-FRα ELISA plate was performed by varying the wash buffer between PBS and PBS with 0.05% Tween20 (PBST) at the different wash steps (Figure 3.9). A higher signal to noise ratio was achieved using PBS, PBS, PBST in that particular order, which had a low background absorbance for FR3 of 0.1595 and positive absorbance of 1.173; compared to PBS/PBS/PBS which had a higher background absorbance of 0.305 and a lower positive absorbance of 1.095. Other combinations of wash buffers such as PBST/PBST/PBST or PBST/PBS/PBS which used Tween20 in first wash step demonstrated an extremely high background, whereas when Tween20 was used in the second wash step for example PBS/PBST/PBS and PBS/PBST/PBST showed an overall reduction in absorbance for both negative and positive wells. Therefore, for all further ELISA protocols, PBS wash was used for both washing before blocking and after primary antibody incubation, with the final wash step after secondary antibody incubation with PBST.

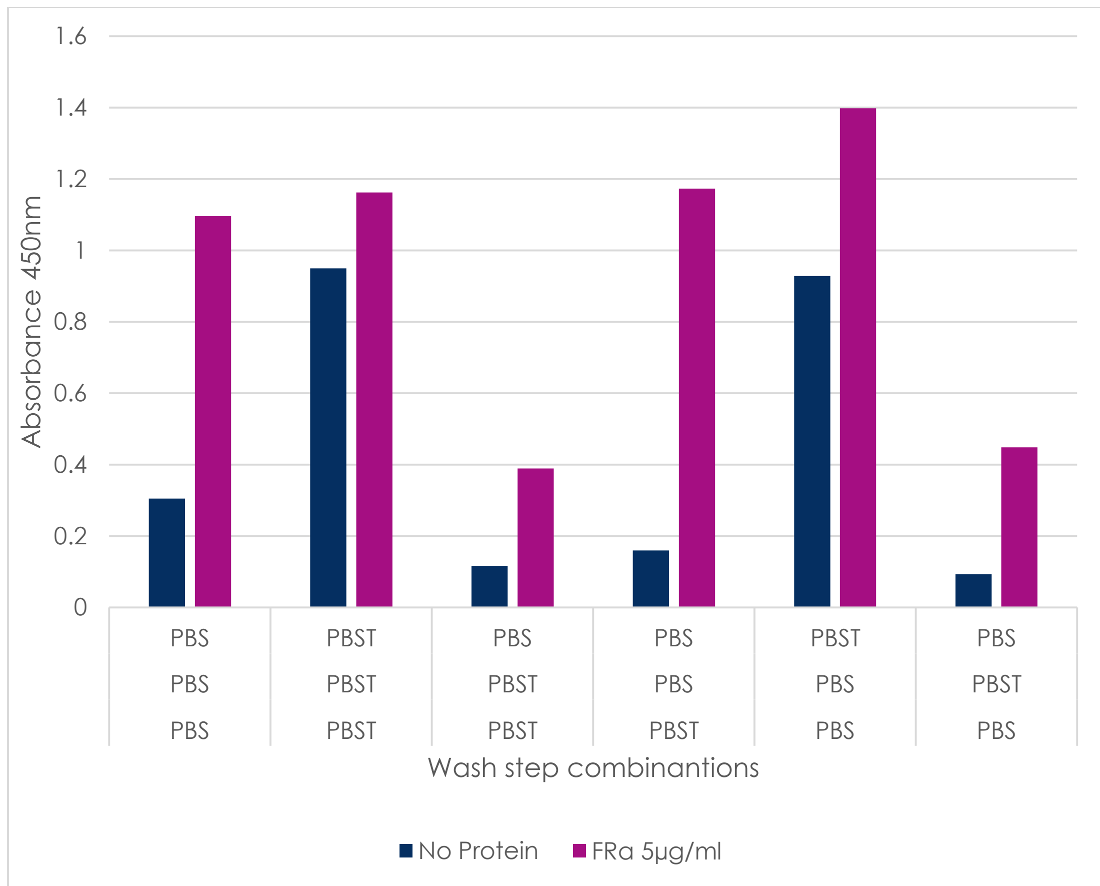


Figure 3.9 Anti-FRα ELISA wash buffer optimisation. (Greiner, Sigma Aldrich). Plates coated with 5µg/ml recombinant FRα. Various combinations of PBS and PBST were analysed for washing buffers. Positive anti-FRα serum FR3 and negetive serum FR0 diluted at 1:50, detected with the secondary antibody anti-human IgG/IgM/IgA at a 1:30,000 dilution.

3.3.5 Final FRα ELISA Protocol

The detailed final anti-FRα autoantibody ELISA protocol after optimization is as below. Overall standard plate setup is demonstrated in (Figure *3.10*)

1. **Coating of ELISA Plate**
    a. Make up a 5µg/ml dilution of recombinant FRα protein (Sino Biological, China) in 0.2M Sodium carbonate/sodium Bicarbonate (pH 9.4) coating buffer.
    b. On a clear flat 96 well polystyrene plate (Greiner, Sigma Aldrich) pipette 100µl/well of 0.2M Sodium carbonate/sodium Bicarbonate (pH 9.4) coating buffer to columns 1-6 using a multichannel pipette.
    c. Pipette 100µl/well of the diluted 5µg/ml recombinant FRα protein to columns 7-12
    d. Cover with a clear ELISA plate seal
    e. Incubate overnight at 4°C
2. **Wash Step 1**
    a. Remove cover and flick out coating buffer from the plate
    b. Dry excess liquid from plate using paper towel
    c. Add 250µl/well of wash buffer 1 x PBS for ELISA
    d. Incubate for 5 mins shaking on a plate shaker
    e. Repeat steps a-d 2 more times.
    f. Remove and dry plate ready for blocking buffer
3. **Blocking**
    a. Add 250µl/well of 3% skim milk powder/PBS
    b. Cover with microplate sealing tape.

c. Incubate at room temperature for 90 minutes shaking at 100rpm on plate shaker
d. Remove blocking buffer and dry using paper towel.

4. Primary Antibody.

a. Thaw samples on ice prior to this step
b. Prepare a 10% FCS dilution in 1x PBS for ELISA
c. Dilute each sample in 10% FCS/PBS at a 1:50 dilution in separate Eppendorf tubes
d. Prepare following controls in 10% FCS/PBS
    i. Positive control antisera FR3 1:50 dilution
    ii. Negative control antisera FR0 1:50 dilution
    iii. Polyclonal antibody positive control anti-FRα IgY (1mg/ml) 1:500 dilution
e. 100µl of each sample and control is pipetted in triplicate in both FRα- and FRα+ wells as demonstrated in (Figure *3.10*)
f. 100µl of 10% FCS/PBS only is also pipetted in triplicate in both FRα- and FRα+ wells as a diluent control
g. Cover with microplate sealing tape
h. Incubate at room temperature for 60 minutes shaking at 100rpm on a plate shaker.

5. Wash Step 2

a. Repeat wash step 1.

6. Secondary Antibody

a. Prepare a 1:30,000 dilution of anti-Human IgG, IgM, IgA HRP conjugated antibody (Rockland, USA) in 10% FCS/PBS.

b. Prepare a 1:2000 dilution of anti-chicken IgY HRP conjugated antibody in 10% FCS/PBS.

c. Pipette 100µl of diluted anti-human IgG, IgM, IgA HRP conjugated antibody into all wells except the wells containing Polyclonal anti-FRα positive Control.

d. Pipette 100µl of diluted anti-chicken IgY into the remaining wells containing the Polyclonal anti-FRα positive Control.

e. Cover with microplate sealing tape.

f. Incubate at room temperature for 60 minutes shaking at 100rpm on a plate shaker

7. Wash Step 3

a. Repeat wash step 1 using 0.05% PBS Tween20.

8. Chromogenic Analysis

a. Prepare a 50:50 mix of TMB Substrate

b. Add 100µl to each well

c. Incubate shaking at 100rpm for 3 minutes

d. Stop reaction with 100µl of 2M H_2SO_4

e. Read absorbance of plate at 450nm

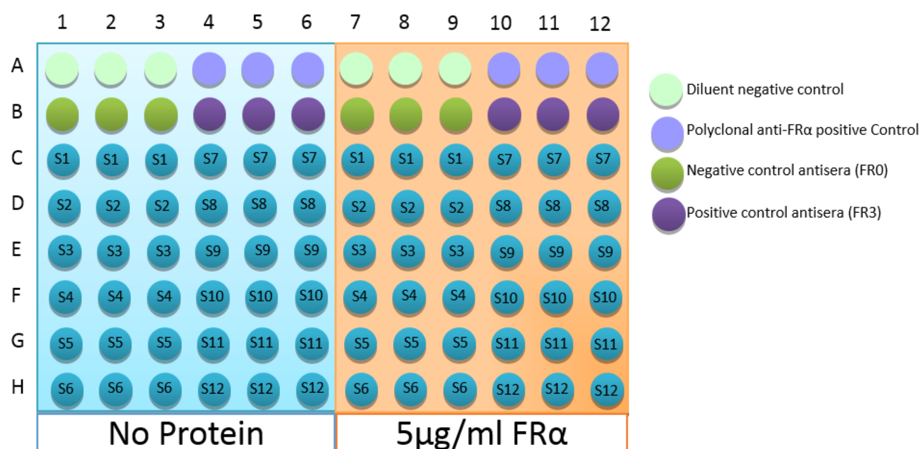


Figure 3.10 Anti-FRα autoantibody standard ELISA set up.

3.3.6 Validation of ELISA

3.3.6.1 Comparison with Flow Cytometry

To confirm that the absorbance readings for the anti-FRα autoantibody ELISA were correct, a series of samples that had previously been tested using flow cytometry were further run on the ELISA: Samples FR0, FR1, FR3, B2 and B27. Figure 3.11 demonstrates that the ELISA was just as effective at detecting the autoantibodies against the FRα protein compared with the previous method of flow cytometry as all samples show similar positivity between sera and plasma in relation to the positive sample FR3. Flow cytometry results demonstrated a higher level of detection for FRα autoantibodies in sample B4 than was detected in the anti-FRα autoantibody ELISA, however there was also a significant amount of background detected for this sera against the negative FRα cell line Cos-7.

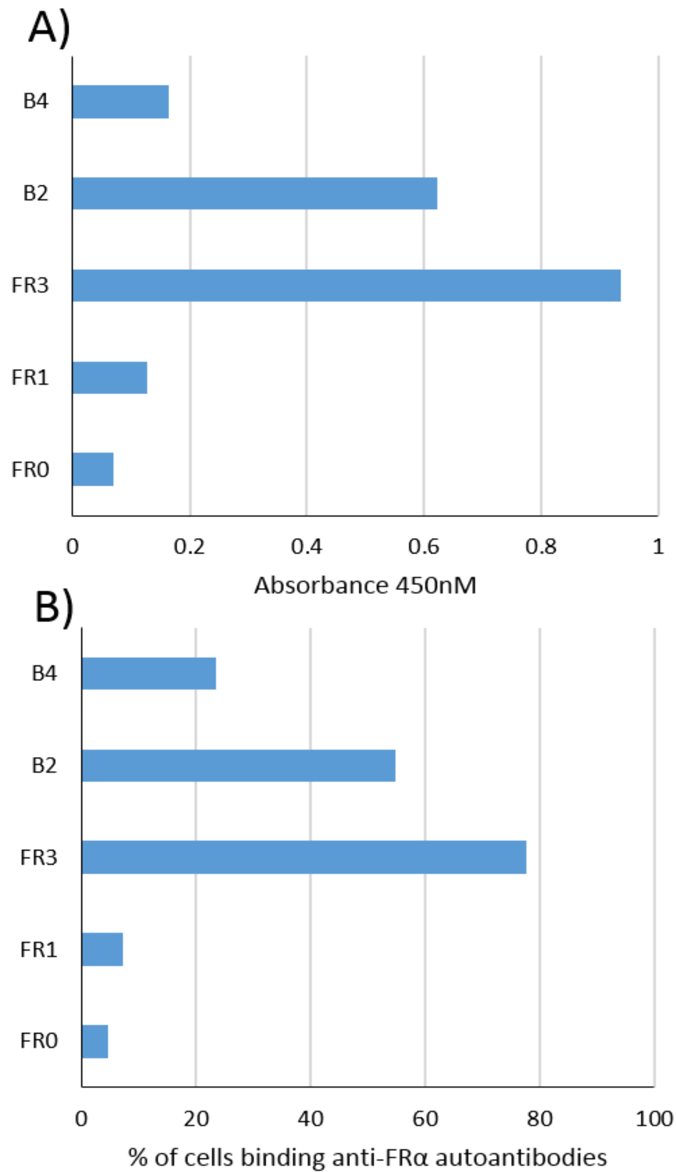


Figure 3.11 Comparison of anti-FRα autoantibody detection with the optimised anti-FRα autoantibody ELISA and the in-house flow cytometry. 5 samples were tested on both methods. A) Anti-FRα autoantibody ELISA following standard protocol with 5μg/ml FRα recombinant protein. B) Flow cytometry detection utilising the FRα stably transfected Cos-7 FRα cell line and autoantibodies detected by anti-Human IgG/IgM/IgA FITC labelled secondary antibody.

3.3.6.2 Inter Assay Variation

Inter-assay variation was performed using a strong positive, a weak positive and a negative anti-FRα autoantibody sera for the optimized anti-FRα autoantibody ELISA (3.3.5) in 10 different ELISA assays to determine plate-plate variation. Data for the inter-assay run is shown in Table 3.3 and demonstrates individual CVs of 6.72, 11.00 and 19.91 for strong, weak and negative serum, respectively. The overall inter-assay CV for the FRα auto-antibody ELISA is 8.86, which was under the recommended CV of 10% for Inter-assay levels. CVs for the negative sera was not included in the overall CV for the FRα autoantibody ELISA as it was too close to zero, and therefore would always have a large variation, however never above the positive cut off level. Table 3.3 demonstrates that each replicate for each sample was within 2 standard deviations of the mean and therefore considered reliable and reproducible.

Table 3.3 Inter-assay statistics for the anti-FRα autoantibody ELISA

	Strong Positive	***Weak Positive***	***Negative***
Number Replicates	10	10	10
Mean	1.192	0.345	0.081
Median	1.176	0.356	0.083
Standard deviation	0.080	0.037	.0162
Minimum	1.099	0.271	0.062
Maximum	1.297	0.384	0.103
Coefficient of variation	6.722	11.00	19.91
	8.86%		

3.3.6.3 Intra-Assay Variation

The Intra-assay variation was performed with four different samples of varying antibody titres, a strong, moderate, and weak positive and a negative anti-FRα autoantibody sera sample. Each sample was repeated on the same plate a total of 8 times. Data for the intra assay is shown in Table 3.4. For the highest FRα autoantibody sera sample there was a small intra-assay variation observed however as the autoantibody level decreases the

intra-assay variation increases. The overall intra-assay variation excluding the negative sample was 9.58%, which is acceptable.

Table 3.4 Intra-assay statistics for the anti-FRα autoantibody ELISA

	Strong Positive	***Moderate positive***	***Weak Positive***	***Negative***
Number Replicates	8	8	8	8
Mean	1.048	0.523	0.271	0.060
Median	1.037	0.523	0.262	0.061
Standard deviation	0.033	0.058	0.038	0.009
Minimum	1.028	0.433	0.217	.048
Maximum	1.126	0.627	0.341	.071
Coefficient of variation	3.18	11.18	14.38	15.47
Overall Intra-Assay		9.58%		

3.3.6.4 Lower Limit of Detection

The limit of blank, limit of detection was calculated for the validation of the ELISA. The limit of blank when utilising the intra-assay results (*Table 3.*) is the absorbance value of 0.1076; while the limit of detection is the absorbance value of 0.168, when using a suspected weak positive sample.

3.3.6.5 Matrix Compatibility

As the type of sample that is available may vary between studies, and even in the same study it is important to determine if the ELISA is suitable for a range of matrices. The two most common matrices that would be used for an autoantibody ELISA is serum and plasma. We had access to three samples that had both sera and plasma collected and therefore were screened on the anti-FRα autoantibody ELISA. Samples tested were the negative control FR0; the positive sera control FR3 and a Thrombosis In Stroke Study (TISS) sample that had a low reaction to initial testing using the sera sample. Samples were assayed at 1:50 dilution with 1:30,000 anti-human IgG IgM, IgA HRP conjugated secondary antibody, as per optimised protocol outlined in section 3.3.5. Figure 3.12 demonstrates that plasma on the ELISA had a slightly higher absorbance value for each sample; however, after performing a Student's t-test (equal variances not assumed) on each sample there was no significant difference demonstrated. Although there were only three samples tested due to availability, results still support that the anti-FRα autoantibody ELISA was suitable for both sample matrix types.

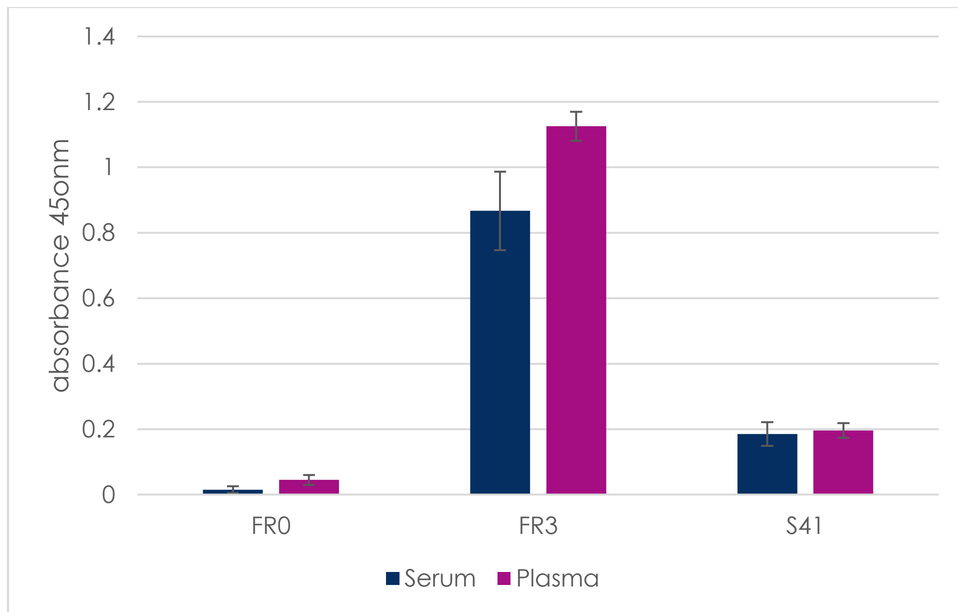


Figure 3.12 Comparison of FRα autoantibody detection in sera and plasma. Three subjects FR0, FR3 and S41 all with both serum and plasma samples were analysed with the anti-FRα autoantibody ELISA at 1:50 dilution. FRα autoantibody detected with 1:30,000 anti-mouse IgG HRP.

3.3.7 FRα Autoantibody Positivity

To determine normal population levels and a baseline cut off for a positive reading for FRα autoantibodies using the ELISA assay. A total of 22 samples were collected from a random population at Royal Perth Hospital over a period of three weeks. Collection of samples was blind and no information was collected in relation to the participants, therefore it was assumed that there was a mix of both male and female, as was this was not discriminated against. Samples were run in triplicate on the optimized anti-FRα autoantibody ELISA, at a 1:50 dilution with the secondary anti-human IgG/IgM/IgA at 1:30,000, in addition, the known positive FR3 and known negative FR0 samples were also tested. Absorbance values were indexed against the known negative and known positive sample, and average and

standard deviations were calculated. It was observed that 2 samples had high readings, which were both above the limit of detection value and above the 99th percentile, therefore are likely to be positive for FRα autoantibodies; they were excluded from further statistical analysis for the baseline level for FRα autoantibodies. The overall mean for the 20 included normal population samples is 1.925 frU with a standard deviation of 3.56 (Table 3.5). The cut off values for FRα negative and positive samples are stated in Table 3.6.

Table 3.5 Statistics on baseline controls using the anti-FRα autoantibody ELISA. All values except number are represented in Index values.

	Baseline Samples
Number	20
Mean	1.925
Median	0
Standard deviation	3.56
Minimum	0
Maximum	12.59
95th Percentile	8.76
99th percentile	11.82

Table 3.6 Positive and negative cut off values for the anti-FRα autoantibody ELISA.

	Indexed Score
Negative frU	<9.065
Low Positive frU	9.065 ≤ X ≥ 19.769
High positive frU	> 19.769

3.4 Discussion

The ELISA is a method that has permitted detection of a range of substances, and is a technique that has been well researched since it was first developed over 40 years ago (Engvall *et al.* 1971). Although much research has gone into the method, detection of a specific analyte always requires a large amount of optimization to get the most accurate result. The major objective of this study was to develop a simple ELISA method that is easily reproducible and accurate in any laboratory for the detection of anti-FRα autoantibodies. Through much optimization a technique was developed, that allows for successful detection of anti-FRα autoantibodies in human sera and plasma using a recombinant FRα protein. The amount of colour developed is proportional to the amount of autoantibody present in the sample. Positive level cut-offs for the presence of FRα autoantibodies was determined by the screening of 20 normal population serum samples; which are negative <9.065 frU, low positive 9.065 frU ≤ X ≥ 19.769 frU while a positive result is an index value over 19.769 frU and then validated to confirm accurate and consistent results.

There are multiple studies looking at the presence of FRα autoantibodies that can affect folate transport in various tissues throughout the body, however there is no commercial test to detect these antibodies. In addition, there are an array of different methods that previous studies have used, from radio-immunoassay to complicated ELISA techniques which are not always reproducible in smaller laboratories, unless available commercially. Due to limitations in our laboratory regarding technology we had available for ELISA

production, the ELISA we developed was designed using materials and equipment that are readily accessible in a majority of research and diagnostic laboratories. This is advantageous over current literature protocols as there is no requirement to print the FRα protein to the plate nor is there any requirement for the use of the hazardous radio-isotope ^{3}[H], which is still used in some laboratories detecting FRα autoantibodies (Sutula *et al.* 1986). As previous papers that have studied FRα ELISAs have not described in detail the procedure, nor assay performance; it is hard to allow for a comparison with the ELISA described in this study.

ELISA's can be very complex with many factors that need to be taken into account to replicate the correct immune complex that is being analysed. The development of the anti-FRα autoantibody ELISA required a significant amount of optimisation from the amount of FRα protein required, to the wash buffers used, due to the complex nature of serum which often results in high background readings. The optimal conditions determined were 5μg/ml of recombinant FRα protein, coated overnight on general 96 well ELISA plates, blocked with 3% skim milk powder/PBS with the dilution of serum at 1:50, and secondary antibody mouse-anti-human IgG/IgA/IgM at 1:30,000 in 10% FCS/PBS. In addition, the wash step regimen of PBS, PBS, and PBST played an important role in obtaining the lowest signal to noise ratio. Throughout the screening of certain serum samples, a significant background has been observed, and has also been observed for the positive control, where background levels are ~0.1-0.2 absorbance. This phenomenon cannot be explained; however it does not appear to affect the overall result as each

sample had the background reading subtracted and was indexed against both a negative and a positive standard sample.

The choice of FRα protein has been discussed over many of the publications analysing FRα autoantibodies, with no one type found to be superior to the other (Cabrera *et al.* 2008, Ramaekers *et al.* 2005, Rothenberg *et al.* 2004, Sequeira *et al.* 2013). Initial studies utilised the bovine FRα, and FRα purified from human placental tissue; with later studies producing recombinant forms of FRα protein expressed using insect cell lines (Billie *et al.* 2010, Boyles *et al.* 2011, Cabrera *et al.* 2008, Ramaekers *et al.* 2005, Rothenberg *et al.* 2004, Sequeira *et al.* 2013). Bovine FRα although demonstrated to have a high cross reactivity with human FRα autoantibodies shares only an 80% homology; consequently some FRα autoantibodies may not be detected (Henderson 1990, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2008). Selection of the type of FRα antigen is critical as the confirmation of the FRα protein has been demonstrated to be crucial in binding of the antibodies against the FRα protein, and the denatured FRα protein has no binding affinity for folate or autoantibodies (Sequeira *et al.* 2013). This study initially aimed to produce a recombinant full-length protein with a N-terminal 6x His tag for purification, however after the stably transfected cell line was developed the 6 x His tag was un-identifiable on the protein; which may have resulted in the tag being folded into the protein during post translational modification. Due to the lack of 6 x His tag, purification was performed using Direct IP kit, which far exceeded the cost of a commercial

recombinant FRα protein, due to the large requirement of monoclonal FRα antibody.

A commercial recombinant FRα protein was purchased from Sino Biological and was then chosen to develop the anti-FRα autoantibody ELISA; where the structure is the same as used by Billie *et al* (2010), which was demonstrated to be antigenic to both monoclonal and polyclonal FRα antibodies. The amount of protein was optimised in two different assays with 5μg/ml being confirmed to be optimal. A concentration of FRα protein greater than 5ug/ml demonstrated the hook effect, while below 5ug/ml the signal was proportional to the amount of FRα protein, above 5ug/ml there was either no change or a decrease was observed in the signal (Byun *et al.* 2013). The commercial recombinant FRα protein was also demonstrated to be similar to the native form when comparing results from the ELISA (recombinant protein) to the flow cytometry method (native protein). Comparison, between the two methods demonstrated the same reactivity profiles of the monoclonal FRa antibody, and Positive and negative serum samples, supporting the use of the recombinant protein from Sino Biological.

Three different plates were trialled in the optimization for the detection of FRα autoantibodies, the simplest 96well polystyrene plate exhibited the highest signal-noise ratio and was selected for the overall ELISA assay. Initial optimization was carried out utilizing the Ni-NTA HisSorb Qiagen plates. These plates were selected due to the advantage of having a 6xHis-tag present on the C-terminal end of the FRα; allowing for uniform binding of the protein to

the plate which would reduce assay variation and increase reproducibility. Unfortunately, significant non-specific binding was observed between both negative and positive FRα autoantibody sera, with and without plate blocking. To date, there is no information published in the scientific literature that can explain why non-specific binding is observed, possible reasons are proteins/antibodies within serum samples cross reacting with the nickel; or there may be anti-BSA antibodies in the serum which may cross react with the pre-blocking step that contains BSA. No real comparison could be identified between the Nunc Immuno Maxi-sorp U bottom plates and standard flat bottom polystyrene as the fish gelatine was the only blocking buffer tested on both plates. Nonetheless the flat bottom polystyrene did demonstrate a reduction in background compared to the Nunc- Immuno Maxi-sorp plates. High background was demonstrated in the majority of the blocking buffers tested in the Nunc- Immuno Maxi-sorp plates, and therefore the flat bottom polystyrene plates were selected for further optimisation. Furthermore low-medium binding plates were selected as complex matrices such as serum contain human IgG that have an increase affinity in "high binding" plates, as the surface has been altered to be slightly ionic and hydrophobic, capturing more protein; therefore increasing the chance of non-specific binding.

