GENETIC PREDISPOSITION OF M2 ANNEXIN A5 HAPLOTYPE IN RECURRENT PREGNANCY LOSS AMONG THE MALAYS IN MALAYSIA

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by

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°C	Celsius
ACOG	American College of Obstetrics and Gynecology
AF	allele frequency
ANXA5	Annexin A5
APC	activated protein C
aPLs	antiphospholipids
APS	antiphospholipid syndrome
AUCs	areas under the curves
BLAST	basic local alignment search tool
CI	confidence intervals
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
DVT	deep venous thrombosis
EDTA	Ethylenediaminetetraacetic acid
FVL	Factor V Leiden
GDM	gestational diabetes mellitus
GHT	gestational hypertension
GW	gestational week
hCG	human chorionic gonadotrophin
HWE	Hardy-Weinberg equilibrium
IQR	interquartile ranges
IVF	*in vitro* fertilization
LDA	low dose aspirin

LMWH	low-molecular-weight heparin
MCMC	Monte Carlo Markov chain
min	minutes
MTHFR	Methylentetrahydrofolate reductase
NOS	Newcastle-Ottawa scale
OR	odds ratio
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PS	phosphatidylserine
PTm	Prothrombin mutation
RE	restriction enzyme
rhPCR	RNase H2- dependent PCR
ROC	receiver operating characteristic
RPL	repeated pregnancy loss
SGA	small gestation age
TAE	Tris-Acetate-EDTA
TFR	total fertility rate

KECENDERUNGAN GENETIK M2 ANNEXIN A5 HAPLOTIP DALAM KEGUGURAN IDIOPATIK BERULANG DI KALANGAN MELAYU DI MALAYSIA

ABSTRAK

Keguguran berulang merupakan suatu keadaan lazim yang berlaku di mana faktor-faktor trombofilia keturunan konvensional yang telah dikenal pasti bukannya punca utama keguguran di populasi Malaysia. Haplotip *M2/ANXA5*, suatu variasi pada kawasan promoter gen Annexin A5 (ANXA5), telah diimplikasikan sebagai suatu faktor kecenderungan bagi keguguran idiopatik berulang. ANXA5 ialah protein antikoagulan plasenta yang terikat pada permukaaan "syncitiotrophoblasts" untuk mengelakkan episod trombosis yang akan menyebabkan keguguran berulang. Di dalam kajian ini, kadar kehadiran haplotip *M2/ANXA5* adalah 42.2% di kalangan populasi melayu, 34.9% untuk kategori kawalan parus. Apabila membandingkan kadar *M2/ANXA5* di dalam kategori pembawa keguguran berulang primer dan sekunder dengan pembawa *M2/ANXA5* di kalangan populasi rawak, nisbah ganjil pada 1.52 (dengan sela keyakinan 95%: 1.1 sehingga 2.1) telah dinilaikan. Nisbah ganjil yang tertinggi ialah 1.97 (dengan sela keyakinan 95%: 1.3 sehingga 3.1) telah didapati apabila dibandingkan dengan kawalan parus. Pertambahan bilangan alel *M2/ANXA5* akan meningkatkan risiko keguguran berulang pada pasangan, terutamanya jika kedua-dua pasangan merupakan pembawa *M2/ANXA5*. Kajian ini telah mengesahkan peranan haplotip *M2/ANXA5* sebagai faktor kecenderungan untuk mengalami keguguran berulang di kategori primer dan sekunder pada awal kehamilan di kalangan populasi Melayu. Berikutan dengan itu, kuantifikasi plasma ANXA5 di kalangan subjek yang mengandung dan tidak mengandung telah dijalankan untuk menentukan potensi nilai diagnostiknya. Namun begitu, keputusan yang didapati menunjukkan

bahawa tiada perbezaan yang ketara dalam paras ANXA5 di plasma di antara wanita yang tidak mengandung, subjek kawalan parus mengandung dan wanita yang mengalami keguguran berulang, walaupun terdapat trend yang menunjukkan perbezaan yang ketara dalam paras ANXA5 di plasma di antara pembawa *M2/ANXA5* dan bukan pembawa (p = 0.02, dengan pembetulan post-hoc Bonferoni) di dalam analisis 'dalam-kumpulan'. Oleh itu, paras ANXA5 di plasma tidak mempunyai nilai diagnostik bagi menilai risiko keguguran berulang jika dibandingkan dengan pendekatan genetik. Seterusnya, ujian tindakbalas rantai polimerase yang bergantung kepada RNase H2 telah dihasilkan sebagai ujian saringan haplotip *M2/ANXA5*. Akhirnya, untuk mengenalpasti hubungan di antara haplotip *M2/ANXA5* dan keguguran berulang, satu analisis meta yang melibatkan 13 laporan kajian telah dilaksanakan. Sesungguhnya, keputusan menunjukkan bahawa haplotip *M2/ANXA5* merupakan faktor kecenderungan untuk megalami keguguran berulang (OR: 1.597, 95 % CI: 1.083 hingga 2.354, p = 0.018). Secara keseluruhannya, kajian ini telah mengesahkan bahawa haplotip *M2/ANXA5* mempunyai kaitan dengan keguguran berulang pada awal kehamilan yang tidak dapat dijelaskan di kalangan populasi Melayu, khasnya apabila kedua-dua pasangan merupakan pembawa *M2/ANXA5*. Oleh itu, ia membuktikan bahawa ujian saringan haplotip *M2/ANXA5* merupakan suatu ujian prognostik bagi penilaian risiko keguguran berulang, di mana ujian tindak balas rantai polimerase yang bergantung kepada RNase H2 telah dihasilkan bagi saringan tersebut.