Multiple blocking and antibody dilution buffers were tested on all three plates to obtain the highest signal to noise ratio. Blocking buffers included skim milk, BSA, cold water, fish gelatine, casein, FCS and a commercial protein free blocking buffer. Through much optimisation it was only when a

combination of 3% skim milk powder for blocking and 10% FCS serum was used for both primary and secondary antibody dilution that an acceptable signal to noise ratio was achieved. With a complex sample matrix being analysed such as serum or plasma the blocking and dilution buffers have to be compatible, and not interfere with the antigen-antibody reaction. Various ELISA's use a combination of buffer types for blocking and dilution buffers to obtain a sufficient signal to noise ratio, as observed in this ELISA. Skim milk is an effective blocking agent in many ELISA's due to the capacity to block both hydrophilic and hydrophobic sites and was successful in blocking non-specific binding with negative sera, however it still resulted low signal to noise ratio for FRα positive sera(Verma *et al.* 2008). When 10% FCS was used as a dilution buffer for both serum and secondary antibody in place of 1% skim milk the signal to noise ratio was greatly increased. Serum as a dilution buffer for human serum analysis is often useful as it mimics the matrix of the serum sample, reducing non-specific binding that can occur, as seen in this study (Cox KL 2012 (updated 2014)). Therefore, the combination of skim milk as a blocking agent, removes non-specific binding seen in negative sera, while the dilution in FCS reduced the non-specific binding in positive sera, leaving a high absorbance value in wells containing FRα protein.

The range of different blocking buffers tested showed various results from having no signal development to extremely high background signals. Certain buffers resulted in low or negative signals for the confirmed positive serum, as seen in protein-free blocking buffer and casein; where the buffers

themselves could cause interference between the autoantibody binding to the FRα antigen, and were therefore deemed not suitable for this ELISA assay. A large amount of background was observed in skim-milk powder, BSA, fish gelatine with high absorbance values also observed in negative serum controls for both BSA and fish gelatine. There may be several reasons non-specific binding may occur in an ELISA utilising serum; especially in patients with a genetic predisposition to auto-reactivity who have been observed to have high background levels, however the cause is not fully understood. Possible reasons include, the serum reacting to the insufficiently blocked plate where serum proteins cause non-specific binding of human IgG, general non-specific binding with anti-human antibodies i.e. rheumatoid factor like antibodies and antibodies against the proteins in the blocking buffer. This has been demonstrated in several studies where patients have antibodies against BSA due to immunisation with a vaccine containing BSA-conjugate (Konishi *et al.* 2010, Maple *et al.* 2004). In addition certain BSA preparations have been shown to have stronger non-specific binding over other preparations for example Xiao and Isaacs (2012) demonstrated that in an ELISA with human serum, 3 out of 4 tested BSA preparations caused non-specific binding, due to impurities in proteins; this may be a possible reason for the high background seen on the NI-NTA HisSorb plates (Konishi *et al.* 2010).

A crucial step in the optimisation of the FRα autoantibody ELISA was the selection of wash buffer at the critical wash step. Wash steps in any immunoassay are important to remove unwanted but non-specifically

bound material, especially seen in complex sample matrices such as serum; without disrupting/interfering with the important binding steps of the assay and detergents are common additives which have blocking and substance removal properties. Final optimization of the 96 well polystyrene ELISA plates was performed using PBS as a wash buffer for all three steps; with a moderate signal to noise ratio observed. Introduction of Tween20 in the PBS at the final wash step only was performed leading to a higher signal to noise ratio than with PBS alone and therefore was included in all further assays. Other combinations of Tween20 in wash buffer steps was performed and resulted in a significant change in the absorbance values depending on where Tween20 was included. Results demonstrated that the presence of Tween20 in the wash buffer prior to blocking resulted in high background absorbance values; Tween20 in this step may interfere with the ability of the blocking buffer to absorb to the plate. Inclusion of Tween20 in the wash buffer after primary antibody incubation, resulted in an overall low absorbance value; which may be caused by the Tween20 disrupting the antibody-antigen binding or affecting the secondary antibody interaction with the primary antibody. What was interesting is that some technical guidelines recommend against the inclusion of Tween20 at the final wash step due to the interference it may have with the enzyme activity reducing the signal; which was not observed in our assay and instead demonstrated no signal reduction in positive wells but a greatly reduced absorbance values in negative control wells, increasing signal-noise ratio.

The results obtained from the three serum samples tested in the anti-FRα autoantibody ELISA are comparable in sensitivity and level of detection as what was demonstrated in the flow cytometry method; however the ELISA has several advantages over the flow cytometry method. Two important advantages are the reduction in patient sample required, and the significant decrease in time; as flow cytometry requires up to three days of cell growth for one day's assays. The number of samples that are able to be analysed in one ELISA exceeds the capability of flow cytometry procedures. The ELISA is capable of running 16 samples in triplicates on one plate, while in our laboratory we have only been successful in running 8 samples, not replicated, in a single procedure using flow cytometry, without affecting the results with delayed sample handling. If you require duplicates/triplicates then the number of samples to be analysed would be greatly reduced. The ELISA showed little to no non-specific binding, whereas the flow cytometry demonstrated high background levels with some samples, likely due to the use of a whole cell rather than pure protein. Furthermore, the amount of monoclonal antibody required to confirm FRα was present in the assay is noted, as the amount of antibody used in one flow reaction is equivalent to 12 samples run on the ELISA.

A limitation in the development of this assay is the lack of standard calibrators for the FRα autoantibody, which are generally required for standardization and determination of analyte concentration. For that reason, both a known negative and known positive sample were included in each ELISA assay to ensure the ELISA performed appropriately and to

account for any plate to plate variation; caused by slight changes in incubation times, room temperatures and pipetting or dilution errors. The known positive sample demonstrated an inter-assay of 3.18% and therefore was deemed an appropriate control to use to standardise each plate. Unknown samples were indexed against both the negative and positive sample as previously described.

Another consequence in the development of this assay without standard calibrators was an inability to determine the true concentration of the FRα autoantibody present in the sample. Therefore, we were only able to create a range of low and positive values by comparison to a randomised normal population cohort. Sera from 20 random sampled subjects were used to establish a baseline for a negative population, and indexed against a standard positive and negative sample. A negative sample was defined as a sample below the index value of 9.065 while a low positive sample was defined as having a value between 9.065 and 19.769. A high positive sample was defined as any level above 19.769. The limit of blank and limit of detection were also calculated for the ELISA to determine if the positive cut-off values are accurate in measurement. The limit of blank is the value which a negative sample will not exceed; while the limit of detection is the lowest value that the ELISA can accurately detect a specific analyte in the ELISA. The absorbance values for these were 0.107 and 0.168 respectively. The low positive range was defined because the negative/positive cut off range in an absorbance value 0.152 was lower than the limit of detection of 0.168.

However, a result in this range still produced a CV below 20% which is acceptable.

The analysis of samples in this assay was highly repeatable and demonstrated as a valuable tool for detecting patients with FRα autoantibodies in both serum and plasma samples. The covariant of variation for both inter-assay and intra-assay is 8.86 % and 9.58%, respectively, falling under 10%, which is a strong CV for a manual ELISA. In the strong positive FRα autoantibody sample the CV was significantly lower at 6.72% and 3.18% than what was observed in a sample with a low level of FRα autoantibodies being 11.00% and 14.38; which is expected nonetheless still under the acceptable CV of 20%. This demonstrates that the ELISA has acceptable reproducibility when manually performed; while plate to plate variation would be reduced by the use of an automated ELISA or automatic plate washer, it is not necessary.

Access to a certain sample type is not always possible, therefore compatibility of the ELISA to detect FRα autoantibodies both plasma and serum is quite important; as differences between a plasma matrix and a serum matrix can enhance or inhibit signal development, altering the result of the assay. A small test was performed analysing three samples that had both serum and plasma samples available. Results demonstrated that there was no significant change in absorbance values observed in any of the three samples tested; conversely plasma samples had a slightly higher absorbance value observed. This may be caused by factors such as

fibrinogen, clotting factors, or tube additives such as EDTA, which is present in plasma samples, but not in serum (Cox KL 2012 (updated 2014)). A larger number of serum and plasma samples collected at the same time is required to confirm these results, however the initial result demonstrates that the ELISA is compatible for both plasma and serum without a sample being categorised falsely.

3.4.1 Conclusion

The development of the FRα autoantibody ELISA was achieved with highly repeatable data and suggests that the assay will be a valuable tool in screening large cohorts of serum or plasma samples of patients suspected of having FRα autoantibodies. Although the ELISA developed uses methods to standardise the results between plates, the need for standard calibrators is evident as demonstrated with any clinical test to attain a true level of autoantibodies within a sample; in no study to date looking at the FRα autoantibodies has standard calibrators been developed.

Chapter 4:

Role of FRα Autoantibodies in Recurrent Miscarriage

4 Chapter 4: Role of FRα Autoantibodies in Recurrent Miscarriage

4.1 Introduction

One can't imagine the grief of losing an unborn child let alone suffering from recurrent miscarriages (RM), yet 1% of all women trying to conceive go through such trauma. What makes the situation worse is that over 50% of these women have no identifiable underlying cause confounding treatment options needed for a successful pregnancy (Chen *et al.* 2010, Christiansen *et al.* 2008, Lund *et al.* 2010, Rai *et al.* 2006, Ren *et al.* 2006, Vinatier *et al.* 2001). When you think about the processes that are required to take a pregnancy to full term, you can understand why it is difficult to determine the exact cause of a miscarriage. It has been well established that RM is a multifactorial disease, so for many women the possibility that a combination of factors is causative for their recurrent miscarriage (Li *et al.* 2002). Because of the multifactorial nature, women suffering RM are often subjected to a multitude of diagnostic testing which may or may not end in the couple having a successful pregnancy. These tests include autoimmune testing, genetic testing, nutrient and hormone testing, protein abnormalities, and occasionally more invasive testing such as a hysteroscopy (Jeve *et al.* 2014, Li *et al.* 2002).

During a pregnancy there are many organs, tissues, and processes that are required to function correctly in order to have a successful live birth, and the immune system is no exception with its complex interactions between the

maternal and a semi-allogenic fetus (Kuon *et al.* 2015). Autoimmune and allogenic disorders have been extensively studied and recognized to play a significant role in the occurrences of recurrent miscarriage, as a slight abnormality in the immune system can have detrimental effects in the developing embryo (Jeve *et al.* 2014). Antiphospholipid syndrome (APS); which is the only autoimmune disorder routinely evaluated; can account for up to 15% of recurrent miscarriage cases (ACOG 2002, Rai *et al.* 2006, Rai, Clifford*, et al.* 1995, Rai, Regan*, et al.* 1995). The pathology consists of numerous antibody targets; including anti-cardiolipin and anti-β2M antibodies; causing thrombotic events, immunological dysfunction and direct toxicity in the placenta, ultimately leading to miscarriage in 90% of women who are left untreated for these autoantibodies (ACOG 2002, Rai *et al.* 2006, Rai, Clifford*, et al.* 1995, Rai, Regan*, et al.* 1995). In addition to APS, other immune disorders that have been shown to increase the risk of recurrent miscarriage are anti-thrombin, anti-thyroid, anti-nuclear antibodies, celiac disease and allogenic abnormalities, involving cytokines, regulatory T-cells, and NK cells (Bansal *et al.* 2011, Kuon *et al.* 2015, Molazadeh *et al.* 2014). A large number of these immune disorders have multiple modes of actions directly targeting proteins, cells and tissues, inhibiting their specific functions, or via indirect effects causing inflammatory responses, activating the complement cascade and/or cell toxicity often at the vitally important maternal-fetal interface. (Adams Waldorf *et al.* 2008)

One potential autoimmune disease that has not been studied in recurrent miscarriage, though has been associated with other pregnancy complications, is autoimmune reactivity to FRα via autoantibodies that bind

to FRα at the maternal-fetal interface, with the possibility of blocking folate uptake leading to reduced fetal folate levels (Berrocal-Zaragoza M. I. *et al.* 2009, Billie *et al.* 2010, Boyles *et al.* 2011, Cabrera *et al.* 2008, Da Costa *et al.* 2003, Frye *et al.* 2016, Frye *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers, Blau*, et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira*, et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004, Sequeira *et al.* 2013, Shapira *et al.* 2015, Vo *et al.* 2015). FRα autoantibodies have been detected in the general population of both male and female, however their overall association with disease is unclear. The very first paper to identify the effects of an autoantibody against the FRα by Da Costa *et al* (2003) demonstrated that pregnant rats who were exposed to anti-FRα serum did not make it to full term highlighting the possibility that they could be an important antibody related to unsuccessful pregnancy. In 2004 the first report of human FRα autoantibodies in humans identified a strong association with neural affected pregnancy in a small cohort of mothers (Rothenberg *et al.* 2004). Subsequently, the presence of FRα autoantibodies have also been associated with increased risk of subfertility which is the failure to conceive after one year of unprotected regular sexual intercourse and preterm birth; being the birth of a baby before 37 weeks (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Taylor 2003, Vo *et al.* 2015). A study by Berrocal-Zaragoza *et al* (2009) analysed 42 women trying to conceive and demonstrated that there was a 12 times higher risk of subfertility in women with blocking autoantibodies compared to women without the antibodies. While Vo *et al* (2015), illustrated the prevalence of FRα autoantibodies in 65% of women who had experienced preterm birth compared to only 28% of full term pregnancies. Both these studies, suggest that reduced fetal folate levels at

the periconception period can affect early stages of cell division and embryogenesis in addition to apoptosis in human cyto-trophoblastic cells (Berrocal-Zaragoza Maria Isabel *et al.* 2009).

The most significant association of FRα autoantibodies to a particular disease was the discovery of these autoantibodies in patients with cerebral folate deficiency, where the autoantibodies were found to be responsible for blocking folate transport across the choroid plexus (Ramaekers *et al.* 2005). Numerous studies have been now been performed identifying a high incidence of FRα autoantibodies with several CFD associated diseases such as Rett syndrome, low function autism with neurological deficits and autism spectrum disorders (Frye *et al.* 2016, Frye *et al.* 2014, Frye *et al.* 2013, Opladen *et al.* 2007, Ramaekers, Blau*, et al.* 2007, Ramaekers, Sequeira*, et al.* 2007, Ramaekers *et al.* 2008, Sequeira *et al.* 2013). Although CFD is not directly linked to pregnancy complications, the strong associations seen between FRα autoantibodies in cerebral folate deficiency have led to further studies to determine the cause of FRα autoantibodies, and to the implementation of a routine FRα autoantibody binding and blocking test to specialised CFD centres in the USA (Berrocal-Zaragoza M. I. *et al.* 2009, Sequeira *et al.* 2013).

The possibility that FRα autoantibodies may be associated with an increased risk of recurrent miscarriage has arisen due to evidence that high fetal folate levels are required to take a pregnancy to full term, and that FRα autoantibodies are a factor that can inhibit transport of folate across the

placenta (George *et al.* 2002, Rothenberg *et al.* 2004).. Inhibition of folate transport by blocking FRα autoantibodies was first demonstrated by Rothenberg *et al* (2004), where KB cells (FRα positive) were incubated with serum containing FRα autoantibodies and $[H]^3$ labelled folic acid. A 95% reduction of cellular folate uptake was demonstrated when compared to FRα autoantibody negative serum (Rothenberg *et al.* 2004). Folate is important to cell development including both DNA synthesis and repair, the conversion for homocysteine to methionine; and a deficiency in folate can lead to cell apoptosis, oxidative stress, impaired cell growth and toxic homocysteine levels (van der Linden *et al.* 2006, van der Put *et al.* 2001, Wouters *et al.* 1993). Folate deficiency can affect pregnancy from the very early stages of gamete formation to implantation and fetal development (Laanpere *et al.* , Nelen W. L. D. M., Blom H. J., Steegers E. A. P., Den Heijer M., Thomas C. M. G.*, et al.* 2000). In addition, high levels of homocysteine can lead to defective chronic villous vascularisation like in APS which may in turn affect embryonic development (Creus *et al.* 2013, Nelen W. L. *et al.* 2000). The idea that reduced folate levels have been thought to increase the risk of miscarriage is documented, however the exact mechanism is not well understood. There are several factors that can lead to folate deficiency and hyperhomocysteinemia including genetic abnormalities in folate metabolism pathways and folate transport i.e. MTHFR yet their role is controversial (Creus *et al.* 2013). However, a recent study has demonstrated the importance of folic acid supplementation before and during pregnancy with reduced risk of miscarriage/spontaneous abortions in women with a higher intake of folic acid compared to women who have a very low folate intake (Gaskins, Afeiche*, et al.* 2014, Gaskins, Rich-Edwards*, et al.* 2014). In

addition to the blocking of folate transport, FRα autoantibodies have also been thought to cause an inflammatory response to tissues that they bind during pregnancy, ultimately leading to damage of the placenta and microvilli which are essential to a successful pregnancy (Gaskins, Rich-Edwards, *et al.* 2014).

With evidence supporting the role of FRα autoantibodies in folate deficiency and pregnancy complications from the early stages (subfertility), embryo development (NTD) right up until the final stages of a pregnancy (preterm birth), it's hard not to hypothesize if they could be a contributing factor to recurrent miscarriage, as well. FRα autoantibodies may play a role in RM by reducing fetal folate levels, or causing an inflammatory response against important tissues for example the placenta which is required to support the developing embryo. To our knowledge no group has studied the relationship between FRα autoantibodies in a recurrent miscarriage cohort, although recently a case study of women suffering from multiple pregnancy complications and recurrent miscarriage demonstrated to have high titre FRα autoantibodies (Shapira *et al.* 2015). Consequently, our study will use the ELISA assay developed in Chapter 3 which looks at total FRα autoantibodies to analyse a cohort of patients that have experienced recurrent miscarriage; that being 2 or more consecutive miscarriages; to identify if there is any association between the two.

4.2 Methods

4.2.1 Subjects

Patient recruitment was carried out at Hollywood Private Hospital (Western Australia) for the recruitment of women suffering recurrent miscarriage and normal pregnant controls. Recurrent miscarriage was defined as having had 2 or more recurrent miscarriages before 20 weeks. Another control group was women with one or more successful pregnancies which were recruited from Royal Perth Hospital. A total of 15 women who suffered recurrent miscarriage and 20 uncomplicated pregnancy controls were recruited. Written informed consent was obtained as per *National Statement on Ethical Conduct in Research Involving Humans* and the study was approved by the ethics committees of Hollywood Private Hospital (HPH310), Royal Perth Hospital (Western Australia) and Murdoch University (2014/002) (Western Australia).

4.2.2 Data Collection

Each participant was required to answer a range of questions that facilitated the capture of information regarding pregnancy history, age, weight, height, previous medical history and risk factors that could lead to recurrent miscarriage (Appendix 2).

4.2.3 Blood Collection

A total of three serum, two EDTA, two citrate and one lithium heparin tubes were collected for each participant. For serum samples, blood was collected in BD Vacutainer SST 11 advance tubes (BD, USA) and allowed to rest for at least 30 minutes to a maximum of 2 hours. Two of the SST tubes were then spun at 1000xg rpm for 15 mins at room temperature, the separated serum removed and aliquoted into 500ul lots. EDTA plasma was collected in BD Vacutainer EDTA tubes (BD, USA). With one EDTA tube an aliquot of 700ul was removed for DNA extraction, and the remaining blood spun at 3,000 rpm for 15 mins at room temperature, the plasma removed and aliquoted into 500ul lots. All serum and plasma samples were stored at -80°C until required. Citrate plasma was collected in BD Vacutainer Citrate Blood collection tube (BD, USA), and the lithium heparin tube was collected in BD Vacutainer Plastic Lithium Heparin tubes (BD, USA). All remaining SST, EDTA, citrate and lithium heparin tubes were sent to Core Clinical Pathology and Biochemistry laboratory at Royal Perth Hospital for specific diagnostic testing.

4.2.4 Diagnostic Testing

Routine testing was carried out at the Core Clinical Pathology and Biochemistry laboratory at Royal Perth Hospital for specific tests relating to recurrent miscarriage and thrombosis. Tests included free protein S, protein C, serum folate, red blood cell folate, and homocysteine levels.

4.2.5 FRα Autoantibody Testing

Binding FRα autoantibodies were analysed for all samples following the protocol detailed in Chapter 3 (section 3.3.5). Briefly 100ul of serum diluted 1:50 in 10% FCS/PBS was analysed in triplicate on the FRα ELISA pre-coated with 5ug/ml of recombinant FRα, and pre-blocked with 3% skim milk powder/PBS. Human FRα autoantibodies were detected with the secondary HRP conjugated anti-human IgG/IgA/IgM antibody (Rockland, USA), and colour developed with TMB substrate, and read on the Asys UVM 340 microplate reader (Biochom, UK).

4.2.6 Antiphospholipid Autoantibodies

Four antibodies commonly analysed in APS were tested using commercial ELISA kits from Orgentec (UK), for each patient and control. These antibodies were Anticardiolipin IgG and IgM, and Anti-β-2-glycoprotein IgA and IgG. The protocol was the same for each antibody; except the plate, and the secondary enzyme conjugate; and was followed as per the manufacturer's instructions, briefly described below. Serum from each case and control patient was diluted 1:100 in supplied sample buffer. 100ul of each calibrator and prediluted patient sample was added in duplicates to the wells of the supplied ELISA plate. The plate was incubated for 30 minutes at room temperature, before it was washed 3 times with 300ul of the supplied wash solution. Following washing, 100ul of supplied secondary antibody enzyme conjugate either anti-IgG, IgM or IgA was added to each well and incubated for 15 minutes at room temperature. The plate was further washed 3 times with 300ul of wash solution. 100ul of supplied TMB substrate

was added to each well and allowed to incubate for 15 minutes at room temperature before 100ul of supplied stop solution was added to each well. Each plate was read at 450nm and the reference at 620nm on the Asys UVM 340 microplate reader (Biochom, UK) plate reader. The mean for each sample and calibrator was calculated and a calibration curve was produced. The results were validated if the optical density of positive, negative and calibrators A-F fell within the range indicated on the quality control certificate enclosed within each kit. An estimate antibody concentration of each sample was calculated from the calibration curve and a sample was deemed negative, borderline or positive according to the manufacturers' specifications (Table 4.1)

Table 4.1. Antibody ranges for antiphospholipid related antibodies using the Orgentec ELISA kits.

	Negative	*Borderline*	*Positive*
Anticardiolipin IgG	<10 GPL U/ml		>10 GPL U/ml
Anticardiolipin IgM	<7 GPL U/ml		>7 GPL U/ml
B2-Glycoprotein I IgG	< 5 GPL U/ml	5-8 GPL U/ml	>8 GPL U/ml
B2-Glycoprotein I IgA	< 5 GPL U/ml	5-8 GPL U/ml	>8 GPL U/ml

4.2.7 DNA Extraction

Two aliquots of 350µl of whole blood, that was collected via an EDTA tube was used for genomic DNA extraction. Genomic DNA was extracted with EZ1 DNA Blood 350µl Kit (QIAGEN, Australia) with the EZ1 advanced instrument (QIAGEN, Australia). This method is a one-step automated process, which uses silica coated magnetic particles to extract DNA form 350µl of whole blood, and is eluted into 100µl. Samples were stored at -20°C.

4.2.8 MTHFR Mutation

4.2.8.1 MTHFR PCR

A Polymerase Chain Reaction (PCR) was performed on each genomic DNA extract from cases and control to analyse the MTHFR mutation c.677C>T. The two PCR primers that were selected from Nair et al. 2012 were the forward primer 5' AGGCTGTGCTGTGCTGTTG 3' and the reverse primer 5' CGCTCTGCAAGTTCTGGA 3' which produce a PCR product of 477bp which contains the c.677C>T mutation site C677T in the MTHFR gene. Amplification was carried out in 5x 20ul reactions for each DNA sample and was set up in 0.2 mL thin-walled PCR tubes (Scientific Specialities Inc., USA). Two micro litres of genomic DNA sample was added to each reaction with 2µl of PCR grade water to each negative control in place of the DNA sample. The remaining reaction containing 1x PCR buffer (Invitrogen™, USA), 1.5mM $MgCl_2$ (Invitrogen™, USA), 2µM dNTPs (Invitrogen™, USA), 1pmole sense and antisense primer (Geneworks, Australia), 1U Platinum® Taq DNA Polymerase (Invitrogen™, USA) and made up to the final volume of 20µl with water for injection (Pfizer®, USA). Cycling conditions for the PCR reaction consisted of

an initial 2 minute denaturation step at 94°C followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and 1 minute extension at 68°C; with a final extension step at 68°C for 7 mins. PCR amplification was carried out on Verti® thermocycler (Thermo Fisher Scientific, USA). Post PCR samples were visualized using 2% agarose gel electrophoresis.

4.2.8.2 Purification of PCR Product

The five PCR reactions for each extracted DNA sample were combined and purified using the QIAquick PCR Purification Kit (Qiagen, Australia), as per the methods. Briefly 80µl of the extracted DNA sample was mixed with 400µl buffer PB, then applied to the QIAquick spin columns. Samples were centrifuged for 1min at 17,800xg, with the flow through being discarded. The columns were washed with 750µl of buffer PE, and centrifuged twice to remove excess buffer PE. The spin columns were then transferred to new 1.5ml Eppendorf tubes, and 50µl of buffer EB was added to the centre of the column. The spin columns were incubated at RT for 1min, then centrifuged for a further 1min at 14,000rpm to collect the purified PCR product.

4.2.8.3 Restriction Digest

Purified PCR was digested with HinfI restriction enzyme (Promega, USA). Digestion of the 477bp PCR product produces two bands at 425bp and 52bp for the wild type CC, four bands at 425bp, 260bp, 165bp and 52bp for the heterozygous mutation CT and 3 bands at 260bp, 165bp and 52bp for the homozygous mutation TT. Each enzyme digest contained 500ng purified PCR product, 1.2U of HinfI, 1x Buffer B (Promega, USA) and 2µg acetylated

BSA (Promega, USA), made up to a total reaction volume of 20µl with water for inject (Pfizer, USA). Digestion was for 3 hours at 37°C with a 15 minute stop reaction at 65°C. Reactions were then visualised on a 1% agarose gel (section 2.1.6.3) to confirm the correct size DNA fragments.

4.2.8.4 Sanger Sequencing

Sequencing was performed on all purified MTHFR PCR products for each patient using the forward primer 5' AGGCTGTGCTGTGCTGTTG 3' and the reverse primer 5' CGCTCTGCAAGTTCTGGA 3' to confirm polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) results. Sequencing was performed by Lottery West State Biomedical Facility Genomics, Department of Clinical Immunology and Biochemical Genetics, Royal Perth Hospital. The sequence was aligned and analysed using the Chromas Lite 2.0 sequencing analysis program.

4.2.9 Factor V Leiden (FVL) and Prothrombin G20210A Mutation

Factor V Leiden (FVL) and Prothrombin G20210 mutations were tested in both the cases and control, with a protocol supplied by Pathwest Molecular Haematology, Royal Perth Hospital (Perth Western Australia), utilising Life Technologies, predesigned TaqMan SNP Genotyping assays. A 25ul reaction was carried out for each DNA sample, containing 12.5µl of 2x TaqMan Genotyping master mix (Applied Biosystems, USA), 0.325µl of the selected TaqMan® SNP genotyping Kit (80x) (Applied Biosystems, USA), 9.685µl of

sterile water and 2.5μl of DNA (~30ng/μL). A known wild type, heterozygous mutation and a homozygous mutation DNA sample was also analysed as a control.

4.3 Results

Table 4.2 Pregnant Cases and RM control Characteristics.

		Pregnancy control	***Recurrent Miscarriage Cases***		
			Total	**Non-pregnant**	**Pregnant**
		20	**15**	**10**	**5**
age in years	**mean (SD)**	32.7 (3.97)	34.5 (4.17)	35.4 (3.03)*	33.2 (6.02)
	range	(26-41)	(26-41)	(29-41)	(26-37)
BMI in kg/m2	**mean (SD)**	25.9 (4.48)	24.3 (5.19)	21.7 (2.16)*	29.4 (5.86)
	range	19-33	19-37	19-26	26-37
pregnant	**n**	18 (90%) ^	5 (33.33%)	0	5 (100%)
Recurrent miscarriages	**mean n (SD)**	0	3.13 (0.99)	3.18 (1.32)	3.6 (0.89)
	range	0	2-5	2-5	3-5
	weeks occurred (range)	0	4-12	5-12	4-10
IVF	**n**	5 (25%)	3(20%)	2 (20%)	1 (20%)
Environmental factors	**hormonal imbalance**	1	1	0	1
	Smoking	2	2	1	1
	Drug Use	1	0	0	0
	Malnutrition	0	0	0	0
	Excessive caffeine	0	0	0	0
	Radioactive Isotypes	0	1	1	0
Folate supplementation in ug/day	**N**	18 (90%)	14 (93.3%)	9 (90%)	5 (100%)
	mean (SD)	619.4 (245.6)	878.6 (1208.4)	522.2 (263.5)	1520 (1951.2)*
	range	250-1000	300-5000	300-800	500-5000
Medical conditions associated with pregnancy#	**N**	2(10%)	9 (60%)	6 (60%)	3 (60%)
					Laboratory findings
Folate Receptor alpha autoantibody in frU	**Mean titre**	11.3	19.2	21.7	13.0
	Negative (≤9.06 frU)	13 (65%)	8 (53.3%)	5 (50%)	3 (60%)
	low (9.06 - 19.76 frU)	3 (15%)	1 (6.67%)	0	1 (20%)
	High (≥19.76 frU)	4 (20%)	6 (40%)	5 (50%)	1 (20%)
Serum folate Level in nmol	**mean (SD)**	54.9 (26.9)	55.6 (22.9)	60.7 (24.4)	43 (14.4)
	range	16.8-64.6	23.8-77.3	35.3-77.3	23.8-58.5
	Low (<7nmol)	0	0	0	0
	Normal (7-40nmol)	3 (15%)	2(14.2%)	1 (10%)	1 (25%)
	High (>40nmol)	17 (85%)	12 (85.7%)	9(90%)	3 (75%)
Red blood cell folate in nmol	**mean (SD)**	1917.9 (521.8)	1736.1 (643.3)	1712.4 (685.1)	1795.2 (616.8)
	range	1061-3221	821-3206	821-3206	1405-2623
	low (<260nmol)	0	0	0	0
	normal (260-1450nmol)	3 (15%)	5 (37.5%)	3 (30%)	2 (50%)
	High (>1450nmol)	17 (85%)	9 (62.5%)	7 (70%)	2 (50%)

Table 4.2 Continued.

		Pregnancy control	Recurrent Miscarriage Cases		
			Total	Non-pregnant	Pregnant
		20	15	10	5
homocysteine in µmol	mean (SD)	4.99(1.44)	7.35 (2.30)*	7.78 (2.42)*	6.3 (1.83)
	range	3.8-8.5	4.4-13.5	5.2-13.5	4.4-8.4
	low (<6µmol)	16 (80%)	3 (21.4%)	1 (10%)	2 (50%)
	normal (6-12µmol)	4 (20%)	10 (71.4%)	8 (80%)	2 (50%)
	High (>12 µmol)	0	(1 (7.14%)	1 (10%)	0
MTHFR C677T mutation	WT	17 (85%)	11 (73.3%)	7 (70%)	4 (80%)
	heterozygous	2 (10%)	3 (20%)	3 (30%)	0
	homozygous	1 (5%)	1 (6.66%)	0 (0%)	1 (20%)
FVL mutation	WT	20 (100%)	14 (93.3%)	9 (90%)	5 (100%)
	heterozygous	0	1 (6.66%)	1 (10%)	0
	homozygous	0	0	0	0
Prothrombin X G20210A mutation	WT	20 (100%)	20 (100%)	10 (100%)	5 (100%)
	heterozygous	0	0	0	0
	homozygous	0	0	0	0
Free Protein S (%)	Mean (SD)	50.1 (14.2)	80.7 (28.5)*	57.5 (19.2)	91.0 (26.3)*
	Low (<55)	14 (70%)	2 (13.33%)	0	2 (50%)
	Normal (55-150)	6 (30%)	13 (86.7%)	9 (100%)	2(50%)
Protein C (%)	Mean (SD)	113.4 (23.1)	104.2 (17.7)*	100.8 (19.8)*	112.0 (9.59)
	Low (<70)	2 (10%)	0	0	0
	Normal (70-150)	18 (90%)	13 (100%)	9 (100%)	4 (100%)
Anti-Cardiolipin antibodies IgG in GPL-U/ml	mean (SD)	1.9 (3.21)	0*	0*	0
	Normal (<10)	20	15	10	5
	high (>10)	0	0	0	0
Anti-Cardiolipin antibodies IgM in MPL-U/ml	mean (SD)	1.80 (2.76)	2.74 (4.27))	3.55 (4.84)	1.11 (2.49)
	Normal (<7)	19 (95%)	13 (86.7%)	8 (80%)	5
	high (>7)	1 (5%)	2 (13.3%)	2 (20%)	0
Anti-beta-2-Glycoprotein IgG in GPL-U/ml	mean (SD)	0	0	0	0
	Normal (<5U/ml)	20	15	10	5
	Borderline (5-8U/ml)	0	0	0	0
	elevated (>8U/ml)	0	0	0	0
Anti-beta-2-Glycoprotein IgA in APL-U/ml	mean (SD)	0.48 (1.83)	0	0	0
	Normal (<5U/ml)	19 (95%)	15	10	5
	Borderline (5-8U/ml)	0	0	0	0
	elevated (>8U/ml)	1 (5%)	0	0	0

*significant difference compared to pregnant controls (p<0.05)
^2 just given live birth
#Medical Conditions associated with pregnancy included, PCOS, endometriosis, hyperthyroidism, low protein S, infertility, Chromosomal Abnormalities, Factor V Leiden mutation, Hypertension, gestational diabetes.

4.3.1 Subjects

There were a total of 15 recurrent miscarriage (RM) case women who had experienced >2 consecutive miscarriages and 20 pregnant control women analysed in this study. Each case and control subject completed a questionnaire related to their pregnancy and medical history, and donated blood for standard laboratory testing to identify factors related to recurrent miscarriage and FRα autoantibody testing.

4.3.2 Subject Characteristics

Table 4.2 describes the subject characteristics, history and laboratory results in both pregnant controls and RM cases. RM cases were further grouped into either non-pregnant or pregnant cases, to identify if there was any difference in these two groups and the pregnant controls. The median number of recurrent miscarriages to occur was three with a range of two up to six recurrent miscarriages. All miscarriages in the RM cases were in the first trimester and ranged between 4-12 weeks.

4.3.2.1 Age, BMI and History

The average age was 32.7 and 34.5 for pregnancy controls and RM cases, respectively (Table 4.2). There was no significant difference between the cases and controls, with an age distribution of 26-41years for both groups. However, when the RM cases were separated into non-pregnant and pregnant cases with mean ages of 35.4 and 33.2, respectively, the RM non-

pregnant cases were significantly older than the pregnancy controls (p=0.04).

There was no significant difference in the BMI of the RM cases and pregnant controls with averages of 25.9 and 24.3 (Table 4.2). However, analysis of the pregnant and non-pregnant RM cases individually showed that RM non-pregnant cases had a significantly lower BMI to both pregnant controls [21.7 vs 25.88, p=0.005] and pregnant RM cases [21.7 vs 29.4, p=0.001].

The questionnaire answered by each of the participants asked simple questions regarding environmental factors and medical history that could relate to pregnancy complications and RM. The incidence of women with environmental factors that could affect the pregnancy outcome was low (Table 4.2). Both pregnancy controls and RM cases had equal numbers of women with hormonal imbalances (n=1) and smoking (n=2). There was one pregnant control that used recreational drugs and one RM case had exposure to radio isotopes from kidney stent. There was no case or control that had malnutrition or excessive caffeine intake.

There was a higher proportion of RM cases (60%) that suffered from medical conditions that could be a risk factor for RM, compared to the pregnancy controls (10%) (Table 4.2). Medical conditions associated with pregnancy included, PCOS, endometriosis, hyperthyroidism, low protein S, infertility, chromosomal abnormalities, FVL mutation, hypertension and gestational

diabetes. The occurrence of these medical conditions individually was only one in all subjects except for endometriosis which occurred in two RM cases and one pregnant control.

4.3.2.2 Folate Status

Folate status including folate supplementation, serum folate levels, red blood cell folate levels and homocysteine levels were all assessed to see if there was any significant differences between cases and controls and to determine if they were an identifiable risk factor for RM in this study (Table 4.2). Folate supplementation was present in almost all cases and controls, 90% for pregnancy controls and 93% for RM cases. There was a difference in the types of folate supplementation taken, from normal multi vitamins to pregnancy vitamin formulations, and folate supplementation alone. As a result, variation in the dose of folic acid taken daily was evident in all subjects, ranging between 300-5000µg/day. The average dose of folic acid in pregnancy controls was 619.4µg/day while the RM cases was 878.6µg/day, which was slightly higher but not significantly different. When the two RM groups were analysed separately the RM pregnant cases had a significantly higher intake of folic acid compared to pregnant controls [1520 vs 619.44, p=0.027]; however, it should be noted that one of the RM pregnant cases was taking 5000µg/day of folic acid.

Red blood cell folate and serum folate levels were also measured in both cases and controls and showed no significant difference between to the two groups. When looking at the serum folate levels, no cases or controls

had levels in the low range (<7nmol), with 80% with levels above normal serum folate levels (>40nmol). RM non-pregnant cases did seem to have a slightly higher serum folate level than the pregnant controls and RM pregnant cases however this was not significant. This was also seen in the red blood cell folate levels, where over 60% in both cases and controls had high levels (>1450nmol) and no subjects fell into the low-level range (<260nmol).

There was only one subject that had a high homocysteine level (>12μmol) which was a RM non-pregnant case. The mean level of homocysteine in pregnant controls was 4.98 μmol, defined as within the low range (<6μmol) of homocysteine, and was significantly different to the RM cases and more specifically the RM non-pregnant cases which had means associated with a normal homocysteine range (6-12μmol) of 7.35μmol and 7.78μmol, respectively [p=0.0003 and p=0.0001]. However, the RM non-pregnant cases were not significantly different to the mean RM pregnant cases (6.3μmol) (Table 4.2). Figure 4.1 demonstrates the distribution of homocysteine levels in RM cases and control. Overall there was a higher number of control subjects that fell within the low homocysteine level (80%) compared to the greater proportion of RM cases which had homocysteine levels in the normal range (71.4%).

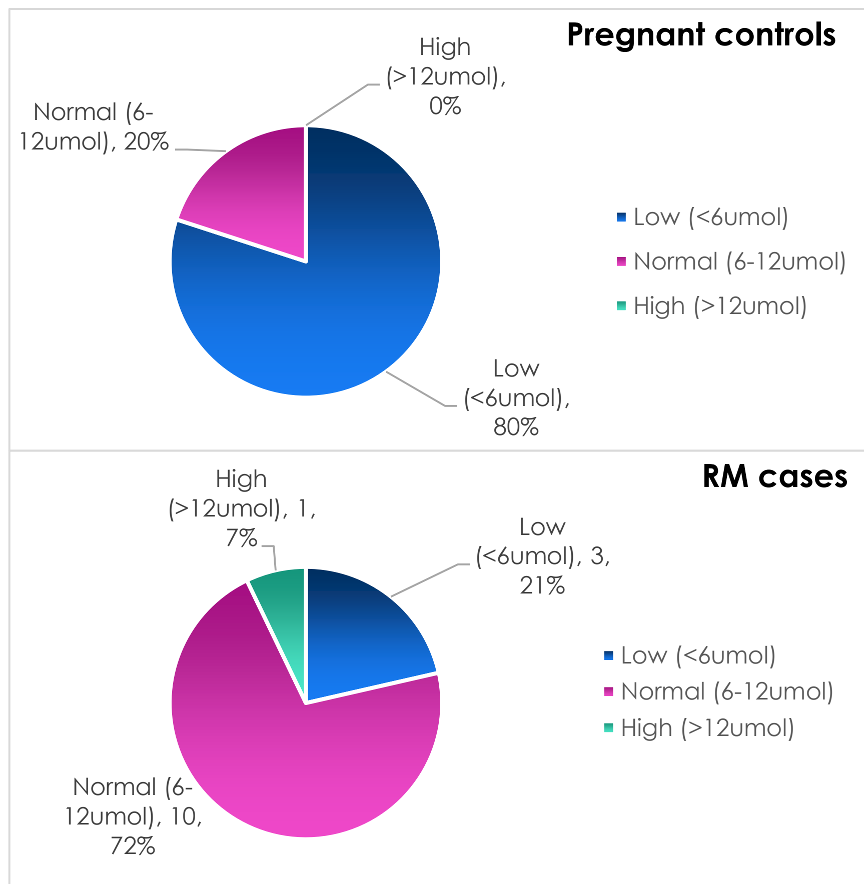


Figure 4.1. Percentage of RM cases and controls in Low, normal and high range homocysteine levels.

4.3.2.3 Laboratory Tests

Several thrombophilia markers that are possible risk factors for RM were analysed in both RM cases and pregnant control subjects (Table 4.2). Variants analysed were the MTHFR c.677C>T mutation, Prothrombin G20210A mutation and Factor 5 Leiden (FVL). The incidence of these mutations were low in both cases and controls, and no significant difference was identified in any of the three mutations. MTHFR c.677C>T homozygous mutation was

identified in 1 case and 1 control subject, where 2 pregnant controls and 3 RM cases had the heterozygous mutation. There was only one FVL heterozygous mutation identified in the whole study which was one RM case, the remaining cases and controls were wild-type. No Prothrombin G20210A mutations were identified in either RM cases or pregnant controls.

The two common thrombophilia proteins, protein C and protein S, were analysed to identify possible deficiencies. Low protein S was identified in 70% of pregnancy controls which is to be expected during pregnancy and only in 13.33% of RM cases yet when the RM cases are analysed into their separate groups, 50% of the RM pregnant cases have a low protein S level. The mean protein S level between both total RM cases (80.7%) and RM non-pregnant cases (91.0%) are significantly higher than the pregnant controls (50.1) [p=0.0001, p=<0.0001] and pregnant RM cases (57.5)[p=0.022]. There was no significant difference in protein C levels between cases and controls, however there were two pregnant controls that were identified as low protein C.

The 15 recurrent miscarriage cases and 20 pregnant controls were investigated for the routinely tested APS antibodies: Anti-Cardiolipin IgG, IgM and Anti-beta-2-Glycoprotein IgG and IgA antibodies. The mean level of anti-cardiolipin IgG was significantly higher in pregnancy controls than RM cases [1.9 vs 0, p=0.012], however they all fell within the normal level for these antibodies (<10 GPL-U/ml) (Table 4.2). The prevalence of high anti-cardiolipin IgM titres in RM cases was 13.33% compared to 5% in pregnancy

controls however the levels were not significantly different. The incidence of Anti-β2-glycoprotein IgG and IgA antibodies in both RM cases and pregnant controls was negligible, with only one pregnant control having a borderline antibody level for anti-β2-glycoprotein IgA antibody.

4.3.3 FRα Autoantibodies

Overall, the incidence of FRα autoantibodies (IgG,A,M) in both RM cases and pregnancy controls was relatively high at 46.7% and 35%, respectively. There was an overall trend showing that RM cases had a higher incidence of FRα autoantibodies compared to pregnant controls, and even more when RM pregnant subjects were omitted from the RM cohort, as seen in figure 4.2 showing the distribution of FRα autoantibody titres. This can also be seen in Figure 4.3 demonstrating the difference in the mean levels of FRα autoantibodies in pregnant controls and RM cases (total, pregnant and non-pregnant). The mean level of FRα autoantibodies in RM cases was 19.2 FrU compared to only 11.3 FrU in the pregnancy controls (Table 4.3). When the RM case group was adjusted to remove pregnant cases from the analysis, the mean for RM non-pregnant cases was higher again at 22.3 FrU. However, when analysed by Cox regression the difference was not significant ($p=0.39$) even when adjusted for age, BMI, smoking and homocysteine levels ($p=0.49$).

The FRα autoantibody titres were categorized into negative (≤9.06 frU), low positive (9.06 - 19.76 frU) and high positive (≥19.76 frU) levels. Pregnant

controls had 13 (35%) negative, 3 (15%) low positive and 4 (20%) high positive, while RM cases had slightly higher antibody levels with 8 (53.33%) negative, 1 (6.67%) low positive and 6 (40% high positive) (Table 4.3). The percent of subjects to have a high level of FRα autoantibodies (≥19.76 frU) in RM cases (6, 40%) was double the incidence in pregnant controls (4, 20%). When RM cases were adjusted to exclude pregnant cases the incidence of high FRα autoantibody titre was increased to 50%. Although this illustrates a higher proportion of RM cases with a higher FRα antibody titre there was no significant difference demonstrated when analysing the data using Chi χ^2 test, nor when it was adjusted for homocysteine, age, and smoking.

Table 4.3 FRα autoantibody titres in serum of pregnant controls and RM cases

		Pregnant Controls	RM Cases		
			Total	Non-pregnant	Pregnant
	Number of patients	**20**	**15**	**10**	**5**
FRα Titre FrU	**Mean (SD)**	11.29 (16.75)	19.19 (20.53)	22.28 (22.74)	13.02 (15.51)
FRα autoantibody Incidence	**Negative (≤9.06 frU)**	13 (65%)	8 (53.33%)	*5 (50%)*	*3 (60%)*
	low positive (9.06 - 19.76 frU)	3 (15%)	1 (6.67%)	0	*1 (20%)*
	High positive ≥19.76 frU)	4 (20%)	6 (40%)	5 (50%)	*1 (20%)*
Statistics	**Minimum**	0	0	0	1.443
	25% Percentile	0.4128	1.443	0.3904	2.279
	Median	4.58	8.259	17.94	8.151
	75% Percentile	16	36.08	40.23	26.21
	Maximum	66.15	60.22	60.22	39.6
	Lower 95% CI of mean	3.451	7.823	6.011	-6.239
	Upper 95% CI of mean	19.13	30.56	38.54	32.29

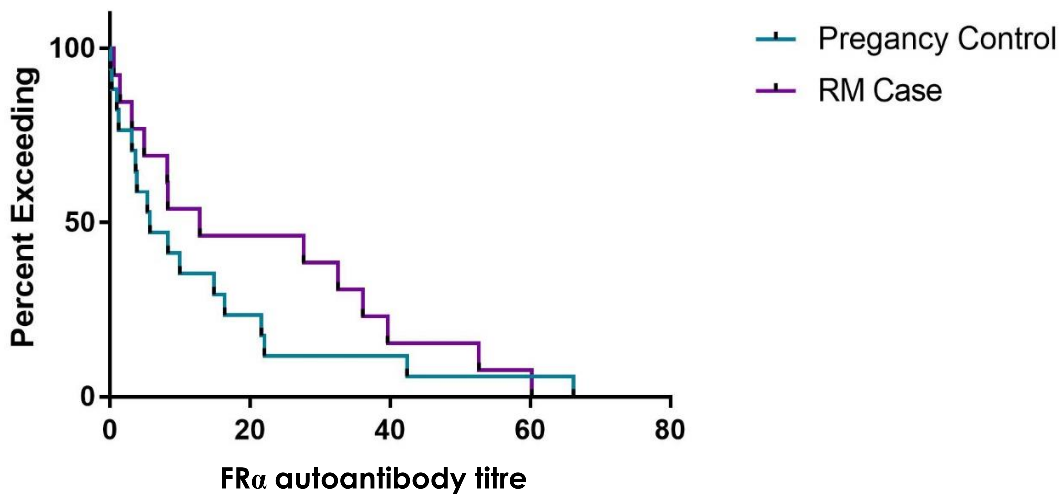


Figure 4.2 FRα autoantibody distribution curves between RM cases and pregnant controls.

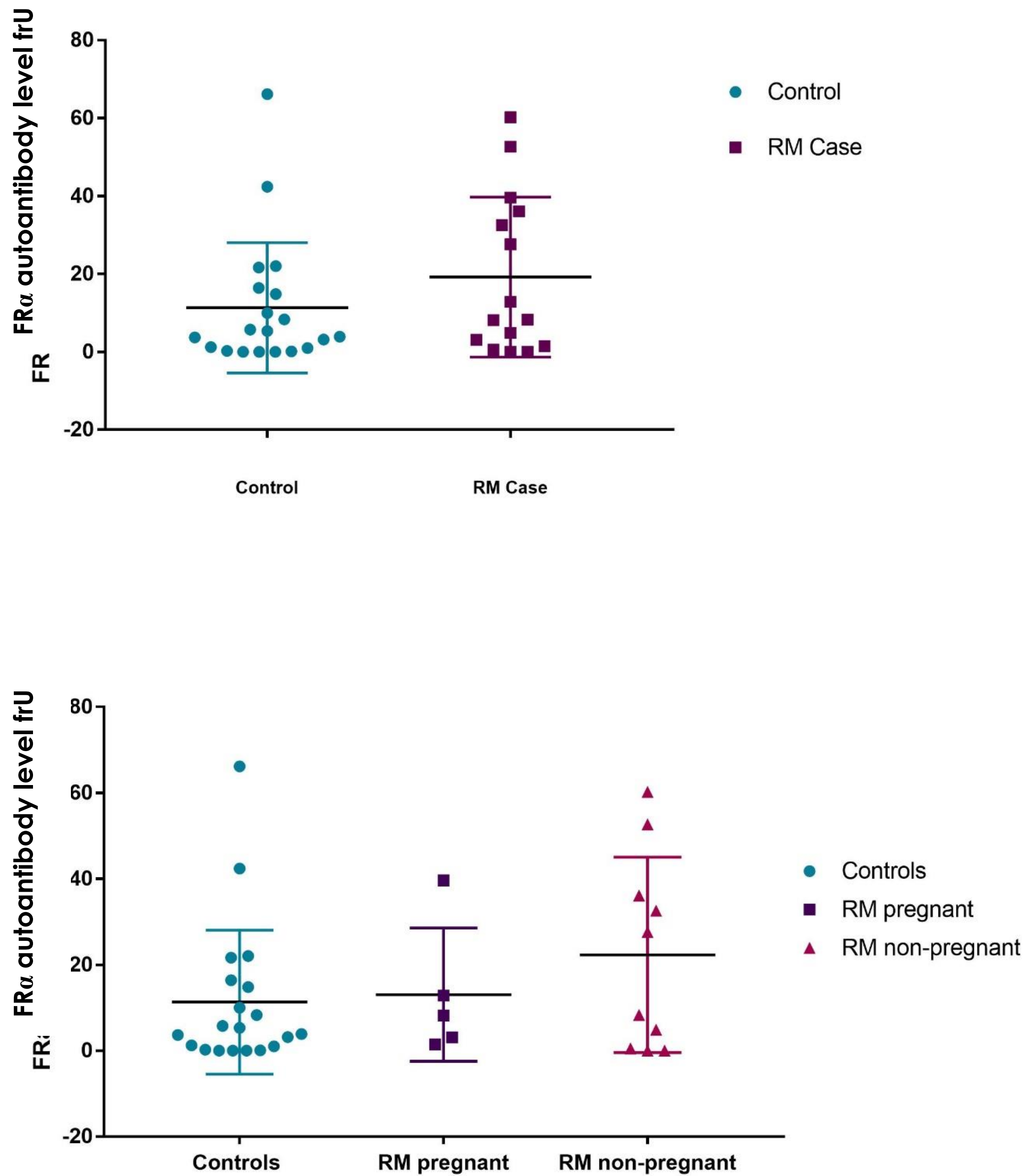


Figure 4.3 Mean levels of FRα autoantibodies. A) pregnancy controls vs RM cases and B) pregnancy controls and RM cases sub groups non-pregnant and pregnant cases.

4.3.3.1 FRα Autoantibodies IgG Isotypes

Further analysis was performed to identify if the positive FRα autoantibodies detected in pregnant controls and RM cases were IgG and the IgG isotype; IgG1, IgG2, IgG3 and IgG4. Figure 4.4 demonstrates the prevalence of FRα IgG compared to the total FRα IgG, A, M autoantibodies in 7 positive pregnant controls and RM cases. IgG FRα autoantibodies were detected in 4 of the pregnant controls and 5 in the RM cases (Table 4.4). There were also two of pregnant controls and 1 RM case that had very low levels of IgG (<5), that were not further investigated to identify IgG isotypes.