GENETIC PREDISPOSITION OF M2 ANNEXIN A5 HAPLOTYPE IN RECURRENT PREGNANCY LOSS AMONG THE MALAYS IN MALAYSIA

ABSTRACT

Repeated Pregnancy Loss (RPL) is a prevalent condition where conventional hereditary thrombophilia factors have been ruled out in Malaysia population. *M2/ANXA5* haplotype, a variation at the core promoter region of Annexin A5 (ANXA5) gene, was suggested to be a predisposition factor for RPL. ANXA5 is a placenta anticoagulant protein which binds on the surface of syncitiotrophoblasts to prevent thrombotic events that would lead to RPL. In this study, the prevalence of *M2/ANXA5* haplotype was 42.2% in Malay population and 34.9% for parous controls. When compared *M2/ANXA5* clinically defined primary and secondary RPL carriers to *M2/ANXA5* carriers in random populations, odds ratio of 1.52 (95% CI:1.1 to 2.1) was determined. The highest odds ratio of 1.97 (95% CI: 1.3 to 3.1) was obtained when compared to parous control. Increasing copy numbers of *M2/ANXA5* alleles contribute a higher risk in RPL couples, especially when both partners were *M2/ANXA5* carriers. This first study confirmed the proposed role of *M2/ANXA5* haplotype as a predisposition factor for early primary and secondary RPL among Malay population. Following that, the quantification of the ANXA5 plasma in pregnant and non-pregnant subjects was carried out to seek its potential diagnostic value. However, the results showed no significant differences in ANXA5 plasma levels across non-pregnant, pregnant parous and RPL women, even though there was a trend of significant difference in the ANXA5 plasma levels between *M2/ANXA5*-carriers and non-carriers (p = 0.02, with post-hoc Bonferoni correction) in the 'within-group' analysis. Hence, ANXA5 plasma levels in maternal does not have a diagnostic value for RPL risk estimation as compared to the molecular approach. Next, a RNase H2-dependent PCR

was developed for the screening of *M2/ANXA5* haplotype. Finally, to ascertain the link between *M2/ANXA5* haplotype and RPL, a meta-analysis involving 13 reported studies was carried out. Indeed, the results indicated that *M2/ANXA5* haplotype is a predisposition factor for RPL (OR: 1.597, 95% CI: 1.083 to 2.354, p = 0.018). Collectively, this work confirmed women with *M2/ANXA5* haplotype is associated with unexplained early RPL. Hence, it warrants the screening of *M2/ANXA5* haplotype as a prognostic test for the risk assessment of RPL, where a single tube RNase H2 dependent PCR was developed for this screening.

CHAPTER 1

INTRODUCTION

1.1 Pregnancy Loss

Pregnancy loss/miscarriage described a loss of the conceptus from fertilized ovum to neonate that failed to progress, resulting in death and expulsion of the embryo or fetus that weigh 500 g or less within 20 gestational weeks. Miscarriage is a major problem in women's health. About 25% of all clinically recognizable pregnancies ended in miscarriage (ASRM, 2008). This is a trauma for couples who are seeking parenthood and remain a formidable clinical challenge to their physician. The couples are affected with significant emotional, social and even the economic impact. It has been reported that 5% of women may encounter two or more losses repeatedly, and 1% of women even have three or more losses throughout their reproductive age (Stirrat, 1990).

1.2 Repeated pregnancy loss

Thus far, there is no consensus achieved on the definition of repeated pregnancy loss [(RPL), (Farquharson et al., 2005, Stirrat, 1990)]. American College of Obstetrics and Gynaecology (ACOG) defined RPL as two or more consecutive miscarriages (ACOG practice bulletin, 2002). In addition, RPL may also be grouped into three sub-conditions: primary RPL; secondary RPL and tertiary RPL. Primary RPL refers to multiple pregnancy losses in which a patient has never had a live birth, while secondary RPL refers to multiple pregnancy losses with at least a live birth previously, and tertiary RPL refers to multiples pregnancy losses in a patient who has had interspersed with pregnancies that have progressed beyond 20 weeks' gestation (Practice Committee of the ASRM, 2008, Carp, 2007).

RPL is a complex and multifactorial obstetric problem with polygenic background (Figure 1.1). It has been shown to be associated with genetics, age, antiphospholipid syndrome (APS), uterine anomalies, hormonal or metabolic disorders, infection and autoimmunity. However about 50% of RPL cases are still unexplained, or idiopathic including non-antiphospholipid syndrome of heritable thrombophilia origin (Ford and Schust, 2009).