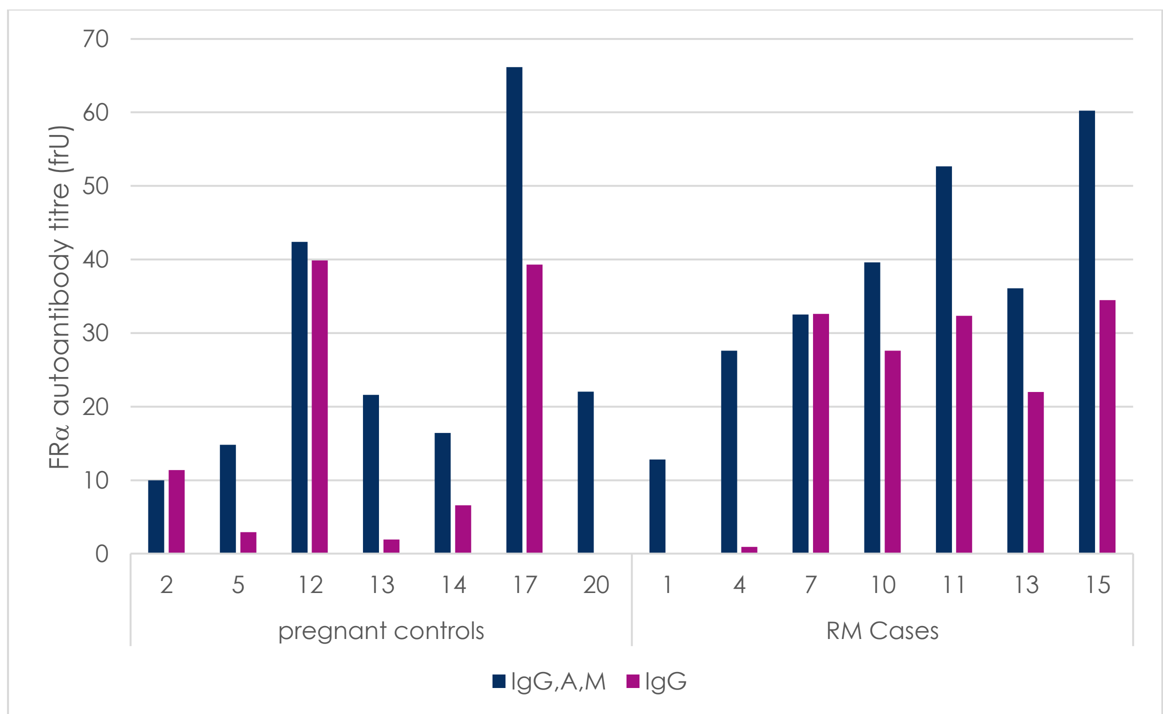


Figure 4.4 Distribution of FRα autoantibody's (IgG,A,M) and IgG subtype alone in pregnancy controls and recurrent miscarriage controls. Values are in mean absorbance.

Table 4.4 Identification of antibody IgG and IgG isotypes in subjects positive for the FRα autoantibody. (Positive classified as an absorbance over 0.100)

	IgG	***IgG Isotypes***			
		IgG1	**IgG2**	**IgG3**	**IgG4**
Pregnancy Control (n=8)	4 (50%)	3 (75%)	1 (25%)	1 (25%)	1 (25%)
RM cases (n=8)	5 (62.5%)	4 (80%)	5 (100%)	0	1 (20%)

IgG isotyping for IgG1, IgG2, IgG3 and IgG4 is detailed in Table 4.4 and Figure 4.5. All IgG isotypes were prevalent in the pregnant control group with 75% of controls having IgG1, 25% IgG2, 25% IgG3 and 25% having IgG4. Figure 4.3 illustrates that one pregnant control (PC2) was positive for 75% of the isotypes being IgG1, IgG3 and IgG4. Pregnant control 17 was also positive for 2 isotypes IgG1 and IgG2, this was also the only control to be positive for IgG2. The last two controls were positive for IgG1 only. RM cases had a slightly different isotype distribution, with IgG3 lacking detection in any of the IgG positive cases (Table 4.4). All (100%) of the RM cases were positive for the IgG2 isotype followed by 80% having IgG1, with only 1 (20%) having IgG4. RM Case 11 was positive for all three isotypes IgG1, IgG2, and IgG4.

There was a significant trend identified for prevalence of IgG1 and IgG2 between cases and controls. IgG2 had a significantly higher mean titre (1.071 vs 0.1226, p=0.0433) and positive detection in RM cases (100%) compared to the pregnancy controls (20%). While IgG1 had an elevated

mean in pregnant controls compared to RM cases, as seen in figure 4.6, however this was not significant.

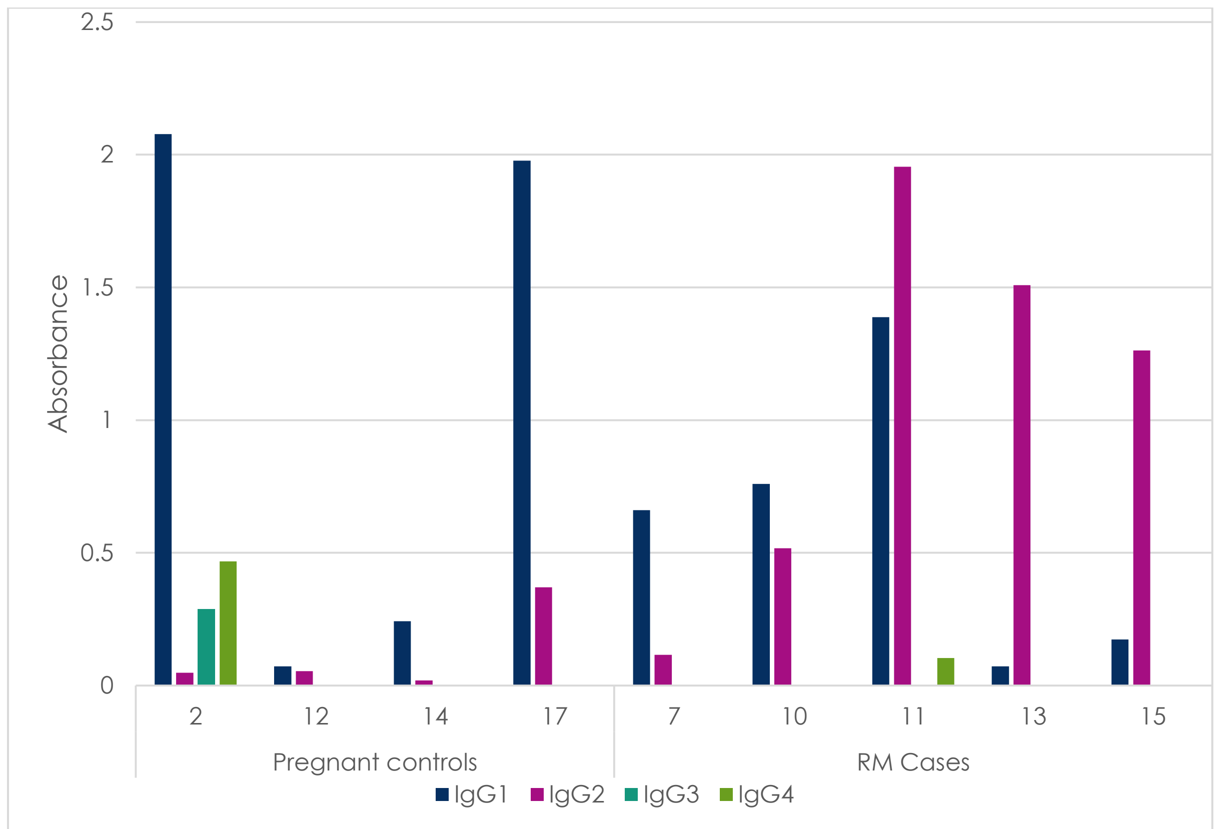
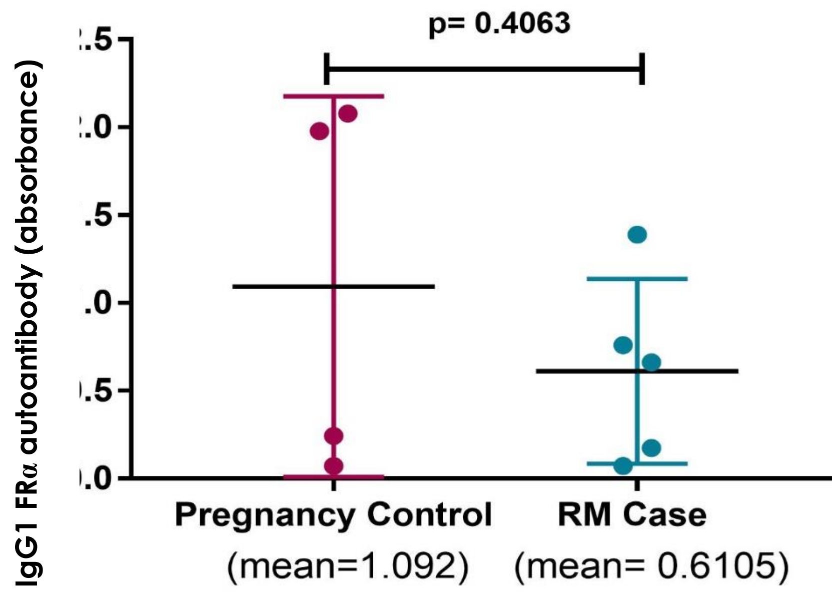


Figure 4.5. IgG isotype distribution between pregnant controls and RM cases.



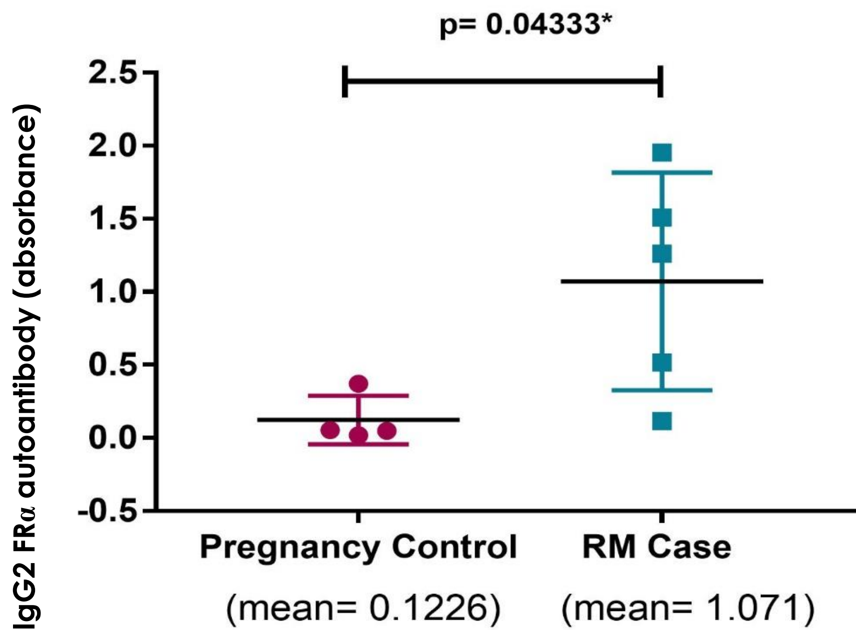


Figure 4.6 FRα autoantibody Isotype IgG1 and IgG2 distribution for pregnant controls and RM cases.

4.4 Discussion

The prevention of RM by folic acid supplementation has been well documented, yet in some studies there are still contradicting results regarding whether or not folate deficiency is a risk factor for RM (Gaskins, Rich-Edwards, *et al.* 2014, Govindaiah *et al.* 2009, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., Den Heijer M., Thomas C. M. G., *et al.* 2000). Notably, most studies focusing on the link between folate deficiency and pregnancy complications test maternal folate levels only; which are generally normal due to folate supplementation; and therefore may mask what is actually happening at the embryonic level (Gaskins, Rich-Edwards, *et al.* 2014, Govindaiah *et al.* 2009, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., Den Heijer M., Thomas C. M. G., *et al.* 2000). Consequently, inhibited folate transport across the placenta could be the reason behind these two opposing results. Inhibited folate transport has been demonstrated with FRα autoantibodies; that target the main folate transporter (FRα) in the placenta, in other folate deficiency related pregnancy complications such as NTD, subfertility and pre-term birth (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Billie *et al.* 2010, Bliek *et al.* 2006, Boyles *et al.* 2011, Cabrera *et al.* 2008, Frye *et al.* 2016, Frye *et al.* 2014, Frye *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004, Sequeira *et al.* 2013, Shapira *et al.* 2015, Vo *et al.* 2015). Yet to date FRα autoantibodies have not been analysed in a recurrent miscarriage cohort. This study investigated the role of maternal FRα autoantibodies in recurrent miscarriage. A cohort of 15 RM cases and 20 pregnancy controls

were collected from Hollywood Private Hospital (Western Australia, Australia), with each patient donating blood for laboratory testing, and completing a survey to identify patient characteristics and medical and pregnancy related history.

Our findings demonstrated a total incidence of FRα autoantibodies in RM cases to be 46.7% compared to only 35% in pregnant controls. The overall trend identified in this study suggested that RM cases had a higher incidence and titre of FRα autoantibodies; the mean antibody titre for RM cases being 19.19 frU while the pregnant controls FRα autoantibody titre was 11.29; although these results were found not to be significant. While analysing the pregnancy history it was discovered that 5 out of the 15 cases were pregnant beyond 12 weeks. As a result, further analysis was performed omitting the pregnant recurrent miscarriage cases, and therefore analysing only the women that suffer from RM and have yet to have a successful pregnancy since there last miscarriage. The results from non-pregnant RM cases compared to pregnant controls strengthened the trend that RM cases had a higher titre and incidence for FRα autoantibodies. This is seen where the mean titre increased from 19.19 frU in total RM cases to 21.7 frU in non-pregnant RM cases, in addition the total incidence of FRα autoantibodies in the RM non-pregnant cases increased to 50% compared to 46.7%. Even though RM non-pregnant cases in this study have a higher incidence of FRα autoantibodies compared to pregnancy controls, it is still not significantly different. The low levels of autoantibodies seen in pregnancy controls may not be enough to cause folate deficiency, unlike the high titre FRα

autoantibodies seen in a higher proportion of RM cases. The level of autoantibody required to deprive the embryo of folic acid or cause issues is unknown, however it has been identified that low levels of FRα autoantibodies do not reduce folate levels, and therefore be one reason why pregnant controls with low levels of FRα autoantibodies have no associated disease (Berrocal-Zaragoza M. I. *et al.* 2009).

Although there was no significant association demonstrated between RM and the presence of FRα autoantibodies, RM cases did however demonstrate a slightly higher titre than what was seen in the pregnancy controls, therefore these autoantibodies should not be ruled out as a risk factor. A case study published late 2015, analysed FRα autoantibodies over several time points in a female who had experienced pregnancy complications (Shapira *et al.* 2015). The patient was negative for prothrombin G20210A mutation, Factor V Leiden, cardiolipin antibody and lupus anticoagulant, however heterozygous for MTHFR mutation A1298C. At the time of initial FRα autoantibody screening the patient had already suffered subfertility, two recurrent miscarriages in the first trimester and twins that were terminated due to encephalocele, hydroplastic heart syndrome and choroid plexus cysts. Initial testing revealed high titres of FRα autoantibodies in the patient's serum, who was subsequently placed on a milk free diet; known to reduce FRα autoantibodies; and demonstrated a reduction in antibody titres. IVF was undertaken once the antibody levels had decreased, but were not completely eliminated, and unfortunately the

following pregnancy also resulted in a miscarriage. A higher dose of folic acid (4mg/day) was later introduced as well as maintaining a milk free diet, resulting in an undetectable level of FRα autoantibodies, and subsequently a normal conception pregnancy and successful live birth at 40 weeks. Granted this was a case-study only looking at one individual's history of RM, and although no direct association can be determined between FRα autoantibodies it does suggest that the elimination of FRα autoantibodies can result in a successful, full-term pregnancy, and raises the possibility that FRα autoantibodies may represent a risk factor for RM.

There has only been one study performed to identify the prevalence of FRα autoantibodies in the general population, while collectively control cohorts have identified healthy patients with FRα autoantibodies levels ranging between 0-30% (Berrocal-Zaragoza M. I. *et al.* 2009, Bliek *et al.* 2006, Boyles *et al.* 2011, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2005, Vo *et al.* 2015). The occurrence of FRα autoantibodies in both cases and controls in this study was relatively high demonstrating 2-3 fold higher incidence compared to FRα autoantibodies identified in the stroke cohort analysed in chapter 4 (35-50% vs 15.5%). Furthermore, a 5-7 fold difference is evident when comparing our results to a study by Berrocal-Zaragoza et al (2009) looking at the prevalence of FRα autoantibodies in the general Spanish population which demonstrated a 7.2% antibody incidence. An interesting finding is that the presence of FRα autoantibodies in our pregnant control population is considerably high compared to the healthy population study (7.2%) or other healthy control groups, however studies which have a pregnant control

group have also presented comparable results to ours. A study carried out by Bliek *et al* (2006) into cleft lip palette demonstrated 30% of their pregnant control population to have FRα autoantibodies, while a more recent study looking at pre-term birth revealed 28% of the pregnant control population to have FRα autoantibodies which is comparable to our pregnant control group (Bliek *et al.* 2006, Vo *et al.* 2015). The reason behind the high incidence of FRα autoantibodies in these pregnant control populations is unknown, however it is known that autoimmunity is up to five times more common in women, especially during pregnancy (Nelson 1996).

There are five antibody classes IgG, IgA, IgM, IgD and IgE, however IgG is the most abundant immunoglobulin in the human body (Janeway CA Jr 2001). IgG can be further categorized into four isotypes IgG1, IgG2, IgG3, and IgG4 which all have different effector functions and distributions within the body, and the pattern of IgG isotypes can often inform you of the type of antigen response (Vidarsson *et al.* 2014). In this study, we analysed the isotypes of IgG in positive FRα IgG autoantibody titres to determine if there was any significant difference between IgG isotypes in pregnant controls and RM cases. IgG1 titres were relatively high in both cases and controls with no significant difference identified. What is interesting is that our results demonstrated that the isotype IgG2 was present in all FRα IgG autoantibody positive RM cases and was significantly different to the pregnant controls, where only one control was positive for IgG2. There is not a lot of data on the differences in IgG isotype classes from other studies analyzing FRα autoantibodies, as they either use an assay utilizing labelled folic acid or

they look and IgG alone with no further subtyping (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Frye *et al.* 2016, Frye *et al.* 2014, Frye *et al.* 2013, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Rothenberg *et al.* 2004, Shapira *et al.* 2015, Vo *et al.* 2015). A number of studies have analysed the presence of IgG and IgM in patient serum, yet only two studies have reported specific IgG1, IgG2, IgG3, IgG4 prevalence (Billie *et al.* 2010, Boyles *et al.* 2011, Cabrera *et al.* 2008, Ramaekers *et al.* 2008, Sequeira *et al.* 2013). Sequeira *et al* (2013) reported the difference in isotypes between NTD, CFD and ASD. All cases of NTD and ASD had IgG1 present while only 68% of CFD cases were positive. However, CFD had a significantly high incidence of IgG4 antibodies (79%). IgG2 was only detected in NTD patients (40%) and ASD (14%) which is a much lower incidence to what we have detected in our RM cases. The overall isotyping of FRα autoantibodies in RM cases does not follow any pattern that is seen reported in Sequeira *et al* (2013) for NTD, ASD or CFD. Nonetheless the high incidence of FRα autoantibodies IgG2 in RM cases yet lacking in pregnant controls, may be indicative as causative for miscarriage in the current cohort.

There are many factors that have thought to be a risk factor for RM including autoimmune disease like APS, genetic and chromosomal abnormalities, deficiencies in addition environmental factors (Chapman *et al.* 2012, Gutaj *et al.* 2013, Kuon *et al.* 2015, Lo *et al.* 2012, Vinatier *et al.* 2001). This study aimed to collect as much information regarding pregnancy health as possible as well as factors related to folate deficiency, and analysis was

carried out to identify any differences between RM cases and controls. In this study, there was no significant associations identified for RM related risks including β2-glycoprotein IgG, or IgA antibodies, MTHFR c.C>T677, FVL, or Prothrombin G20210A mutations, however there was a negative association seen for both anticardiolipin antibodies IgG and IgM, however the antibody titres fell within the negative range.

Environment factors that can affect a pregnancy include, age, weight, smoking, recreational drug use and caffeine intake (Chapman *et al.* 2012, Ford *et al.* 2009, Lo *et al.* 2012). When analyzing total RM cases compared to pregnancy controls, there was not significant difference in these factors. However, when pregnant RM cases were excluded from the analysis, the mean age and BMI of non-pregnant RM cases was significantly higher than pregnant controls. The significant difference seen in the mean BMI between pregnant controls and non-pregnant cases could possibly be due to the fact that the pregnant controls are carrying a baby, and they are expected to have put on weight. Ideally information on the BMI of the pregnant control before they fell pregnant would be useful in order to see if this is a true association.

Folate status in RM miscarriage cases has been well studied and its association to RM is still controversial. This study analysed serum folate levels, red blood cell (RBC) folate levels and homocysteine levels in both cases and controls. No pregnant control or RM cases had a low level of either serum or RBC folate levels, and data identified that a high proportion of both cases

and controls had high levels of folate. The reason behind this is that over 90% of both cases and controls used folic acid supplementation daily at concentrations ranging between 300-5000µg/day. Furthermore, multiple studies analyzing FRα autoantibodies in either NTD, subfertility or CFD have demonstrated that the presence of FRa autoantibodies have not affected normal circulating folate levels (Boyles *et al.* 2011, Cabrera *et al.* 2008, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2005, Ramaekers, Sequeira*, et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004, Vo *et al.* 2015) .Yet this may not tell the story for what is actually happening in the placenta and the developing embryo, which is the most important. What would be a more appropriate determination of folate status in RM would be to detect folate levels in the fetal circulating blood, however accessing these blood samples is very impractical and risky for the baby.

At high levels, homocysteine; which is known to increase due to folate deficiency; can cause adverse effects to the developing embryo including impaired chronic villous vascularisation (Creus *et al.* 2013). Results demonstrated that there was in fact a significantly higher level of homocysteine in RM cases compared to pregnant controls. Nonetheless the mean level of homocysteine found in RM cases was well within the normal range of acceptable levels whereas in the pregnancy controls the mean was below the normal range. Because the mean level in RM cases was not high, we do not propose that it is a risk factor for RM in our study. Like, with folate levels, what would be beneficial would be the ability to analyse the homocysteine level in the developing embryo to see its true significance and

also if the presence of FRα autoantibodies does have an association with hyper-homocysteinemia.

The need to study all risk factors in a RM cohort we believe to be important, as the FRα autoantibodies themselves may not be enough to cause RM. However, the presence of another or multiple underlying factors; such as APS or any genetic disorder affecting embryo development; may be just enough to cause a miscarriage when working in combination with each other. Frye *et al* (2016), also proposed this to be the case with the presence of FRα autoantibodies in ASD (Frye *et al.* 2016). The possibility that this could be the case in our cohort is demonstrated by the higher percentage of RM cases having a pregnancy related medical issue than identified in pregnant controls, 60% vs 10%, respectively. The factors included endometriosis, PCOS, infertility, low protein S, FV Leiden, and chromosomal abnormalities. In addition, although not significant in our study, MTHFR mutations and the presence of APS associated antibodies were also identified and could have the potential to work in conjunction with FRα autoantibodies in causing RM.

To understand more accurately if there is any RM risk associated with the presence of FRα autoantibodies, improvements to this study design or further studies are required. The recruitment of cases and controls in this study was difficult as we were not directly involved in the recruitment process, in addition recruitment was carried out at only one small private hospital, and fertility clinic. As a result, over the time frame of this study only 15 RM cases and 20 pregnant controls were collected, 5 of the RM cases were further

identified as pregnant reducing the number of RM non-pregnant cases to 10.. Due to the small sample size in this study a power calculation using the data collected was performed and it was determined that 69 cases and controls are required. Unless there is a distinctive difference identified between cases and controls, as seen with the first study of FRα autoantibodies in NTD, with such a small sample number it is hard to identify if FRα autoantibodies are a true risk factor for RM and therefore this is only a pilot study. Therefore, a much larger cohort is required, which we hope to collaborate and either collect a larger cohort of RM cases or analyse a pre-existing cohort for RM patients.

The time of data and blood collection has the potential to alter the outcome of any study. Unfortunately, in this study time of sampling the RM cases and sometimes pregnant controls was out of our control. Often in RM cases, like with other diseases or disorders, the patient will present after the event, as obtaining a blood sample from the patient before a miscarriage has occurred is difficult, unless the patient returns when they fall pregnant again. A blood sample collected after the miscarriage occurred may not give you a clear understanding of what was happening at the time of the miscarriage. Factors such as folate levels, folate supplementation, homocysteine levels, weight and environmental factors can change regularly. FRα autoantibodies can also fluctuate; this observation is supported by a number of longitudinal studies. These studies demonstrated that antibody titres could fluctuate from undetectable levels to high antibody titres, in samples collected over different stages of disease

(Berrocal-Zaragoza Maria Isabel *et al.* 2009, Ramaekers *et al.* 2014, Shapira *et al.* 2015). The time since miscarriage was also not recorded in this study so it is hard to identify how relevant the presence or absence of FRα autoantibodies are in identifying the risk of RM. In an ideal world the collection of multiple samples from a patient prior to conception, after conceiving, directly after a miscarriage event has occurred, and a follow up sample would be beneficial. However, if only one sample can be collected ideally it should be as close to a miscarriage event, which would strengthen the outcome of the study.

Lastly the detection of FRα autoantibodies in this study does not discriminate between a binding autoantibody and a folate blocking autoantibody. A large proportion of studies analysing FRα autoantibodies do in fact detect blocking autoantibodies in addition to binding autoantibodies; however, some studies just look at blocking antibodies alone. Identification of a blocking FRα autoantibody in some studies demonstrated an increased risk of disease compared to the presence of binding antibodies. Therefore, further work into detecting blocking FRα autoantibodies in this RM cohort is required to identify if the presence of FRα autoantibodies in a patient is a risk factor for RM.

Several studies have linked FRα autoantibodies to folate dependent pregnancy complications including subfertility, NTD and pre-term birth; however no one has studied their association with RM until this study. In this pilot study, there was a high incidence of FRα autoantibodies detected in

both pregnant controls and RM cases; however a trend was identified showing that RM cases had a higher level of FRα autoantibodies compared to pregnant controls which was strengthened when pregnant RM cases were excluded from the data, although this was also not significant. The role FRα autoantibodies have in RM is unknown; however we propose that they could block folate transport across the placenta, increase fetal homocysteine levels or cause complement activation and inflammation when binding to the FRα receptor. Like the mechanism seen with APS antibodies, the increase in homocysteine and activation of the complement cascade by binding FRα autoantibodies can both affect thrombosis in the developing placental vasculature and decidua; where the presence of micro thrombi have been associated with RM (McNamee *et al.* 2012, Raziel *et al.* 2001). The possible role FRα autoantibodies have in causing RM should not be disregarded just yet, as this is only a small pilot study, and a larger cohort, with more defined collection parameters is needed to truly understand if there is a link.

Chapter 5:

FRα autoantibodies in the Thrombosis in Stroke Study

5 Chapter 5: FRα autoantibodies in the Thrombosis in Stroke Study

5.1 Introduction

Stroke is the second most prevalent disease to affect Australians, with over 60,000 people a year suffering from a stroke (1 every 10 minutes) (Cadilhac *et al.* 2011). The brain requires constant flow of oxygenated blood to function, and if this does not occur then serious damage can arise (Rink *et al.* 2011, Stastics. 2010). There are two main arterial blood systems that supply the brain, the carotid and the vertebral arteries (Purves D 2011). Blockage or damage of any sort to these arterial systems towards the brain is defined as cerebrovascular disease, with stroke classified as a severe or complete blockage of blood to the brain (Mackay *et al.* 2004). There are two types of stroke, ischemic stroke and haemorrhagic stroke, which cause the same damage to the brain; however they have two entirely different aetiologies, and risk factors (Gomes *et al.* 2013). Ischemic stroke, which is the most common, occurs from a blockage of blood in the artery, whereas haemorrhagic stroke results from the bleeding of the artery reducing the blood flow to the brain (Gomes *et al.* 2013, Sacco *et al.* 2013). Transient ischemic stroke also falls under cerebrovascular disease, which is an event known as a mini stroke, where blood supply is only temporally blocked causing only short term effects; however this can often lead to ischemic stroke if preventative measures aren't taken (Johnston 2002, Sacco *et al.* 2013). This chapter will therefore investigate a possible risk factor for stroke, as a much wider understanding of risk factors is required.

The prognosis of stroke is associated with a high mortality rate, with approximately 11,000 deaths a year in Australia caused by stroke (Stastics. 2010), and severe morbidity with a large proportion of patients experiencing immediate and often severe long term disability. The severity of the stroke depends on the proximity of the event to the brain and the amount of area that is affected by hypoxia; where the type of disability depends on the section of the brain affected. Stroke often causes paralysis with loss of muscle movement, affected speech, loss of sight, loss of the ability to swallow, and can also affect the person by change in personality or triggering depression. Symptoms often include dizziness, loss of balance, unexplained falls, severe headaches and difficulty swallowing (Hinkle *et al.* 2007, Senes 2006). Stroke is a very serious disease that needs more research into biomarkers identifying stroke risk and preventative measures as not all people that experience a stroke are able to live independently after the event.

Stroke like recurrent miscarriage is a multi-factorial disease (Goldstein *et al.* 2011, O'Donnell *et al.* 2010). Risk factors for ischemic stroke include genetic, biochemical, physiological, anatomic, and histological factors, which may individually be the cause or work together to trigger a stroke (Goldstein *et al.* 2011, O'Donnell *et al.* 2010). General lifestyle factors such as, smoking, diet, alcohol consumption, and fitness can all increase the risk of stroke; as well as other health problems including high blood pressure, diabetes, high blood cholesterol and arterial fibrillation (O'Donnell *et al.* 2010). A major cause of stroke is the presence of antiphospholipid and anticardiolipin autoantibodies

(Espinosa *et al.* 2010, Panichpisal *et al.* 2012). The condition known as Antiphospholipid Syndrome (APS) is clinically recognised by the presence of these autoantibodies in two consecutive tests in combination with arterial and/or venous thrombosis or pregnancy morbidity (Espinosa *et al.* 2010, Panichpisal *et al.* 2012). The presence of antiphospholipid/anticardiolipin antibodies induces thrombosis by several possible means, for example inhibition of the protein C pathway, inhibition of anti-thrombin, binding and activation of platelets, activation of the complement cascade, and induction of adhesion molecules and tissue factor (Espinosa *et al.* 2010, Katikaneni *et al.* 2015, Panichpisal *et al.* 2012).

As stroke is easier to prevent than to treat, due to the irreversible damage that is caused to the brain, much research has focused on identifying biomarkers that pre-dispose people to having a stroke. Recent studies have looked at increases in total homocysteine levels in addition to folate deficiency as a risk factor for stroke and recurrent stroke (Ashjazadeh *et al.* 2013, Doshi *et al.* 2002, Fallon *et al.* 2003, Nakamura *et al.* 2002, Study of the Effectiveness of Additional Reductions in *et al.* 2010)). As early as 1969 it was thought that elevated homocysteine levels could increase the risk for stroke as a study demonstrated that children with the inborn metabolic error causing homocystinuria also had a high incidence of premature occlusive vascular disease; however homocysteine as a risk factor for stroke is still debated in the literature with many studies demonstrating a relationship while others reject the idea (Boysen *et al.* 2003, Goldstein 2000, Homocysteine Studies 2002, McIlroy *et al.* 2002, Ntaios *et al.* 2009, Saposnik *et*

al. 2009, Smulders *et al.* 2011, Study of the Effectiveness of Additional Reductions in *et al.* 2010, Toole *et al.* 2004). The major differences in studies are due to threshold cut-offs for high levels of homocysteine and also whether the levels were retrospective or prospective of the stroke event. Two large meta-analyses were performed in 2002 demonstrating an association between stroke and elevated homocysteine levels. The Homocysteine Studies Collaboration analysed a prospective stroke study demonstrating that a 25% lower homocysteine level was associated with 19% lower risk for stroke, where Kelly *et al* (2002) found that the risk of stroke was increased by the OR of 1.79 with elevated levels of homocysteine (Homocysteine Studies 2002, Kelly *et al.* 2002). Interestingly, a study by Boysen *et al* (2003) was the first study to demonstrate that elevated levels of homocysteine are associated with ischemic stroke only, and not haemorrhagic stroke, highlighting the vascular difference between the two diseases (Boysen *et al.* 2003). Therefore, this chapter will focus on the pathogenesis of ischemic stroke rather than haemorrhagic.

Even though homocysteine is often the final factor in the cause of stroke, elevated levels of homocysteine is often the result of another deficiency or inborn error in homocysteine metabolism. These include folate deficiency, and homozygous defects in the genes that encode the Methyltetrahydrofolate Reductase (5-MTHFR) enzyme, or the Cystathionine β-synthase enzyme; which are associated with the increase in ischemic stroke risk via elevation of homocysteine (Ganguly *et al.* 2015, Kelly *et al.* 2002). As dietary folate 5-MTHFR is an important co-factor for the conversion

of homocysteine to methionine a decrease in folate results in the elevation of homocysteine (Ganguly *et al.* 2015). However a deficiency in circulating folate can also independently increase the risk of stroke in some patients (Aydin 2015, Selhub *et al.* 1995). A large prospective study into men looking at folate and stroke, demonstrated that men in the highest quintile of folate intake had an approximately 30% lower risk of ischemic stroke. What's more, studies demonstrate that treatment with folate supplementation reduces the risk of stroke, by reducing homocysteine levels, as high doses of folate can act as a protective barrier against oxidative stress in the arteries to reduce the risk, and progression of carotid plaques leading to atherosclerosis (Alsulaimani *et al.* 2013, Ashfield-Watt *et al.* 2001, Doshi *et al.* 2002, Lonn *et al.* 2006).

How folate deficiency leads to an independent increased risk factor for stroke is not fully understood. However, folate is a major factor in one carbon transfer metabolism and plays a role in many biological reactions and processes including DNA synthesis and repair, and is responsible for methylation reactions for both DNA and proteins (Bailey *et al.* 1999, Selhub 2002). Therefore, there may be multiple ways that folate deficiency causes downstream vascular injury by affecting cell mechanisms and disruption in the coagulation system. One mechanism that has been postulated relates to the observation that high levels of folate help maintain adequate levels of nitric oxide, which is an important factor in reducing the risk of atherosclerosis; if folate is deficient a decrease in nitric oxide may lead to an

increased risk of stroke (Ashfield-Watt *et al.* 2001, He *et al.* 2004, Selhub *et al.* 1995).

What is known is that folate deficiency is a major factor in elevating homocysteine levels in the blood, which has several means of causing vascular injury further leading to stroke (Study of the Effectiveness of Additional Reductions in 2010). Mild-high elevation of homocysteine has been demonstrated to cause large vessel atherosclerotic disease and cerebral small vessel disease leading to stroke (Ashfield-Watt *et al.* 2001, Yang *et al.* 2012). Such ways that homocysteine levels can affect the vascular system include, toxic accumulation in endothelial cells causing dysfunction of vascular endothelium, reduced ability to inhibit platelet aggregation, deregulation and increase of clotting factors and tissue factors, and in addition the inhibition of the anticoagulant protein thrombomodulin (Alsulaimani *et al.* 2013, Ashfield-Watt *et al.* 2001, Goldstein 2000, He *et al.* 2004, Lonn *et al.* 2006). This demonstrates that folate deficiency can have an effect on the vascular system in several different ways all leading to a possible increase in stroke, independent, and dependent on the effects of elevated homocysteine levels. In addition, the effect of folate deficiency/elevated homocysteine levels on the vascular system has many similarities to the mechanism which the autoimmune disease APS exerts on the vascular system, suggesting a possible relationship between the two and also the possibility of increasing the risk of stroke further in a patient that has both conditions.

Another level to this cascade effect increasing the risk of stroke, is how folate may be deficient within the blood. This can also have multiple causes with genetic errors or even errors in folate transport mechanisms. This is where the FRα may play a role, as it is a major transport protein of folate. In 2004 the first autoantibody to bind and block folate binding to the FRα protein in humans was described (Rothenberg *et al.* 2004). Since then FRα autoantibodies have been studied in many folate deficient diseases, such as neural tube defects, cerebral folate deficiency, and congenital heart disorder, with strong associations seen in the first two diseases (Bliek *et al.* 2006, Cabrera *et al.* 2008, Lewandowski *et al.* 2013, Ramaekers, Blau*, et al.* 2007, Ramaekers, Sequeira*, et al.* 2007, Rothenberg *et al.* 2004). We therefore propose that the presence of FRα auto antibodies in ischemic stroke patients may in fact cause folate deficiency, further increasing the risk of stroke, and, therefore be an important biomarker in the prevention of stroke.

The aim of this chapter is to identify if there is any association between the presence of FRα autoantibodies and ischemic stroke, by investigating if there is any association between the presence of these FRα autoantibodies, with changes in homocysteine levels, folate status and/or another auto-immune disease such as APS.

5.2 Methods

5.2.1 Study Population

This study utilised 142 cases and controls from the Thrombosis in Ischemic Stroke Study (TISS) cohort, with samples collected at Royal Perth Hospital in Western Australia, between March 1996 and June 1998. Ethics was obtained at Royal Perth Hospital (Western Australia) (EC497). The study consists of patients with first ever ischemic stroke, where stroke is defined as a clinical syndrome characterised by rapidly developing clinical symptoms and/or signs of focal and at times global loss of brain function, with symptoms lasting >24 hours or leading to earlier death, with no apparent cause other than that of vascular origin. Ischemic stroke was defined as a stroke with either normal CT brain scan or evidence of recent infarct in the clinically relevant area of the brain on a CT or MRI brain scan preformed within 3 weeks of the relevant event or at autopsy. The control subjects consist of age (within 5 years range), sex and postal code matched controls. This cohort has extensive baseline demographic data, and history of conventional vascular risk factors collected, in addition a range of other suspected risk factors for stroke including folate/homocysteine levels, genetic mutations, pro-coagulant factors, and autoimmune diseases.

5.2.2 FRα ELISA Analysis

The assay procedure that was used to identify the presence or absence of FRα autoantibodies was as described in section 3.3.5. As the TISS cohort was collected prior to this study not all cases and controls had serum samples

remaining, and therefore EDTA plasma samples were used to maintain uniformity for analysis. EDTA samples were previously stored at -80°C until required, and a small aliquot was taken out to reduce freeze/thawing of the sample. 6 samples with their 6 matched controls were analysed on an ELISA plate that was prepared the previous day in triplicates, in addition to the positive, negative and monoclonal antibody controls. Two plates were assayed per day until all samples were screened.

5.2.3 Data Collection

This cohort has an extensive range of clinical data that has been collected. These tests were all preformed at the central core laboratory Path West, Royal Perth Hospital (Western Australia) when initial samples were collected. Information pertaining to serum folate levels, red blood cell folate levels, homocysteine levels, antiphospholipid autoantibody presence, and lupus anti-coagulant was extracted from the database for each case and control.

5.2.4 Statistical Analysis

Baseline differences between cases and controls were examined by means of the χ^2 test for categorical data and an unpaired t-test for continuous data.

For each ELISA, the unknown samples were indexed against a known positive control, and a known negative control which was analysed on each

plate. This was performed to standardise the absorbance reading variation between each plate. To calculate the index the formula below was used:

$$\frac{OD_x - OD_n}{OD_p - OD_n} \times 100$$

Where OD_x is the optical density of unknown serum sample, OD_n is the optical density of the negative control, and OD_p is the optical density of high positive control. The ischemic stroke case group was compared to the control group using the Wilcoxon matched pairs signed rank test to assess for differences in the mean values. The samples were then categorised either negative, low positive or high positive, as per section 3.3.7. The χ^2 analysis of contingency tables was performed to determine if there was statistical difference between matched cases and control samples. Statistical analysis was performed either using Minitab16 or Graphpad prism6 and p value <0.05 was considered significant.

Data has previously been collected for this cohort on other risk factors related to vascular disease, including antiphospholipid autoantibodies, homocysteine, serum/red cell folate levels and the MTHFR gene C677T variants. Analysis was performed comparing the presence of FRα autoantibodies with each individual's previously investigated factors for this cohort. A χ^2 test for categorical or an Anova test for continuous data was carried out to determine if there is an association between the above factors in relation to the presence of FRα autoantibodies.

5.3 Results

142 ischemic stroke cases and 142 age/sex/postcode matched controls were studied and analysis was performed on demographic and conventional risk factors for cardiovascular disease that were previously collected for this cohort. There was no significant difference between the cases and controls for hypercholesterolemia, or previous venous vascular events. However there was a significant higher difference for hypertension, diabetes, hypercholesterolemia, smoking and previous arterial vascular events in cases compared to controls (Table 5.1).

Table 5.1. Baseline Demographics and Conventional Vascular Risk Factors in Ischemic Stroke Cases Controls.

	Case (n=142)	*Control (n=142)*	*odds ratio*	*95% CI*	**P*
Mean age (sd)	65.52 (12.7)	66.26			0.617
Male sex	89 (63%)	89 (63%)			1.00
Hypertension	76 (54%)	43 (30%)	2.651	1.268 to 2.011	$<$ 0.0001
Diabetes	38 (27%)	15 (11%)	3.094	1.612 to 5.935	0.0005
Hypercholesterolemia	37 (26%)	29 (20%)	1.373	0.7890 to 2.390	0.261
Current Smoker	50 (35%)	24 (17%)	2.672	1.529 to 4.669	0.0004
Previous Venous vascular event	4 (2%)	5 (4%)	0.7942	0.2088 to 3.022	0.7348
previous Arterial vascular event	40 (28%)	17 (12%)	2.884	1.543 to 5.387	0.0007

*χ^2 for categorical data, unpaired *t*-test for continuous data.

5.3.1 FRα Autoantibodies

142 ischemic stroke cases and 142 age/sex/postcode matched controls were analysed using the FRα autoantibody ELISA for IgG/IgM/IgA antibodies. Each sample was indexed against the known positive and negative sample for the specific ELISA plate to reduce variation between ELISA runs. If a sample had a negative index value it was designated zero. The mean index value for ischemic stroke cases was 4.63 ± 10.56 and for the matched controls 4.31 ± 10.41. The histogram Figure 5.1demonstrates a positive skew, as a high number of samples are negative for FRα autoantibodies which is expected, and therefore the data for both cases and controls was continuous, but was not reflective of a normal distribution pattern (Figure 5.1). As this is the case the differences between the mean was analysed by the Wilcoxon matched pairs signed rank test. Presence of the FRα autoantibodies in the 142 ischemic stroke cases showed no significant difference (p=0.8319) compared to the age/sex/postcode matched control group.

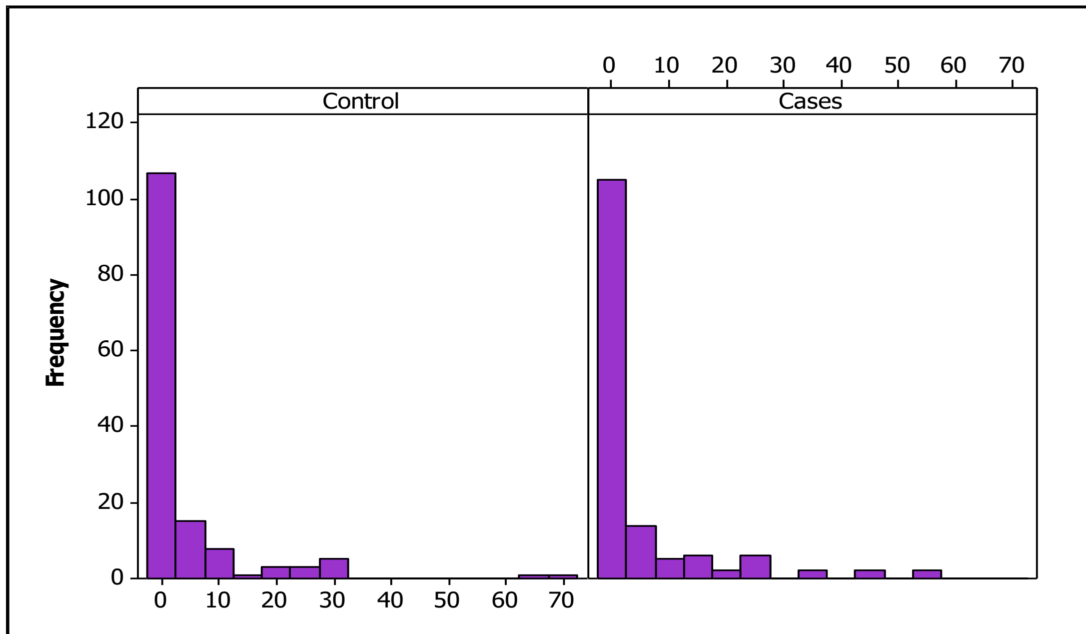


Figure 5.1. Distribution of Ischemic Stroke Cases and Control for the Level of FRα autoantibodies present in EDTA samples.

The FRα autoantibody ELISA is utilised to determine whether the EDTA sample of either the ischemic stroke cohort or age/sex/postcode matched controls was present for antibodies against the FRα protein; negative, low positive and high positive samples were analysed. As described in section 3.3.7 a negative sample is defined as below ≤9.06 frU, low FRα autoantibody sample is greater than 9.06 frU, but less than 19.769 frU, and a high positive is classified as a sample ≥19.769 frU. Table 5.2 demonstrates the distribution of FRα levels in cases and controls. The numbers of ischemic stroke cases and controls in the different categories with FRα autoantibody present are very similar to controls, but have a slightly higher percent than cases for low autoantibody presence. However, ischemic stroke cases have a slightly higher number (9.15%) compared with matched controls (7.0%) for high

levels of FRα autoantibodies. The total incidence of FRα autoantibodies in EDTA samples is 14.8% for cases and 14.0% for controls. There is a slightly higher percent of cases having a higher autoantibody level than the controls however comparing ischemic stroke cases with their age/sex/postcode matched control samples using a χ^2 test no significant difference in the presence of FRα autoantibodies was demonstrated.

Table 5.2. Number of negative, low positive, high positive FRα autoantibody samples for ischemic cases and control subjects.

	Case (n= 142)	***Control (n=142)***
Negative (≤9.06frU)	121 (85.2%)	122 (85.9%)
low positive (9.06≥ X ≤19.769 frU)	9 (5.63%)	10 (7.0%)
High positive (≥ 19.769 frU)	12 (9.15%)	10 (7.0%)

5.3.2 FRα autoantibodies with APS and Other Factors

In this study, we set out to determine if the presence of FRα autoantibodies may be associated with the presence of antiphospholipid antibodies, adding to the risk of vascular events, as if one immune disease is present others are often a cause as well. Data was previously obtained for the presence of antibodies that contribute to APS, in both cases and controls for the TISS cohort. The antibodies analysed include anti-lupus anticoagulant antibodies (LA), anti-cardiolipin antibodies IgG and IgM (ACA) and anti-β2-

glycoprotein 1 antibodies (aβ2GP1). In addition, the presence of any anti-phospholipid antibodies compared to the presence of FRα autoantibodies was also analysed. However, results analysed by Pearson χ^2 test in Table 5.3 demonstrate that there was no significant association independently between any of the APS antibodies or collectively and the presence of FRα auto-antibodies.

As the FRα is responsible for transport of folate within cells, it is important to analyse if folate, homocysteine and other vitamins are affected by the presence of FRα autoantibodies in serum. Table 5.4 shows the means and prevalence of homocysteine, serum folate, red cell folate, vitamin B 12 and vitamin B6 in addition using a choice of cut points compared to the prevalence of FRα autoantibodies; with ANOVA analysis performed for mean levels and Pearson χ^2 tests for categorical levels. Normality and homogeneity of variance assumptions, which were assessed for each variable by examining histograms and the skewness and kurtosis test, were not violated. A slight trend was identified when analysing vitamin B6, which demonstrated the only significant association between the mean and presence of FRα autoantibodies when comparing negative (M=35.9, SD=27.5) to low FRα autoantibody prevalence (M=52.05, SD= 37.0) only ($p= 0.026$, $\alpha=0.5$); with low levels of FRα autoantibodies showing the highest level of vitamin B6. However overall, for all variables analysed there was no association demonstrated between the means or various cut points and the presence of FRα autoantibodies.

Table 5.3: Frequency of various APS antibodies compared to FRα autoantibody presence in all case and control subjects combined for the TISS cohort. Association was determined using Pearson's χ^2 squared test.

		FRα autoantibody presence			*P value*
		Negative (≤9.06frU)	**low positive (9.06 X 21.96 frU)**	**High positive (≥ 21.96 frU)**	
Anti-cardiolipin IgG antibodies	Negative ≤2Ku/L	176 (72.7)	16 (80)	16 (72.7)	0.811
	Low 2-8 Ku/L	46 (19)	2 (10)	5 (22.7)	
	High 8≥Ku/L	20 (8.2)	2 (10)	1 (4.5)	
Anti-cardiolipin IgM antibodies	Negative ≤2Ku/L	160 (66.1)	13 (65.0)	18 (81.8)	.410
	Low 2-8 Ku/L	58 (23.9)	6 (30.0)	4 (18.2)	
	High 8≥Ku/L	24 (9.9)	1 (5.0)	0 (0.0)	
anti-β2-glycoprotein 1 antibodies (aβ2GP1)	Negative	202 (84.1)	15 (75.0)	17 (77.2)	.439
	Positive	38 (15.9)	5 (25.0)	5 (22.7)	
anti-lupus anticoagulant antibodies (LA)	Negative	232 (96.3)	18 (90.0)	20 (90.9)	.253
	Positive	9 (3.7)	2 (10.0)	2 (9.1)	
Any Antiphospholipid antibodies	None	99 (40.9)	8 (40.0)	9 (40.9)	0.995
	1 APS antibody	103 (42.6)	8 (40.0)	9 (40.9)	
	2 ≥ APS antibodies	40 (16.5)	4 (20.0)	4 (18.2)	

Table 5.4: Prevalence of various cut points for high homocysteine, low serum folate, red cell folate, serum vitamin B12 and B6, and the proportion who had various levels of FRα autoantibodies in both case and control subjects combined from the TISS cohort.

		FRα autoantibody presence				*P value*
		Total	**Negative (≤9.06frU)**	**low positive (9.06> X <21.96 frU)**	**High positive (≥ 21.96 frU)**	
Homocysteine	N	257	219	17	21	
	Average (Std Dev)	12.09 (5.1)	12.20 (5.1)	11.31 (4.3)	10.95 (6.0)	0.438†
	Elevated Level (>15µmol/L)	50	44	4	2	0.459
Serum Folate	N	259	221	17	21	
	Average (nmol/L)	17.11 (7.5)	17.18 (7.8)	17.48 (5.3)	16.08 (6.3)	0.799
	Very low ('<6.8)	6	4	0	2	
	Moderately low (6.8-<11)	49	44	2	3	0.173
	Normal (≥11)	204	173	15	16	
Red Cell Folate	N	249	212	17	20	
	Average (nmol/L)	688.02 (323.0)	699.66 (335.3)	642.05 (232.2)	603.8 (239.7)	0.373
	Very low ('<370)	28	23	2	3	
	Moderately low (370-<513)	45	40	2	3	0.921
	Normal (≥513)	176	149	13	14	
B12	N	257	219	17	21	
	Average (pmol/L)	333.36 (328.8)	338.55 (346.6)	328.64 (263.5)	283.04 (127.7)	0.761
	Very low ('<125)	19	15	2	2	
	Moderately low (125-<185)	43	39	1	3	0.702
	Normal (≥185)	195	165	14	16	
B6	N	249	213	17	19	0.074
	Average (nmol/L)	37.44 (28.7)	35.9 (27.5)*	52.05 (37.0)*	40.63 (31.6)	

5.4 Discussion

This chapter aimed to identify if there was an association between the incidence of FRα autoantibodies and increased risk of ischemic stroke. No significance difference was identified between cases and controls for this study suggesting that the presence of FRα autoantibodies is not an independent risk factor for stroke. FRα is a highly tissue specific membrane receptor that is present only in the placenta, choroid plexus, kidney proximal tubules, and in a variety of cancer cells. Therefore, autoantibodies that affect folate transport via the FRα will not ultimately affect the levels of folate/homocysteine within the blood or within the cells that make up the arteries. This is supported, by the analysis between the presence of FRα autoantibodies and levels of folate and homocysteine. It would be expected, as published by other studies, that the presence of these autoantibodies to reduce both serum folate and red blood cell folate levels, and thus in turn elevate levels of homocysteine. This was not the case in the study, where levels of homocysteine, and folate either serum or red blood cell levels, remained unaffected by the presence of FRα autoantibodies. This correlates with a 2009 study by Berrocal-Zaragoza *et al*, which demonstrated that the presence of FRα autoantibodies in both men and women did not affect levels of folate in the blood. This observation is seen because there is no transport via the FRα in the blood stream or red blood cells, and folate levels are only affected by dietary deficiencies, or problems with absorption in the gut.

Of note is the overall percent of cases and controls that have FRα autoantibodies present at any positivity is 15.5%. A population study looking at both men and women in Spain demonstrated overall 7.2% prevalence of FRα autoantibodies in serum, while other studies have reported a higher overall level of FRα autoantibodies (Berrocal-Zaragoza M. I. *et al.* 2009). For example, a 2009 study by Molly *et al* demonstrated the presence of FRα autoantibodies in control samples at a level 13%. It is important to note that this cohort was of a higher age group, (mean age 65 years) as the risk of stroke increases with age (Sequeira *et al.* 2013). Immunosenescence, which is the gradual deterioration of the immune system occurs as we get older; older people have more autoantibodies against a wider range of antigens (Sequeira *et al.* 2013). This may explain the higher prevalence of FRα autoantibodies in the stroke cohort as the overall age is greater than previous studies.

Analysis of the present cohort highlights not only the differential distribution of FRα, but also the pathogenetic niche of FRα autoantibodies with the results of this study suggesting that the presence of FRα autoantibodies may only be clinically relevant to tissues that have a high demand of folate, and/or are reliant on FRα to transport the folate, which is not the case for tissues and cells of the circulatory system. For example, thromboses in the developing embryo during pregnancy as these autoantibodies have been shown to decrease transit of folate into the placenta, thus if folate is highly deficient it may not only be a driver for conditions such as NTD, but may also cause thrombosis leading to death of the fetus (Rothenberg et al. 2004).

Within blood and the vascular system there are other alternatives for folate transport, such as the reduced folate carrier (RFC) or proton coupled folate transporter (PCFT), unlike in the placenta or choroid plexus where FRα is the major transport protein (Gorman1 et al, McMartin et al. 1992, Barber et al. 1999), supporting this study's finding that FRa autoantibodies are not associated with stroke .

Like with all diseases, it is better to prevent than treat, and stroke is no exception. If anything it is more important to have biomarkers that are able to identify if people are at a high risk of stroke; as there is not a large amount of treatment available if intervention is not carried out within hours of the stroke event occurring (Goldstein *et al.* 2011). Although this study did not demonstrate any association, it allowed us to eliminate FRα autoantibodies as a possible biomarker allowing further research to investigate other risk factors for stroke. In addition, analysis of the TISS cohort further validated the FRα specific ELISA; demonstrating specificity, sensitivity and its ability to screen a large cohort fairly quickly compared to other methods previously adopted.

Chapter 6:

Cell Proliferation and Anti-FRα antibodies

6 Chapter 6: Cell Proliferation and Anti-FRα antibodies

6.1 Introduction

Autoantibodies can exert their effect in many ways throughout the body either directly or indirectly leading to various different diseases and tissue damage (Cervera *et al.* 2007) Examples of the mechanism of an autoantibody include; binding to a protein to change the structure, affecting downstream processes; blocking a receptor from releasing/transporting various particles; stimulation of the immune-response toward the cell/tissues that the antibodies bind to (Lleo *et al.* 2010). The exact mechanism of action that a circulating FRα autoantibody has on a cell or a tissue is not well understood. Nonetheless, as FRα is responsible for the transport of folate across a cell and often a barrier, for example the placenta, the obvious mechanism would be the inhibition of folate binding to the receptor ultimately reducing the intracellular folate level and increasing homocysteine; but a process of activation cascades has furthermore been proposed (Antony 1996, Rothenberg *et al.* 2004, Sequeira *et al.* 2013). The analysis of how an autoantibody like the FRα autoantibody can damage a cell or tissue is important to understand the disease and the treatment that may be required. Additionally, a greater understanding of the processes involved could enable identification of symptoms related to the presence of the autoantibody, paving the way for diagnostic applications.

The presence of an autoantibody does not always mean a person will have an autoimmune disease at the time of autoantibody detection; they may present with symptoms a couple years later or at not at all in the future. The reason for this is that an autoantibody is required to bind at the exact location on an antigen to elicit an immune response or alter the function of a protein enough that the cells and associated tissues are damaged; for example, an autoantibody directed against ADAMTS13 is required to bind to a specific site which will stop the protein binding to the vWF protein therefore inhibiting its cleavage (Bettoni *et al.* 2012, Igari *et al.* 2012) This is thought to be the case with FRα autoantibodies, as seen in a population study carried out in Spain where 7.2% of the population both men and women were positive for FRα autoantibodies, yet not all cases had folate deficiency, as was demonstrated in our study into both RM and Stroke (Chapters 4 and 5) (Berrocal-Zaragoza M. I. *et al.* 2009). If all circulating FRα antibodies present had an effect on the body a high number of people would have issues related to folate deficiency such as cerebral folate deficiency, which is not evident.

There is significant variation into the type of autoantibodies analysed in many studies to date, with earlier studies analysing binding autoantibodies only, while other studies analyse both blocking and binding, or blocking alone (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Billie *et al.* 2010, Bliek *et al.* 2006, Cabrera *et al.* 2008, Frye *et al.* 2014, Frye *et al.* 2013, Molloy Anne M. *et al.* 2009, Opladen *et al.* 2007, Ramaekers, Blau, *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007,

Ramaekers *et al.* 2008, Rothenberg *et al.* 2004, Sequeira *et al.* 2013, Vo *et al.* 2015). Determination of the different antibodies in a patient is critical to understand the possible outcome for their presence. An initial study carried out by Caberra *et al* (2008) analysing FRα autoantibodies in association with neural tube defects (NTD), identified a number of patients with high titres of FRα autoantibodies that did not block folic acid binding to the receptor ; demonstrating that an individual may have FRα autoantibodies yet have no clinical presentation (Cabrera *et al.* 2008). A large number of studies into CFD investigate both types of FRα autoantibodies, and have identified patients that have either blocking or binding antibodies alone or a combination of both blocking and binding antibodies (Frye *et al.* 2014, Frye *et al.* 2013, Opladen *et al.* 2007, Ramaekers, Blau*, et al.* 2007, Ramaekers, Sequeira*, et al.* 2007, Sequeira *et al.* 2013). For example, Frye *et al* (2013), identified 31% of CFD patients to have blocking only FRα autoantibodies, 15% of patients to have binding only FRα autoantibodies, while 29% of the cohort had both binding and blocking FRα autoantibodies. It has also been identified that blocking FRα autoantibodies are associated with an increased risk of disease, supporting the requirement to analyse both blocking and binding antibodies (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Boyles *et al.* 2011, Cabrera *et al.* 2008, Frye *et al.* 2016, Rothenberg *et al.* 2004, Shapira *et al.* 2015, Vo *et al.* 2015).

The mechanism of how FRα transports folate across the cell membrane is complex, and requires binding of folic acid to the receptor, internalization via an endosome, where the folic acid is released in response to a shift to a

more acidic environment within the cell (Chen *et al.* 2013, Della-Longa *et al.* 2013). The overall structure and binding sites of FRα are important to how this process works, however up until recently the crystal structure has been unknown due technical difficulties in expression, purification and crystallization of FRα (Chen *et al.* 2013). In 2013, two different groups published the crystal structure and folic acid binding site, identifying a deep folic acid binding pocket dependant on several amino acid residues across the receptor (Chen *et al.* 2013, Wibowo *et al.* 2013). Wibowo *et al* (2013) identified six distinct crystal models which can be involved in the transport of folate, with pH dependent conformational changes depending on the stage of the folate trafficking in addition to a conformational change which occurs when folate binds to the receptor (Wibowo *et al.* 2013). Results from these two studies illustrated just how complex the binding and transport of folic acid is to the FRα, which further complicates analysis of FRα autoantibodies.

Wibowo *et al* (2013) and Chen *et al* (2013), while analysing the crystal structure, demonstrated that the overall structure is important for binding folate with all of the amino acids associated with the binding site shown to be critical. A study carried out looking at FRα autoantibodies has also demonstrated that antibodies do not bind to denatured or small fragments of the FRα protein (Sequeira *et al.* 2013). Therefore, it is difficult to just identify antibodies that bind to the binding site alone. The only means of determining if the autoantibody binds in the binding site inhibiting folic acid transport is by analysing the inhibition of folic acid binding to the whole FRα, or the effect

the autoantibody has on a cell if it binds to exact location that inhibits folic acid transport analysed by growth assays. There have been various methods utilized to study the blocking capacity of the FRα autoantibodies, with two main methods being investigated (Billie *et al.* 2010, Boyles *et al.* 2011, Sequeira *et al.* 2013, Vo *et al.* 2015). The two methods work on a similar principle, where folic acid is depleted from the serum sample; the serum is then incubated with the FRα protein, followed by addition of labelled folic acid (Billie *et al.* 2010, Boyles *et al.* 2011, Sequeira *et al.* 2013, Vo *et al.* 2015). The level of folic acid blocked will then be inversely proportional to the colour/radioactivity detected depending on the method. The differences between the two methods is how the folic acid is labelled for detection (Billie *et al.* 2010, Boyles *et al.* 2011, Sequeira *et al.* 2013, Vo *et al.* 2015). The protocol commonly used in the CFD studies carried out at the SUNY Downtown University (New York, USA) employs [H^3] labelled folic acid; whereas a number of the studies investigating NTD and other pregnancy related complications have developed their assay with folic acid labelled with horseradish peroxidise (HRP), eliminating the use of radioactivity (Billie *et al.* 2010, Boyles *et al.* 2011, Sequeira *et al.* 2013, Vo *et al.* 2015). There has been no consensus as to the correct way of analysing the samples nor has there been a comparison between the two methods in assessing the accuracy in detecting folic acid blocking; however, the use of radioactivity in a majority of laboratories is not feasible and a simple ELISA using HRP conjugated folic acid may be more beneficial in a clinical setting of determining blocking FRα autoantibodies.

As it is known there are blocking and binding FRα autoantibodies present in patients and how they are important in disease state; the aim of this chapter is to analyse the different effects of anti-FRα antibodies have on the overall proliferation. It is of importance to this study to identify blocking autoantibodies because of the high number of both cases and controls that have FRα autoantibodies present yet we cannot determine if there is any association with recurrent miscarriage. The use of a growth assay has not been performed to determine if the FRα autoantibodies are capable of blocking folic acid binding; however, the method may be a useful tool in identifying the overall affect the autoantibodies has on a FRα dependent cell, by blocking folic acid binding or by activation cascades initiating apoptosis.

6.2 Methods

6.2.1 Polyclonal FRα Antibody Production

A polyclonal antibody against the recombinant FRα protein was developed for two reasons; a positive control in the anti-FRα ELISA and for use in growth kinetics analysis of FRα inhibition by FRα antisera. Anti-FRα antibodies were raised in a chicken as the egg yolk yields high titres of IgY, and the purified IgY does not cross react with mammalian IgG nor mammalian Fc receptors as IgY antibodies lack Fc-domains. Ethics approval was obtained at Murdoch University (Western Australia) (R2287/09).

6.2.1.1 Immunization

One chicken was purchased from a commercial chicken supplier (Altona) at one day old, and allowed to grow to maturity. The antigen used was a commercial recombinant FRα protein which is 220 amino acids (aa) in length spanning 25-233aa with a polyhistidine tag on the C-terminus (Sino Biological Inc., PRC). For the initial immunization, the chicken was injected subcutaneously with a total of 100µg antigen resuspended in 500ul of sterile phosphate buffered saline pH 7.2 (Gibco, Life Technologies Australia, Australia) and emulsified with 500ul Freund's Complete Adjuvant (Sigma Aldrich, USA). Two subsequent subcutaneous injections were carried out using 100µg protein emulsified with Freund's Incomplete Adjuvant (Sigma Aldrich, USA) to a total volume of 1ml, 10 and 20 days after the first injection.

6.2.1.2 Purification

IgY antibodies are the only immunoglobulins present in the yolk of chicken eggs and 1-10% of IgY purified is specific to the immunization antigen. All eggs were collected and stored in 4°C, and purified within 2 months of collection. IgY from each egg yolk was purified using the Pierce® Chicken IgY Purification Kit (Thermo Fisher Scientific Inc., USA), as per instructions provided. Briefly egg yolks were separated from the egg white using an egg separator. The yolk sack was washed with sterile H_2O for irrigation (Baxter) to remove excess egg white, and then dried on paper towel. The yolk sack was pierced to allow the yolk only to be collected. The volume of egg yolk was measure and then delipidated using 5 x egg yolk volume of cold delipidation solution mixing well, and incubated for 2 hours at 4°C. The delipidated-yolk solution was then centrifuged for 15 minutes at 4,000 x g at 4°C. The supernatant was collected and an equal volume of precipitation solution was added while stirring. The solution was incubated at 4°C for one hour to precipitate the IgY from solution, followed by centrifugation at 4,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pelleted IgY was resuspend in equal volume of sterile PBS (Gibco) as to the original volume of egg yolk. Purified IgY was stored at 4°C until tested, then sterilized using a 0.45μm filter and either stored at 4°C for short term use or 10% glycerol was added and stored at -20°C for long term storage.

6.2.1.3 IgY Confirmation

Successful purification, and purity of IgY from the egg yolk was carried out by Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-page) followed by Coomassie blue staining or western blotting. 10μl of a 1:10

dilution of purified IgY run on a NuPage Novex 4-12% Bis-Tris Gel as per section 3.2.1.2. For Coomassie blue staining the gels were washed with deionised water and then placed in a small staining container. The gels were incubated overnight in 25mls Acqua Stain (Acqua Science) for 1 hour to stain the protein bands blue. The gels were then rinsed with deionised water and dried overnight using GelAir Cellophane Support (Biorad). For confirmation of IgY antibody a western blot was performed using anti-chicken IgY HRP antibody (Santa Cruz Biotechnology, Inc. Dallas, USA). The purified IgY was run on an SDS-Page gel as described above, and then transferred onto a nitrocellulose membrane as in section 3.2.1.4. The membrane was blocked using 3% skim milk TBST solution for 1 hour then incubated in 1:2000 anti-chicken IgY HRP antibody (Santa Cruz Biotechnology, Inc. Dallas, USA) diluted in 1% skim milk TBST solution of 1 hour. After three 15 minute washes the membrane was incubate in 1ml of SuperSignal West Pico Chemiluminescent Substrate solution (Pierce, Thermo Fisher Scientific Inc.), and developed.

6.2.1.4 IgY Titre Determination

The FRα specific IgY antibody titre levels produced in the individual egg yolks purified were determined by an in-direct ELISA. The concentration of each purified IgY was measured using a NanoDrop 1000 (Thermo Fischer Scientific Inc.), and diluted to a final concentration of 1mg/ml total IgY in sterile PBS. The optimized ELISA protocol for anti-FRα detection was used as section 3.3.7 with recombinant FRα (Sino Biological, PRC) at a 5μg/ml concentration. 100μl of purified IgY (1mg/ml) diluted in 10% FCS-PBS at series of dilutions 1:100,

1:500. 1:1000 and 1:2000, with a 1:5000 secondary antibody anti-chicken IgY HRP (Santa Cruz Biotechnology, Inc. Dallas, USA) used to detect the anti-FRα IgY antibodies. A positive control was run of the plate using 1:1000 mouse anti-hFRα IgG primary antibody, 1:15000 anti-mouse IgG HRP (Santa Cruz Biotechnology, Inc. Dallas, USA) for the secondary antibody.

6.2.1.5 IgY Immunoblotting

The binding affinity and specificity of the chicken anti human FRα IgY antibodies was demonstrated by immunoblotting (western blotting). The IgY was analysed against three different sources of FRα protein to test for affinity, while whole cell lysates for cells expressing the FRα were used to test for specificity of the antibody. These antigens included pure recombinant FRα protein (Sino-biological, PRC), Cos-7 FRα cell line which is an African green monkey cell line expressing a recombinant form of FRα and the KB cell line which is a human cell line expressing native FRα protein. 0.5µg of pure FRα and 10µl each of cell lysate were separated via SDS-PAGE gels as described in section 3.2.1.2, then transferred to a nitrocellulose membrane (section 3.2.1.4.1). General western blotting protocol was followed as section 3.3.1.4 with a 1:5000 dilution of 1mg/ml purified IgY for primary antibody, and 1:2000 dilution of MOv18 (Enzo Life Sciences Inc., Australia) as a positive control. Primary antibodies were detected with a 1:5000 dilution of both goat anti-chicken IgY HRP (Santa Cruz Biotechnology, Inc. Dallas, USA) for IgY, and goat anti-mouse IgG HRP (Santa Cruz Biotechnology, Inc. Dallas, USA) for the MOv18 antibody.

6.2.2 MTT Assay

6.2.2.1 Preparation of Cells for MTT assay

A MTT standard curve was performed using the KB cell line to calculate the number of cells and to ensure that the number of cells used for each experiment were within the exponential growth phase. Cells were trypsinized and seeded in a 96 well plate in quadruplicate in 200ul of folate free RPMI/10% (v/v) Charcoal stripped FCS/25nM folate, in increasing cell numbers from 7.5×10^3 cells/ml to 50×10^4 cells/ml, with a negative control (media only). Cells were allowed to adhere for 24 hours at 37°C/5% CO_2, before the MTT assay was performed (section 6.2.2.2). For each proliferation assay KB cells were seeded at 2.5×10^4 cells/ml in quadruplicate in folate free RPMI/10% (v/v) charcoal stripped FCS with the addition of filter sterilised folic acid at a concentration of 25nM, 100nM, or 1000nM, in addition 0.1M NaOH alone as a vehicle control. A 96 well plate was seeded for each time point of analysis and at 24 hours 100ul of media was removed and replaced with 100ul of fresh folate free RPMI/10% (v/v) charcoal stripped FCS containing treatment of choice. MTT assay was performed at 24 hours (pre-treatment), 48 hours, 72 hours and 96 hours.

6.2.2.2 MTT Assay

24 hours post seeding for the standard curve and at each time point for the proliferation assay of KB cells, the MTT assay was performed. In all experiments, a negative control of media only was also assayed. For each well 100ul of media was removed using a multichannel pipette carefully, and 20ul of MTT reagent was added to each well, then wrapped in aluminium foil

to protect from light. To permit uptake of the MTT reagent cells were incubated for 4 hours at 37°C/5% CO_2. 100ul of MTT solvent was added to each well to dissolve the purple formazan crystals that are formed by viable cells when they uptake the MTT reagent; solution was pipetted vigorously to ensure all crystals are dissolved. Colour development was analysed at the OD_{560} and the background at OD_{720} on FluorStar Optima spectrophotometer. The background reading at OD_{720} of each well was subtracted from the reading at OD_{560} to determine the final absorbance. The MTT standard curve was created by plotting the seeded cell number against the average of final absorbance values for each cell number. A line of best fit was generated, and the equation was used to determine the number of cells from absorbance values generated for all following cell proliferation assays using KB cells. All steps were performed in low light conditions.

6.2.3 Manual Cell Counting Proliferation Assay

KB cells were trypsinized and seeded at 2.5 x 10^4 cells/ml in 500ul of folate free RPMI/10% (v/v) charcoal stripped FCS with the addition of filter sterilised folic acid at a concentration of 25nM, 100nM, 1000nM or with 0.1M NaOH as a vehicle control, in 48 well plates. An individual plate was prepared with all treatments for each time point that was required to be counted, 24 hours, 48 hours, and 72 hours. Cells were incubated for the required time at 37°C/5% CO_2. Cells were then washed with 250ul of sterile PBS, trypsinized with 50ul of 0.25% Trypsin EDTA and returned to incubator for 5 minutes until cells become detached. 250ul of fresh in folate free RPMI/10% (v/v) charcoal stripped FCS was added to each well and pipetted gently until the cell

suspension became homogenous. Cells were then counted with a haemocytometer (section X). Quadruplicates for each assay was performed and averages and standard deviations were calculated. Treatment was added at 24 hours and the cell count at 24 hours was classified at time point 0. Cells were treated with either the anti-FRα MOv-18 monoclonal antibody with the IgG mouse isotype as a control or the anti-FRα chicken IgY polyclonal antibody (section 6.2.4)

6.2.4 Treatment for Cell Proliferation Assay

All growth assays were replicated on the MTT assay and manual cell counting assay. For all assays, the cells were cultured in folate free RPMI/10% (v/v) charcoal stripped FCS, 24 hours prior to cell assay being performed. Initial studies performed analysed different concentrations of folic acid on the growth of the cell line KB, therefore 25nM, 100nM, and 1000nM of filter sterilised folic acid was supplemented back into folate free RPMI/10% (v/v) charcoal stripped FCS. Cell proliferation assays analysing the inhibition of cell proliferation with an anti-FRα antibody was all performed at the physiological folic acid level of 25nM folic acid. Two different anti-FRα antibodies were used to analyse the effects. The first antibody was the monoclonal mouse anti-FRα antibody MOv18, with the control antibody Leaf™ Purified Mouse IgG1, κ Isotype Control (Biolegend®, USA). The second antibody tested was the polyclonal anti-FRα chicken IgY 26 antibody with the IgY 0 antibody as the negative control.

6.3 Results

6.3.1 Production of Polyclonal Anti-FRα IgY

Validation of the polyclonal anti-FRα antibody required ascertaining both the specificity and sensitivity of the preparations for detecting FRα protein. Production of the chicken anti-FRα IgY polyclonal antibody was produced in only one chicken. One egg was collected prior to the first immunization of the chicken with the recombinant FRα protein and designated Day 0. The IgY was purified from the egg yolk which was demonstrated by running on a SDS-Page gel in addition analysed on a western blot Figure 1. Day 0 IgY was also screened for the presence of anti-FRα antibodies using the indirect ELISA at dilutions of 1:100, 1:500, 1:1000 and 1:2000 to confirm that the chicken had no prior exposure to human FRα protein. At Day 0, IgY was negative for anti-FRα IgY antibodies with an absorbance <0.1 OD (figure 2) for all dilutions. Day 0 IgY was also used as a primary antibody against the pure recombinant FRα protein, and the cell lysates of Cos-7 FRα and KB cells, which have a high expression of the human FRα protein, demonstrated that no IgY present in the chicken cross-reacted with the FRα nor other proteins in the Cos-7 and the human KB cell lysates. Day 0 IgY was used for all further experiments as a negative IgY control.

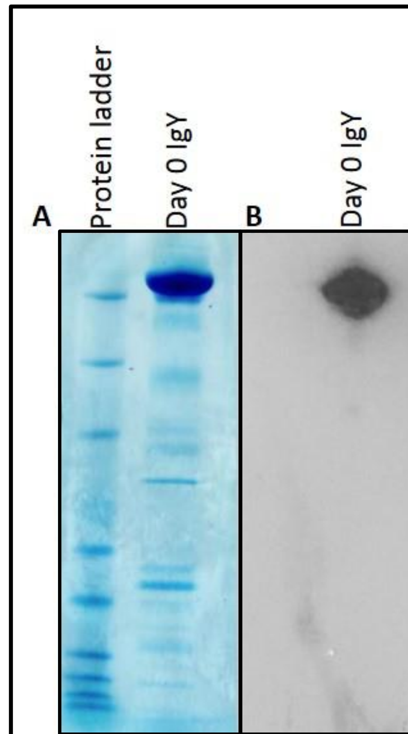


Figure 6.1 Day 0 IgY confirmation. A) Coomassie blue stain of purified IgY from day 0. B) Western blot detecting purified IgY from day 0, with 1:5000 anti-chicken IgY HRP

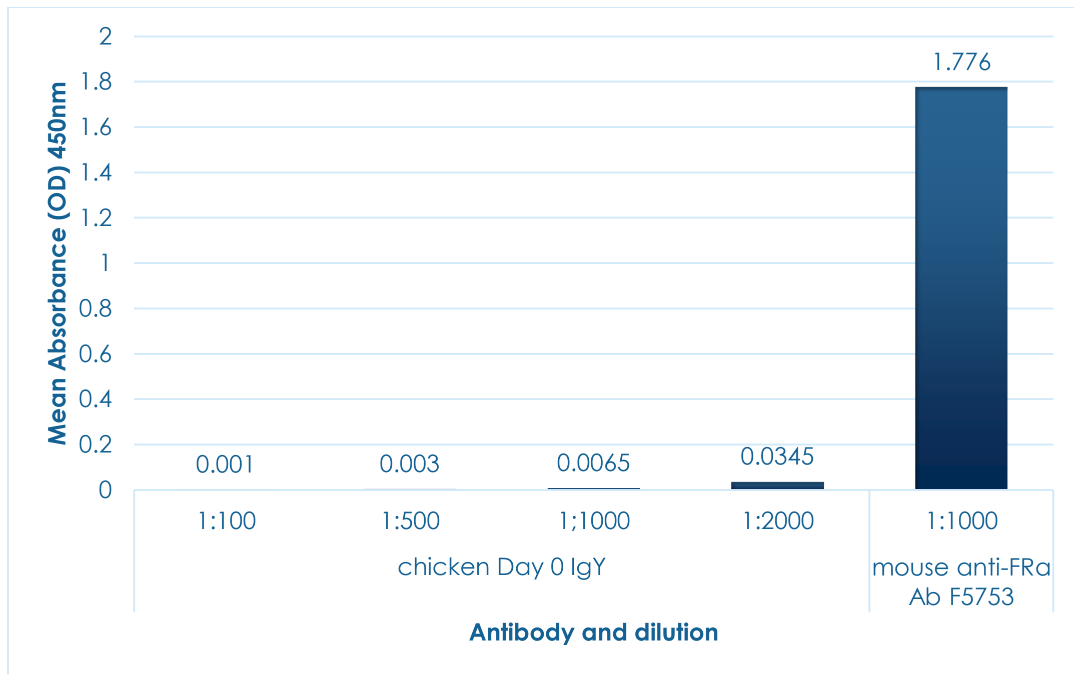


Figure 6.2 Screening of Day 0 IgY purified antibody vs mouse monoclonal anti-hFRα IgG using anti-FRα antibody ELISA represented as mean absorbance values. 5µg/ml of recombinant FRα protein was coated onto microtitre plates. A series of dilutions 1:100, 1:500, 1:1000, 1:2000 of Day 0 IgY and a 1:1000 mouse anti-hFRα IgG was used for positive control primary antibody, with a 1:5000 anti-chicken IgG HRP or 1:15000 anti-mouse IgG HRP used as the secondary antibody, respectively.

6.3.1.1 Titre Determination

Eggs from day 14, 17, 26 and 34 were initially screened to test if anti-FRα IgY antibody had been produced. Negative control IgY day 0 and positive control MOv18 were also screened on the ELISA plate. Total concentrations of IgY yielded for each egg was 3.97, 6.48, 5.39, 4.15mg/ml respectively; then diluted to 1mg/ml before analysis. It was observed that at Day 14 (three days after the second injection) there was no immune response with no anti-FRα IgY being detected at any IgY dilution; however, three days later IgY

purified from day 17 egg yolk produced a high titre of anti-FRα antibodies and even at a dilution of 1:4000 there were still anti-FRα IgY antibodies detected at an absorbance of 0.66. This was increased slightly for the IgY purified from Day 26, though there was a significant decrease in anti-FRα IgY antibodies detected in Day 34, as seen in figure 6.3.

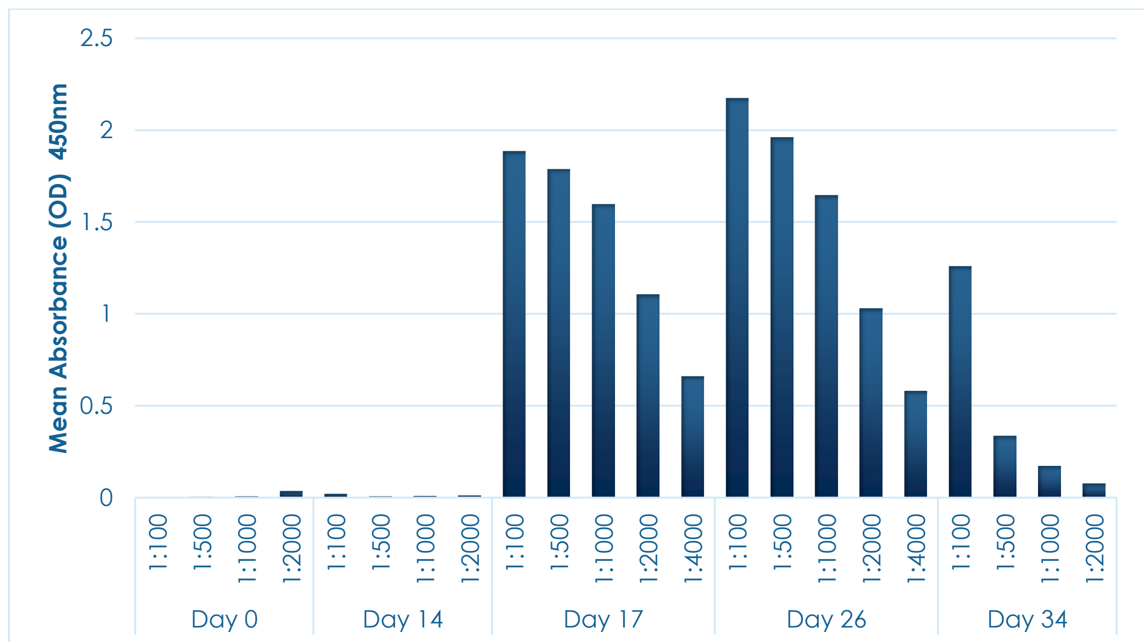


Figure 6.3. Screening of 5 days of purified chicken IgY. Mean absorbance values for each day screened by anti-FRα antibody ELISA detecting presence of anti-FRα chicken IgY antibodies. 5μg/ml of FRα recombinant protein (Sino Biological) was coated onto microtitre ELISA plates. 1:100, 1:500, 1:1000, 1:2000 dilutions for purified chicken IgY Days 0, 14, 34 (1mg/ml) and 1:100, 1:500, 1:1000, 1:2000, and 1:4000 for Day 17, and Day 26 (1mg/ml) were used. Chicken IgY was detected with 1:5000 dilution of anti-Chicken IgY HRP. Each absorbance value represents the mean of three individual readings.

A selection of the remaining eggs after inoculation were also purified and screened using the anti-FRα antibody ELISA to determine presence of anti-FRα IgY antibody titres. Each purified IgY was diluted to a final concentration of 1mg/ml and then used at a 1:500 dilution with the secondary anti-chicken IgY HRP (Santa Cruz Biotechnology, Inc. Dallas, USA) antibody at a dilution of 1:5000. Figure 6.4 demonstrates the overall immune response over the three months of egg collection from the one chicken. Production of anti-FRα IgY in the egg yolk was first seen at Day 17 which was 7 days after the first injection, however as there were no eggs produced between Day 14 and 17 it is unclear to the exact date of anti-FRα IgY production. The highest absorbance for anti-FRα IgY antibodies was at Day 26, and remained stable throughout egg collection, except on Day 34 where the absorbance dropped below 0.3.

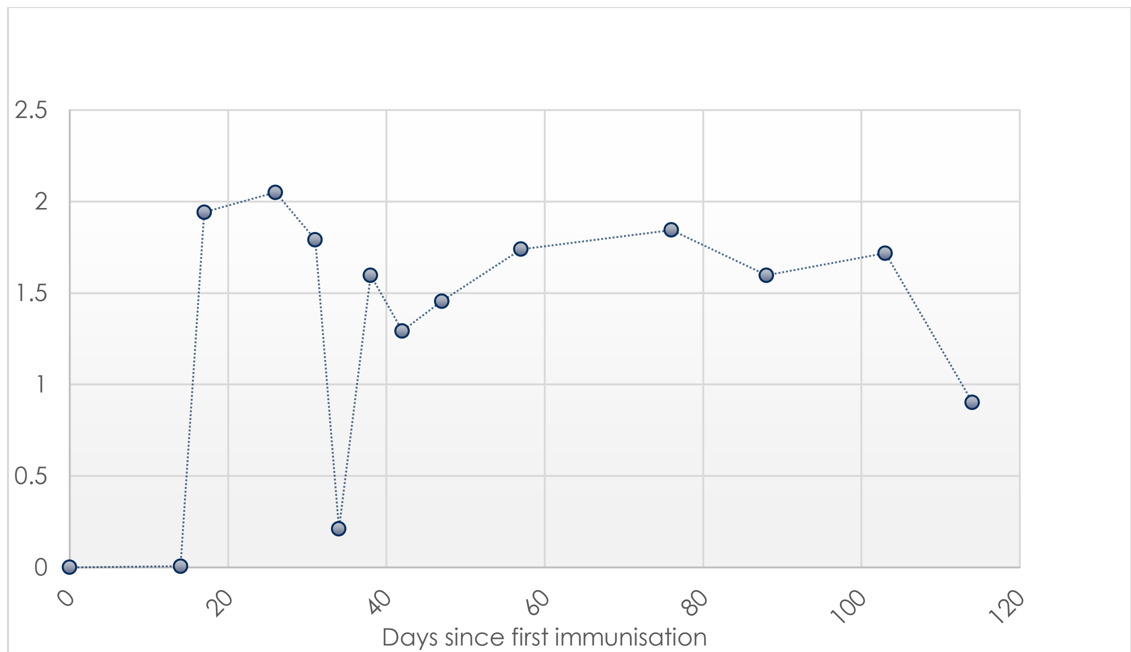


Figure 6.4. Immune response individual chicken to recombinant FRα over 4month period. Mean absorbance values for each day screened by anti-FRα antibody ELISA detecting presence of anti-FRα chicken IgY antibodies. 5µg/ml of FRα recombinant protein (Sino Biological) was coated onto microtitre ELISA plates. A 1:500 dilution of each purified IgY antibody (1mg/ml) and 1:5000 dilution of anti-Chicken IgY HRP (SantaCruz Biotechnology Inc.) was used. Each absorbance value represents the mean of three individual readings.

6.3.1.2 IgY Confirmation, Specificity and Affinity

SDS-Page gels and Coomassie blue staining was performed on purified IgY from Day 0, Day 17, Day 26, Day 34 and a pooled IgY from all eggs purified (figure 6.5a). All purified IgY showed a distinct band at 150kDa. The western blot (figure 6.5b) demonstrates that the protein purified was in fact IgY with a clear band being present at 150kDa, however there was some degradation of IgY, as evident by faint bands at 75, 50 and 25kDa. Specificity was also tested using a western blot where a concentration of 1:5000 for each IgY

(Days 0, 17, 26), and pooled positive IgY was analysed against three different FRα antigens Cos-FRα cell lystate, KB cells lysate and commercial recombinant FRα protein (Sino biological, China). All positive purified IgY antibodies day 17, 26 and pooled IgY demonstrated detection of all three antigens, while the negative IgY day 0 showed no detection.

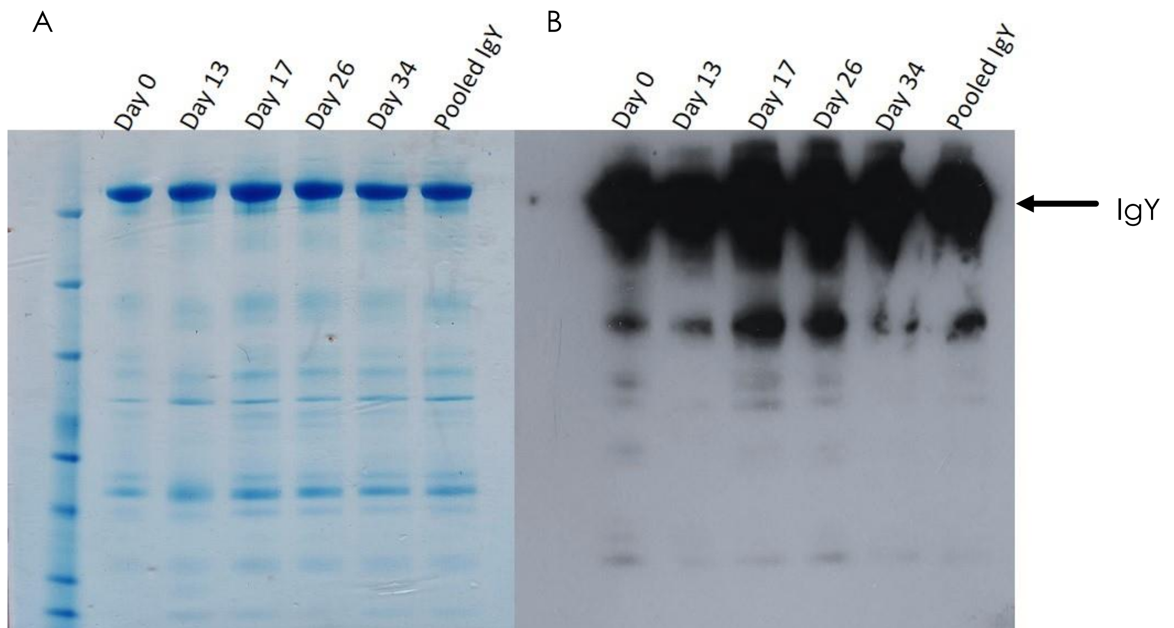


Figure 6.5. Confirmation of IgY from eggs collected at Days 0, 14, 17, 26, 34, and pooled IgY. A) Coomassie blue stain: each purified IgY diluted 1:10 with PBS and 10µl was run on a 4-10% Bis-Tris Gel, then stained overnight with Aqua stain Acqua Stain. B) Western blot of purified IgY, with 1:5000 dilution of anti-chicken IgY HRP.

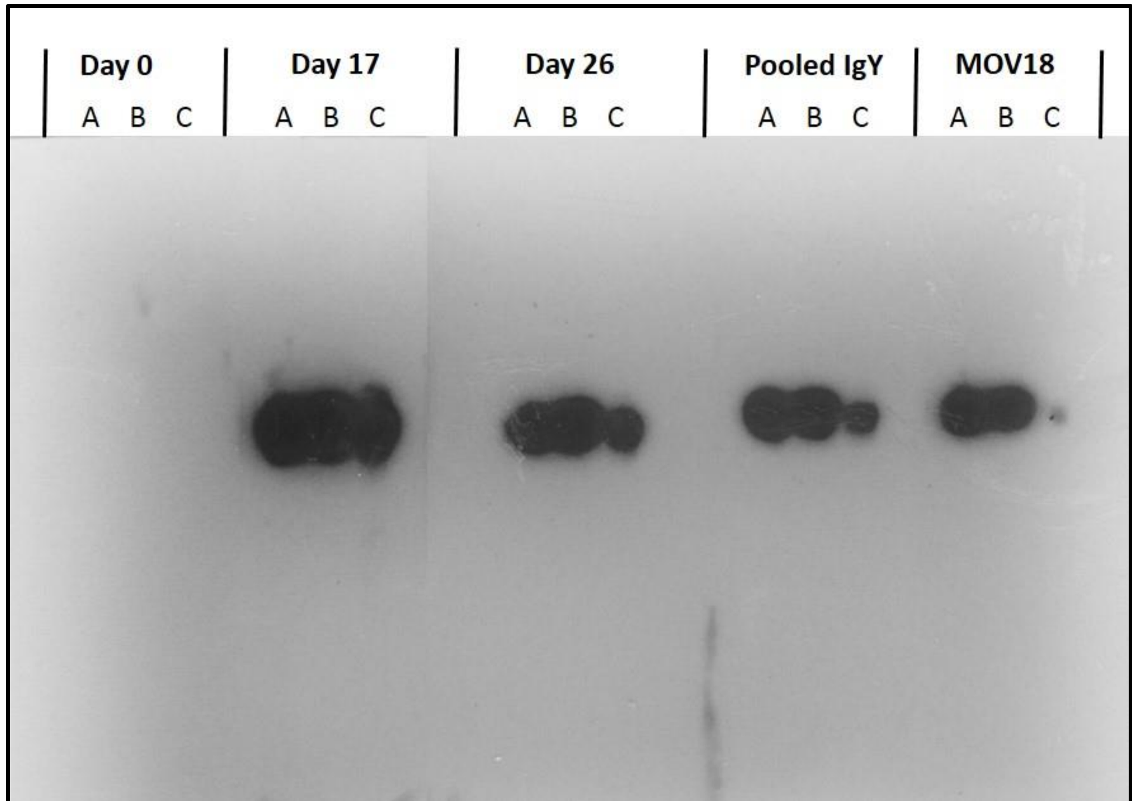


Figure 6.6. Affinity and specificity of purified anti-FRα IgY. 5ul of each a) KB cell lysate b) Cos-7 FRα cell lysate and c) recombinant pure FRα was run on a SDS-PAGE gel 5 times and transferred overnight onto a nitrocellulose membrane. A western blot was performed using 1:5000 dilution of purified IgY from Days 0, 17, 26, and a pooled IgY sample; then with a 1:5000 anti-chicken IgY HRP. Positive control of the presence of FRα in the three samples was the monoclonal anti-FRα antibody MOv18 at a 1:2000 dilution with a 1:5000 anti-mouse IgG HRP secondary antibody.

6.3.2 Growth Assays

Experiments analysing folate concentrations, and anti-FRα autoantibodies were repeated using both manual cell counting assay in a 48 well plate and MTT assay in a 96 well plate.

6.3.2.1 Folate Concentration

The optimum level of folic acid in the media was identified by analysing, no folate, physiological concentration 25nM, and excess concentration of 1000nM of folic acid in folic acid free DMEM. Figure 6.7 reveals 25nM folic acid to show no decrease in proliferation in both MTT and manual growth assay. 1000nM of folic acid showed no significant increase in growth compared to 25nM folic acid. When folic acid is removed from the media completely by folic acid free media and charcoal stripped there was a significant reduction in cell proliferation demonstrated compared to 25nM and 1000nM folic acid.

6.3.2.2 Anti-FRα Antibody Exposure

Inhibition of cell proliferation was examined by exposing cells to both a monoclonal anti-FRα IgG antibody (MOv18) and a polyclonal IgY (Day 26) anti-FRα antibody. Results are illustrated in figure 6.8. Cells were also exposed to matching isotype controls for both monoclonal and polyclonal antibodies. A significant decrease in cell proliferation was seen for both the monoclonal antibody MOv18 and the polyclonal antibody IgY in both growth assays. Proliferation was decreased by roughly 20-30% for both MOv18 and IgY, with no significant difference in growth observed between the two different types of antibodies directed against the FRα. Both negative isotype controls exhibited no growth inhibition and confirmed that the inhibition of cell proliferation seen in antibodies directed against the FRα are due to the antibodies binding to the FRα protein on the cells.

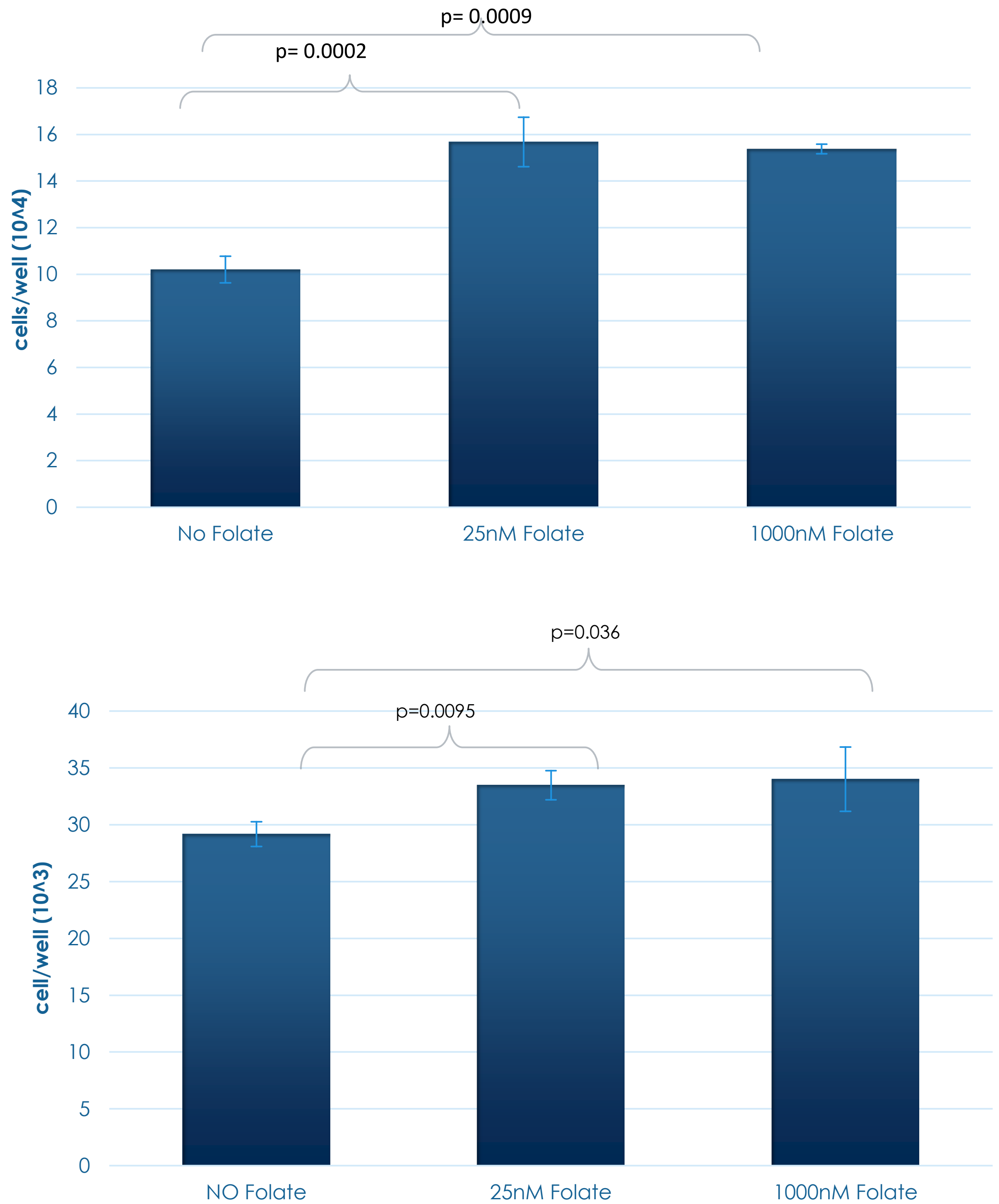
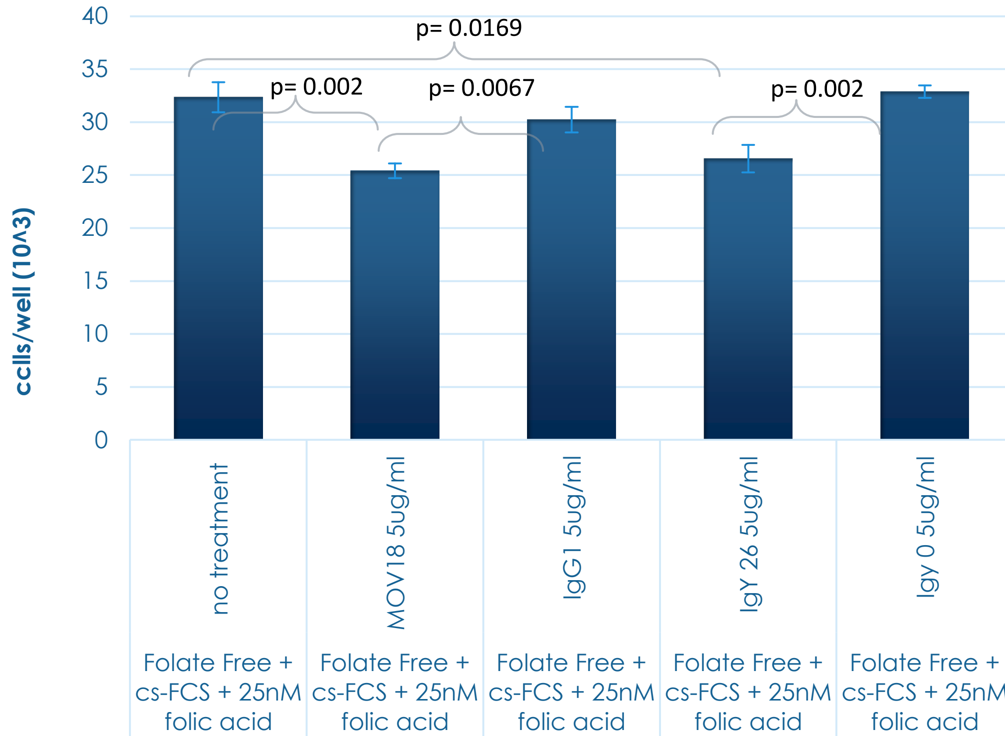


Figure 6.7 Growth inhibition demonstrated when KB cells are grown in folate free media in comparison to physiological folic acid level 25nM and excess folic acid level of 1000nM. A) Inhibition demonstrated using the manual cell proliferation assay in a 48 well plate. B) Inhibition demonstrated using the MTT assay in a 96 well plate.



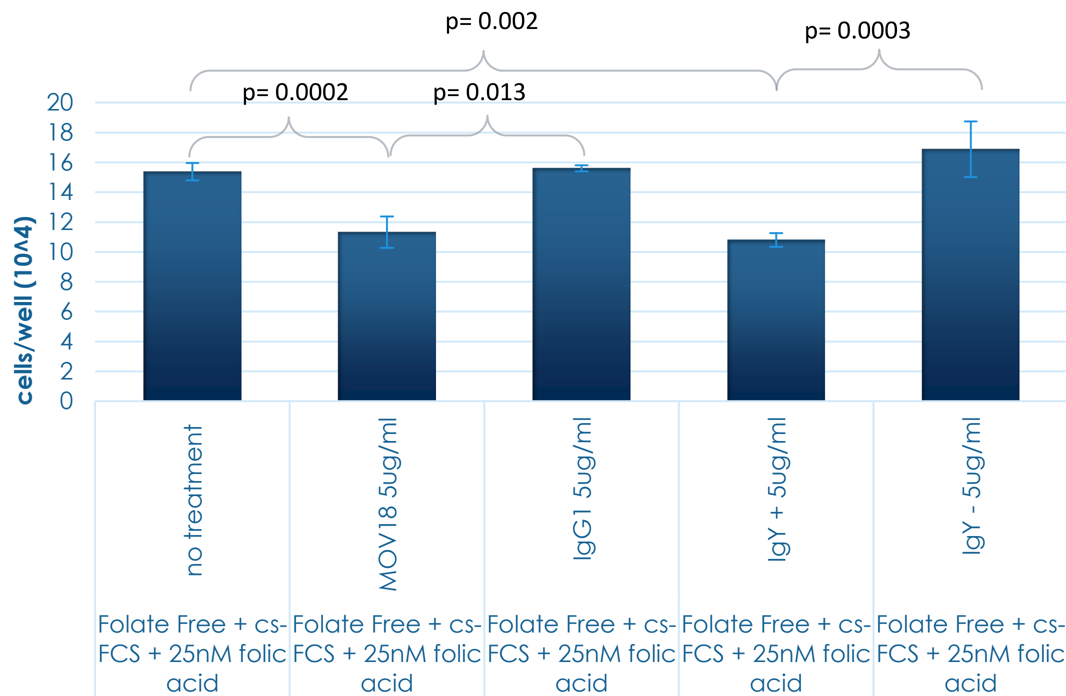


Figure 6.8 Growth inhibition demonstrated when KB cells are grown 25nM folic acid, and exposed to either anti-FRα autoantibodies MOv18 and IgY (Day 26) and their matching negative isotype control antibodies A) Inhibition demonstrated using the manual cell proliferation assay in a 48 well plate. B) Inhibition demonstrated using the MTT assay in a 96 well plate.

6.4 Discussion

It is well established that FRα autoantibodies inhibit the transport of folic acid transport across the cell membrane and into intravillous circulation and the cerebral spinal fluid (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Boyles *et al.* 2011, Cabrera *et al.* 2008, Frye *et al.* 2014, Frye *et al.* 2013, Opladen *et al.* 2007, Ramaekers *et al.* 2005, Ramaekers, Sequeira*, et al.* 2007, Ramaekers *et al.* 2008, Rothenberg *et al.* 2004, Vo *et al.* 2015). However, what is not established in relation to FRα autoantibodies and pregnancy complications is how the binding of FRα autoantibodies to the receptor may cause other factors of inflammation and apoptosis. The majority of research into FRα autoantibodies focuses on both blocking and binding autoantibodies, however when analysing blocking autoantibodies, it merely demonstrates the inhibition of folic acid transport via the FRα. Our study proposed examining anti-FRα antibodies in a growth assay setting, hoping to identify the overall affect the presence of anti-FRα autoantibodies have on the cell and not just on folate transport.

Results from manual and MTT growth assays demonstrated that cells have a normal growth rate at 25nM of folic acid, compared to excess folic acid concentration of 1000nM. 25nM folic acid is the average folic acid level in patient serum, and therefore no growth inhibition should occur. A significant inhibition of cell proliferation was demonstrated when cells were grown in folate free media and charcoal striped FCS. This confirms what is well established in the literature, that folic acid is an important factor in cellular growth and formed the basis for using the growth assay to assess anti-FRα

antibody effects on cell proliferation (Blount *et al.* 1997, Elnakat *et al.* 2004, Laanpere *et al.* , Pitkin 2007, Steegers-Theunissen *et al.* 2000, Tamura *et al.* 2006).

To assess the effects of an antibody binding to the FRα on a cell highly expressing the FRα, two different antibodies were examined; the commercially available monoclonal antibody MOv18; and an in-house polyclonal IgY antibody. We chose different types of antibodies, monoclonal and polyclonal, to identify if their binding had any different effect on the cells, the MOv18 antibody is specific to a particular region of the FRα protein whereas the polyclonal IgY should bind to multiple sites of the FRα including the folate binding site within the receptor. Between 20-30% inhibition of cell proliferation was identified with exposure of both antibodies compared to the isotype controls and no antibody control. This inhibition was shown to be significant for both manual and MTT assays, however there was no significant difference demonstrated between the monoclonal MOv18 antibody and the polyclonal antibody IgY. What is interesting is inhibition of cell proliferation is very similar between anti-FRα antibodies compared to the no folic acid media. Whether or not this means the anti-FRα antibodies are mimicking the reduction in proliferation due to complete inhibition of folate transport or another mechanism is yet to be determined.

The MOv18 antibody was developed against the FRα in an ovarian carcinoma tissue. The specific antigen binding site of the antibody to the FRα is between amino acids 31 (Thr) and 43 (His) of the receptor (Selhub 2002).

This binding site has no overlap with known amino acids required for folate binding which have been identified as Y76, F78, D97, Y101, W118, R119, G153, W154, W156 and W187 (Wibowo *et al.* 2013). Because of this we do not believe that the MOv18 antibody binding to the FRα has an inhibiting effect on folic acid transport in the cell inhibiting proliferation, and is likely affecting cell proliferation by other means.

What is important to highlight from the literature, is there is a significant amount of research into utilizing the FRα as a target for immunotherapy treatment in cancer (Armstrong *et al.* 2013, Basal *et al.* 2009, Elnakat *et al.* 2006, Kelemen 2006, Knutson *et al.* 2006, Low *et al.* 2009, Pinard *et al.* 1996, van Zanten-Przybysz *et al.* 2002, Walters *et al.* 2013). It has been well established that the binding of a monoclonal antibody to the FRα can cause passive immunotherapy, where the host immune system is not required to provoke an immune response (Clifton *et al.* 2011). Binding of the anti-FRα antibody to the receptor may elicit antibody dependent cytotoxicity, activate the complement pathway, and in addition effect molecular signalling. A recent study has demonstrated the FRα is not only responsible for folate transport but can also act as a transcription factor, therefore binding of autoantibodies to the receptor may inhibit this pathway (Clifton *et al.* 2011, Mayanil *et al.* 2014). The passive immunotherapy described in the literature, may be the reason for the decrease in cellular growth seen in our assays, particularly MOv18 whose cognate FRα epitope doesn't map to the folate binding site, supporting our theory that the binding of an anti-FRα

autoantibody may not just affect the cell by folate transport inhibition but also by eliciting an immune response.

A polyclonal IgY antibody was developed in this project to represent what might be seen with an FRα autoantibody. A chicken IgY was selected to eliminate cross reactivity with Fc receptors on the cells, in addition to the simplicity of IgY purification. Successful production of a negative IgY antibody collected from an egg the day before FRα antigen exposure and positive anti-FRα IgY was obtained from eggs collected 17 days after the first inoculation right up until Day 114 when the last egg was collected. The anti-FRα IgY was found to be pure and specific for the FRα. The results demonstrate that the FRα specific IgY can inhibit cell proliferation, however we cannot determine if it is from blocking folate transport via the FRα or by causing a passive immune response.

These growth assays have allowed the overall effect of an antibody binding to the FRα to be demonstrated, which we believe to be important. An advantage of the growth assay model allows the interaction between autoantibodies and the receptor to be examined under native protein conditions and physiological folic acid concentrations. The importance of both these factors is illustrated in the study identifying the three conformational states of the FRα, which established that binding of folic acid is pH dependent, and binding of folic acid further changes the conformation of the receptor (Wibowo *et al.* 2013). These conformational changes in the

receptor depending on pH and folic acid binding, may affect autoantibody binding and the effect they have on the cell.

Although the growth assays are a good model for overall effect of the autoantibodies the method does not discriminate between a blocking effect and a passive immune response to the antibody binding. Further research in conjunction with these growth assays is required to determine the specific mechanism. Looking at blocking FRα autoantibodies as well as growth assays will determine if the inhibition of growth is cause by inhibition of folate transport and/or another mechanism. We also suggest analysis of intracellular markers after exposure to anti-FRα autoantibodies specific for cell toxicity and apoptosis to determine if the growth inhibition is an inflammatory response.

We propose that the mechanism of action of the FRα it is not as simple as a blocking and binding FRα autoantibodies, and there is a possibility of binding FRα autoantibodies to elicit an immune response affecting cell proliferation. The growth assays in this chapter established an ideal model to evaluate the overall effect of FRα autoantibody binding, and will be a useful tool in analysing positive FRα autoantibody patient serum, in conjunction with a blocking assay and markers identifying cell cytotoxicity and apoptosis.

Chapter 7:
General Discussion

7 Chapter 7: General Discussion

7.1 Overview and Major Outcomes

Much research is needed to fully understand the pathologies of recurrent miscarriage (RM) as up to 2% of couples trying to conceive experience RM (Ford *et al.* 2009, Rai *et al.* 2006). With a significant number of these couples having no identifiable cause behind their loss, despite thorough investigation, it can be a distressing time (Jeve *et al.* 2014). There are a number of well-established risk factors for RM including autoimmune disease, thrombophilia and genetic and chromosomal abnormalities, however there is the possibility of many more, as it is a multifactorial disease (ACOG 2002, Bansal *et al.* 2011, Ford *et al.* 2009, Jeve *et al.* 2014, Kavalier 2005, Rai *et al.* 2006). Folate deficiency and associated hyperhomocysteinemia are both known risk factors for RM, although sometimes controversial (Bozard *et al.* 2010, Govindaiah *et al.* 2009, Kumar *et al.* 2003, Nair *et al.* 2012, Nelen *et al.* 1998, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., den Heijer M., & Eskes T. K. A. B. 2000, Nelen W. L. D. M., Blom H. J., Steegers E. A. P., Den Heijer M., Thomas C. M. G., *et al.* 2000). A common reason for folate deficiency is dietary deficiencies, and inhibited absorption in the gut, however often is the case that the maternal folate levels are normal yet the fetal folate levels are deficient. A possible cause of fetal folate deficiency are genetic mutations within the folate transport proteins, although recently a new alternative is the FR□ autoantibody which was first discovered in 2003 (Barber *et al.* 1998, Da Costa *et al.* 2003, Nilsson *et al.* 2004). In 2004 the presence of FR□ autoantibodies was demonstrated to be associated with inhibition of folate

transport and associated with an increased risk of NTD, a disease that is well established to be caused by folate deficiency, due to the high requirement of folate needed for cell proliferation and neural tube closure (Rothenberg *et al.* 2004). Therefore, this project aimed to identify if the presence of FR□ autoantibodies increases the risk of RM. The following outcomes demonstrate the development of an ELISA to detect FR□ autoantibodies and there role they have within folate deficiency related disorders.

- Successful development and validation of a specific, rapid, robust and reproducible ELISA in detecting FRα autoantibodies in both serum and plasma samples.
- Analysis of the TISS cohort a large cohort of Stroke cases and controls.
    - No significant difference was demonstrated between cases and controls.
    - Prevalence of FRα autoantibodies in a healthy population was established (14%).
- RM cohort
    - Collection of 15 cases and 20 pregnant controls
    - Occurrence and titre of FRα autoantibodies was demonstrated to be higher in RM cases compared to pregnant controls, however not significantly
    - Significantly higher Incidence of IgG2 FRα autoantibodies in RM cases
    - Significantly higher level of homocysteine found in RM cases
- Production of a polyclonal IgY antibody specific to FRα protein
- Demonstrated the presence of a monoclonal and polyclonal anti-FRα autoantibody can inhibit growth of cell line that expresses FRα at high levels.

7.2 Development of an ELISA to Detect FRα Autoantibodies

The first aim of this study was to develop a robust, rapid and reproducible assay to detect FRa autoantibodies in patient serum. To date there is no commercial kit available, and two different assays are described in the literature (Cabrera *et al.* 2008, Lewandowski *et al.* 2013, Sequeira *et al.* 2013). A considerable number of studies analyse FRα autoantibodies by means of radioisotope labelled folic acid, a method not feasible in our laboratory, or an ELISA assay, however no standardised method has been defined (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Lewandowski *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Rothenberg *et al.* 2004, Sequeira *et al.* 2013) (30, 94, 99, 105, 106, 476, 429, 3, 2, 103). FRα autoantibody screening in our laboratory was initially performed by flow cytometry, however the method that was developed was time consuming and expensive as it used a large amount of monoclonal antibodies, therefore a new method was required. An ELISA for the detection of FRα autoantibodies was designed, developed and validated allowing screening of 12 patient samples at one time, with normalisation against a known positive and known negative control. A baseline cohort was analysed, and FRα autoantibody titres were determined for negative samples (<9.065 frU), low positives (9.065 frU≤X≥ 19.769 frU) and high positive samples (>19.769 frU), respectively.

The ELISA required a substantial amount of optimisation including ascertaining optimal blocking, diluent, and wash buffers, antigen and antibody concentrations in addition to plate type, to obtain the high signal to nosie ratio. It is known that serum can cause considerable background and matrix effects, which was demonstrated in the development of the FRα autoantibody ELISA. Nickel coated and high binding plates as well as a blocking buffer containing BSA seemed to have the highest cross reactivity with serum, with or without the presence of FRα. The cause of this high background is unknown, but it is proposed that proteins/antibodies within serum can cross react with nickel; or in fact the blocking buffer protein themselves (Xiao *et al.* 2012). An interesting find during the development of FRα autoantibody ELISA was the combination of wash steps between PBS and PBS/Tween, required to reduce background and increase signal. Once the protocol was finalised validation of the ELISA was preformed, establishing and intra-assay of 9.58%, and inter-assay of 8.86% which are below the acceptable level of 10%. This assay was shown to be reliable, sensitive and specific, and will make a significant contribution to studies related to folate deficiency and the presence of FRα autoantibodies for example in the investigation of RM.

The type of antigen used in an ELISA may be significant in the overall outcome of determining FRα autoantibodies. The reason for this, is that recently the crystal structure of the FRα protein has been discovered, and, illustrated that folate binding is highly dependent on structure, which is subject to pH levels (Chen *et al.* 2013, Della-Longa *et al.* 2013, Wibowo *et al.*

2013). Additionally, the conformation changes when folic acid binds subsequently affecting the binding of conformation-specific autoantibodies (Wibowo *et al.* 2013). A range of FRα proteins have been previously used across a number of different studies analysing FRα autoantibodies (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Lewandowski *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Rothenberg *et al.* 2004, Sequeira *et al.* 2013). While initial studies used FRα purified from placental tissues or the FBP, the assay developed by the SUNY Downstate Medical Centre (Brooklyn, NY) and used to analyse a majority of the CFD cohorts, now utilizes purified FRα from human milk (Sequeira *et al.* 2013). Still two other studies utilize a recombinant FRα protein (Billie *et al.* 2010, Boyles *et al.* 2011). Possible differences between assays, however cannot be determined as there is no standardised or commercial assay available, and further studies are required to determine the effects the FRα antigen have on detecting both blocking and binding autoantibodies.

7.3 FRα Autoantibodies, Prevalence and Role

The FRα autoantibody ELISA was utilised to screen a small RM and pregnant control cohort to identify if there was a risk of RM when FRα autoantibodies are present. The cohort consisted of 15 RM cases, 10 non-pregnant and 5 pregnant, and 20 pregnant (>20weeks) controls. This study demonstrated that although the association was not significant there was a clear trend showing a higher incidence and titre of FRα autoantibodies in RM cases compared to pregnant controls. This was increased when pregnant RM

cases were excluded from the analysis although still no significant association was established. What was identified in the RM cohort was a significantly higher incidence of IgG2 isotype compared to IgG1 that was seen in pregnant controls. The significance of a higher incidence of IgG2 in RM cases is not well understood however other studies demonstrate a lower prevalence of IgG2 in NTD, CFD, ASD, and therefore may present a possible FRα autoantibody signature related to RM, nonetheless a much larger study is required to confirm this observation (Sequeira *et al.* 2013).

One of the most recent studies investigating FRα autoantibodies in pregnancy complications was demonstrated in a case study. The patient had high titres of FRα autoantibodies, suffered recurrent miscarriage and other pregnancy complications (Shapira *et al.* 2015). Yet when the FRα autoantibodies were eliminated from the system, and a high dose of folinic acid was administered, the patient had a successful full term pregnancy. We believe that there is still might be an increased risk of RM when a patient has FRα autoantibodies, as FRα autoantibodies are associated with other pregnancy folate related disorders including subfertility, NTD and pre-term birth, further supporting the idea that further studies are required to determine the true association (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Sequeira *et al.* 2013, Vo *et al.* 2015).

A large number of factors associated with RM and thrombophilia in pregnancy were analysed in the RM cohort such as APS, folate and homocysteine levels, common genetic mutations, environmental and pre-

existing medical conditions. The only factor to show significant difference between RM cases and pregnant controls was homocysteine levels, however the levels were still within the normal range. What was interesting in this study was that large proportion of RM cases had another possible risk factor for pregnancy complication, ranging from endometriosis, diabetes, FVL mutation of high homocysteine. Although this cohort is not large enough to analyse if there is any significant relationship, we propose that the presence of FRα alone may not be enough to cause a miscarriage, however if another factor is present then the combination of these risks is enough to cause miscarriage or RM is the factors persist.

There are several plausible mechanisms by which FRα autoantibodies can lead to RM. The first and most well studied mechanism is the blocking of folate across the placenta, where the FRα is the main folate transport protein (Rothenberg *et al.* 2004, Sequeira *et al.* 2013). This in turn inhibits proliferation and can lead to increased homocysteine levels resulting in thrombosis of the placental vasculature (Creus *et al.* 2013). A substantial number of studies detect blocking FRα autoantibodies as well as binding antibodies, to identify the level of autoantibodies capable of blocking folate transport (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Bliek *et al.* 2006, Cabrera *et al.* 2008, Lewandowski *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2005, Ramaekers, Sequeira, *et al.* 2007, Rothenberg *et al.* 2004, Sequeira *et al.* 2013). However, the study of blocking FRα autoantibodies alone, in RM or other pregnancy related conditions might not be ideal. We suggest the presence of FRα autoantibodies in addition to

causing low fetal folate levels by blocking folate transport but may also affect the cells and tissues they bind to via activation of the complement cascade and/or causing an inflammatory response and apoptosis within the placental tissue.

Growth inhibition assays in this study were designed to look at the overall effect the autoantibodies *in vitro*. The study demonstrated that the presence of both a commercial monoclonal and the IgY polyclonal anti-FRα antibodies generated in this study, were able to inhibit growth of a cell line highly expressing the FRα, at a physiological serum folate level. These results support the idea that the presence of FRα autoantibodies blocks folate uptake reducing intra-cellular folate levels and restricting growth, as high folate levels are required to maintain DNA replication and repair as well as methylation which is of critical importance in the developing fetus. Conversely, it can also demonstrate that the binding of anti-FRα antibody to the receptor can cause cell damage and apoptosis. What would also be of interest, although was not analysed in this study, is the level of folate or homocysteine that is present in the cell after being exposed to anti-FRα antibodies, in addition to analysis of markers that detect if a cell is under stress and in an apoptotic state. This would allow identification of the exact mechanism the FRα autoantibodies have on the cell, or developing embryo in the case of RM.

To date there has only been one population study to examine the incidence of FRα autoantibodies in the general population, which examined 787 subjects, and revealed 7.2% of the population had FRα autoantibodies

(Berrocal-Zaragoza M. I. *et al.* 2009). If you examine the control groups of all FRα autoantibody studies, although they are often specific to the disease of interest, a large range of FRα autoantibody incidence is identified (0-~30%) (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Berrocal-Zaragoza M. I. *et al.* 2009, Bliek *et al.* 2006, Boyles *et al.* 2011, Frye *et al.* 2014, Frye *et al.* 2013, Lewandowski *et al.* 2013, Molloy Anne M. *et al.* 2009, Ramaekers *et al.* 2005, Ramaekers, Sequeira*, et al.* 2007, Rothenberg *et al.* 2004, Sequeira *et al.* 2013). Our pregnant control group fell within the upper range, with an incidence of 35%, which was similar to what was identified in another pregnant control group looking at pre-term birth. In this study, we analysed a large stroke cohort, 142 cases and 142 controls, to determine if FRα autoantibodies are a risk factor for stroke. Additionally, this work also allowed us to analyse a general, healthy population in the control cohort, adding to the validation of our ELISA, and also enabling a possible association between the presence of FRα autoantibodies and levels of serum folate, red blood cell folate and homocysteine levels in the circulating blood stream. The overall incidence of FRα autoantibody positivity in the stroke cohort control group was 14%, which is 2-fold higher than what is seen in the Spanish population study, however we attributed this to the older age group of our cohort (66.2) (Berrocal-Zaragoza M. I. *et al.* 2009, Sequeira *et al.* 2013). This illustrates that there is still great variation of FRα autoantibody levels in the general population, as well as demonstrating that the presence of these autoantibodies may not always cause complications.

FRα autoantibodies were found not to be a risk factor for stroke in this study, with an incidence of 14.8% compared to 14.0% in healthy controls. This result illustrates that the presence of FRα autoantibodies is not a risk factor for a

folate deficient/hyper-homocysteine related disease that occurs within the normal circulating blood. The presence of FRα autoantibodies did not affect the overall levels of serum folate, red blood cell folate nor homocysteine levels in the blood stream, compared to cases/controls that were negative for FRα autoantibodies. We believe this to be the case, because the FRα protein is not responsible for any folate transport within the blood stream, levels are only dependent on dietary, absorption and folate metabolism pathways. It supports the idea that the presence of FRα autoantibodies only affects folate/homocysteine levels within tissues that are highly dependent on folate transport via the FRα, for example the choroid plexus and the placenta leading to cerebral folate deficiency and NTD, subfertility, and preterm birth, respectively (Berrocal-Zaragoza Maria Isabel *et al.* 2009, Cabrera *et al.* 2008, Sequeira *et al.* 2013, Vo *et al.* 2015).

7.4 Limitations and Future Directions

There are several limitations identified with the FRα autoantibody ELISA developed and the analysis of the RM cohort in this study. The following points will describe some of the limitations recognized and future research required to improve the understanding of FRα autoantibodies in RM and other pregnancy related complications.

7.4.1 Limitations

- The RM cohort, cases and control was small (n=15, n=20), reducing the power of the study in identifying any significant risk factors. Although there was a trend identified between the presence of FRα autoantibodies and RM, it was not significant. The size of the RM cases was further reduced if you excluded pregnant RM cases and analysed non-pregnant cases individually. Not only does a small cohort reduce to ability to discriminate between cases and controls for FRα autoantibodies it also affects the analysis needed to identify if pre-existing RM risk factors played any role in FRα autoantibodies causing RM.
- The time of collection for RM cases varied and was uncontrolled, in addition no data was collected for how far the patient was out from their last miscarriage; Whereas pregnant controls were all collected after >20weeks pregnant. Because of this it is hard to determine the true relationship FRα autoantibodies have on RM as FRα autoantibodies can fluctuate over time.

- FRα autoantibody ELISA does not discriminate between blocking or binding FRα autoantibodies, and therefore when analysing the RM cohort, we were unable to identify If there was a higher level of level folate transport inhibition in RM cases compared to pregnant controls. In addition, although our growth assays demonstrated cellproliferation inhibition with monoclonal and polyclonal anti-FRα autoantibodies no positive FRα autoantibody patient serum was analysed.

7.4.2 Future Research

- Collection of a larger cohort of RM cases and pregnant controls, or collaboration with another group to analyse a pre-existing RM cohort. This will allow for a better understanding of the effect FRα autoantibodies have during pregnancy.
- More stringent collection of samples and data, in relation to the time since the last miscarriage event. An ideal study would be to collect a blood sample before the miscarriage occurs to identify if the autoantibodies are present and likely to cause the miscarriage, however often the patient presents after the event has occurred, therefore collection of the sample directly after the miscarriage occurs would be optimal.
- A longitudinal study of RM patient's over time to detect fluctuations in FRα autoantibodies and associated outcomes.
- The collection of information relating to factors that may cause FRα autoantibodies, for example milk intake which has been demonstrated to increase titres of FRα autoantibodies.

- Screening of positive FRα autoantibody RM case and control serum in the growth assay to determine the overall effect the autoantibody has *in vitro*, whether it is folate transport inhibition, or cell apoptosis, or inflammation. In addition, analysis of the cells after autoantibody exposure to identify specific markers related to apoptosis, activation of the complement cascade and inflammation.
- Development of identification of blocking FRα autoantibodies in positive FRα autoantibody patient serums.

7.5 Conclusions

This study has developed and validated an ELISA specific for FRα, the main folate transport protein in the placenta, in order to identify if the presence of these autoantibodies during a pregnancy increases the risk of RM. A higher incidence and titre of FRα autoantibodies was demonstrated in RM cases compared to controls, yet this result was not significant. What was interesting and significant when further analysis of the FRα autoantibody isotypes was preformed, IgG2 had a higher incidence in RM cases than what was detected in pregnant controls, and we propose that this may be a key factor increasing the risk of RM compared to IgG2 negative serums. Growth inhibition was demonstrated when cells were exposed to anti-FRα antibodies. However, this study failed to identify if the positive FRα autoantibody serum contained blocking or binding autoantibodies, which is important when determining if the presence of FRα autoantibodies are an associated risk factor. Granted this study did not demonstrate significant association we still propose that FRα autoantibodies may in fact be a risk factor for RM, however further research is required to identify if this is true.

Chapter 8: References

8 Chapter 8: References

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Appendix 1:
Buffers and Solutions

Appendix 1. Buffers and solutions

1. Agarose gel (1, 2, 3 %)

1g, 2g, 3g	Agarose powder
100ml	1x TAE buffer[26]
10ul	Ethidium Bromide[7]

Agarose powder was added to TAE buffer and microwaved to dissolve. Ethidium bromide was added after.

2. 1% BSA/PBS

1g	BSA
100ml	PBS (for ELISA)[15]

BSA was dissolved in PBS and stored at 4˚C

3. 3%BSA/PBS

1g	BSA
100mL	PBS (for ELISA)[15]

BSA was dissolved in PBS and stored at 4˚C

4. Coating buffer (0.2M sodium carbonate/bicarbonate coating buffer (pH 9.4)

70mls	0.2M Sodium bicarbonate $NaHCO_3$[22]
30mls	0.2M Sodium Carbonate Na_2CO_3[23]

Combine together and then autoclave

5. 0.5M Dipotassium phosphate K_2HPO_4

57.05	Dipotassium phosphate
500mls	ddH_2O

Dissolve Dipotassium phosphate in water. Autoclave and store at 4˚C 0.5M

6. EDTA pH 8.0

186.12g	EDTA
~20g	Sodium Hydroxide Pellets
500ml	ddH_2O

Dissolve EDTA into 400ml water. Add sodium hydroxide pellets and pH to 8.0. make up to 500ml, and autoclave. Store at room temperature.

7. 10mg/ml Ethidium Bromide

0.1g	Ethidum Bromide
10mL	ddH_20

Dissolve ethidium bromide in water and stored protected from light at 4˚C.

8. 10% FCS/PBS

10ml	FCS
90ml	PBS[14]

9. Flow Buffer

1ml	FCS
99ml	PBS[14]
100mg	Sodium Azide

Add FCS to PBS and then dissolve sodium azide in to solution. Store at 4˚C.

10. Folic acid Stock (1M)

1.765g	Folic acid
100ml	0.1M Sodium hydroxide[24]

Dissolve folic acid in sodium hydroxide. Store at room temperature protected from light.

11. Folic acid working concentration 1500µM

750ul	40mM Folic acid[10]
19.25 ml	ddH_2O

Dilute folic acid in water. Store protected from light at room temperature.

12. 2.5mg/ml MTT reagent

125mg	Thiazol Blue Tetrazolium Bromide
50ml	ddH_2O

Dissolve Thiazol Blue Tetrazolium Bromide in water, aliquot into 5mls tubes store protected from light at -20˚C.

13. MTT solvent

495ml	Isopropanol
5ml	Concentrated HCL

HCL was added to isopropanol and was stored at room temperature.

14. 1x PBS (for cell culture)

8g	Sodium Chloride
2g	Potassium chloride
1.4g	Disodium Phosphate
240mg	Potassium Dihydrogen Orthophosphate
1000ml	ddH_2O

Dissolve all reagents in about 900mL millipore water, PH to 7.2 Make up to 1L. Autoclave or filter sterilise and store at RT or 4°C

15. 1x PBS (for ELISA)

71.7ml	0.5M Dipotassium phosphate K_2HPO_4 [5]
28.3ml	0.5M Potassium Dihydrogen Orthophosphate KH_2PO_4 [17]
8.57g	Sodium Chloride
900ml	ddH_2O

Mix Both Dipotassium phosphate and Potassium Dihydrogen Orthophosphate together, dissolve sodium chloride and make to to final volume of 1000ml. Store at 4˚C.

16. 1 x PBST (0.05%) (for ELISA)

500ml	PBS (for ELISA)
250ul	Tween20

Dissolve Tween20 in PBS and store at 4˚C

17. 0.5M Potassium Dihydrogen Orthophosphate KH_2PO_4

31.02g	Potassium Dihydrogen Orthophosphate
500mls	ddH_2O

Dissolve Potassium Dihydrogen Orthophosphate in water. Autoclave and store at 4˚C

18. 1% Skim milk powder/PBS

100ml	PBS (for ELISA)[15]
1g	Skim milk powder

Dissolve skim milk powder in solution. Make up the day of Use.

19. 1% Skim milk powder/TBST

100ml	TBST[28]
1g	Skim milk powder

Dissolve skim milk powder in solution. Make up the day of Use.

20. 3% Skim milk powder/PBS

100ml	PBS (For ELISA)[15]
3g	Skim milk powder

Dissolve skim milk powder in solution. Make up the day of Use.

21. 3% Skim milk powder/TBST

100ml	TBST[28]
3g	Skim milk powder

Dissolve skim milk powder in solution. Make up the day of Use.

22. 0.2M sodium carbonate Na_2CO_3

16.8g	sodium carbonate
1000ml	ddH_2O

Dissolve sodium carbonate in water. Autoclave and store at 4˚C

23. 0.2M Sodium bicarbonate $NaHCO_3$

21.2g	Sodium bicarbonate
1000ml	ddH_2O

Dissolve Sodium bicarbonate in water. Autoclave and store at 4˚C

24. 0.1M Sodium hydroxide

4g	Sodium hydroxid pellets
1000ml	ddH_2O

Dissolve sodium hydroxide in water. Store at room temperature

25. 2M Sulfuric acid H_2SO_4

111.1ml	Sulfuric acid concentrated
889.9ml	ddH_2O

Add sulfuric acid slowly to water.

26. TAE buffer (50x)

242g	Tris Base
57.1ml	Glacial Acetic Acid
100ml	0.5M EDTA pH 8.0[6]
	ddH_2O

Combine all ingredients and make up to 1000ml of water. Autoclave and store at room temperature.

27. 1x TBS

3g	Tris
8.8g	Sodium chloride
0.2g	Potassium Chloride
1000ml	ddH_2O

Dissolve reagents in 800ml of water for irrigation (Baxter) and adjust pH to 7.4. Bring the volume up to 1L with water and autoclave. Store at room temperature

28. TBST

1000ml	TBS[27]
500ul	Tween20

Dissolve Twen20 into TBS. store at room temperature

29. Transfer buffer

6.06g	Tris
30.02g	Glycine
400ml	Methanol
1600ml	ddH_2O

Combined all ingredients. Autoclave and store at 4˚C.

Appendix 2. Data Collection Form

HOLLYWOOD PRIVATE HOSPITAL

DATA COLLECTION SHEET

Title of Study: Investigation of inhibitors preventing transport of folate in recurrent miscarriage

Age:___________________
Height:________________cm Weight:________kg

Pregnancy History
Number of pregnancies:____
Number of live births: ____
Number of miscarriages:____
Number of consecutive miscarriages:____
How many weeks did miscarriage occur:____
Other relevant history:____

Risk Factors for Miscarriage
Is there any history of:

Risk factor	Yes	No
Hormonal problems		
Recent/current infection		
Smoking		
Drug Use		
Malnutrition		
Excessive Caffeine		
Exposure to radiation or toxic substances		
Other:		

Past Medical History/Current Health Concerns:____
____
____
____
____

Family History of Clotting Disorder, Miscarriage, DVT/PE:________________________________
____
____
____

Current Medications/Medication History (including over the counter and vitamin supplements): __
____
____
____
____

Folate Supplementation YES NO Brand:_____________________________________