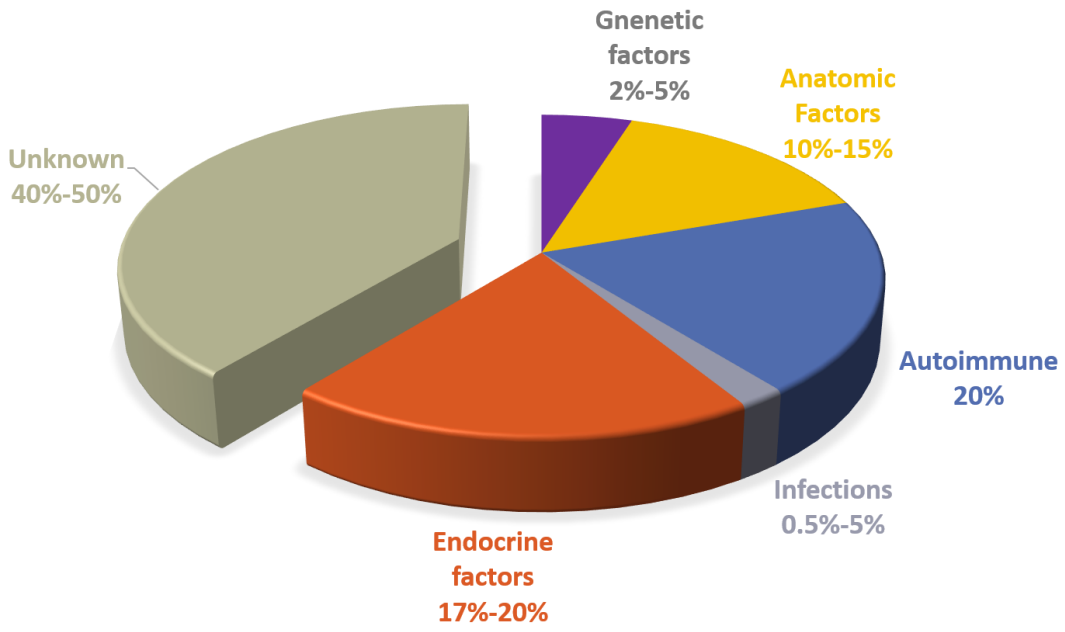

Figure 1.1 Etiology of RPL.

1.3 Heritable thrombophilia and RPL

Heritable thrombophilia has been reported to be one of the major cause for unexplained RPL (Kupferminc et al., 1999, Preston et al., 1996). Predisposing factors of hereditary thrombophilia leading to venous thrombosis are discussed below.

1.3.1 Factor V Leiden mutation

A missense mutation at the nucleotide position 1691 of Factor V gene (from G to A), resulted in a substitution of arginine (R) to glutamine (Q) at position 506 of the procoagulant protein. The site where activated protein C (APC) would normally cleave and inactivate procoagulant factor Va. This substitution partially causes resistant of the activated protein C (APC) anticoagulation properties, resulting in increased thrombin generation leading to a prothrombotic state. This prolong prothrombotic state would lead to venous or arterial thrombosis or RPL.

Factor V Leiden (FVL) is one of the most common factor for heritable thrombophilia. However, its prevalence varied among the different ethnic groups. For example, it is common in Whites [3 to 15%, (Ridker et al., 1997b, Rees et al., 1995)] but rare in Africa and Asia descendant [≤ 1%, (Kujovich, 2010, Irani-Hakime et al., 2000, Fujimura et al., 1995)]. Patients with FVL usually have an increased risk of venous thrombosis (from 3- to 100- fold) depending on their zygosity (Ridker et al., 1997a, Griffin et al., 1993, Koster et al., 1993); the associations of FVL and RPL were reported to have odds ratio ranging from 0.5 to 9 (Said et al., 2010, Clark et al., 2008, Lindqvist et al., 2006, Dizon-Townson et al., 2006, Rai et al., 2001, Murphy et al., 2000, , Wramsby et al., 2000, Foka et al., 2000, Kutteh et al., 1999, Brenner et al., 1997, Balasch et al., 1997), indicating that carrier of FVL will have 0.5 to 9 times higher risk in experiencing RPL when compared to non-carrier.

Indeed, a meta-analysis carried out by Rey et al. (2003) successfully showed that FVL was truly associated with early recurrent fetal loss (OR 2.01, 95% CI: 1.13 to 3.58); and even higher in late non-recurrent fetal loss (OR 3·26, 95% CI: 1·82 to 5·83). A year later, another meta-analysis (involving 16 reported studies) that was carried out by Kovalesky et al. (2004) also reported an OR of 2.0 (95% CI: 1.5 to 2.7)

in support for the association between FVL and RPL. Following that, Robertson et al. (2006) performed systematic review of 40 studies linking the associations of **(a)** FVL and RPL in first trimester loss (OR 1.91; 95% CI: 1.01 to 3.61); **(b)** FVL and non-RPL in second trimester loss (OR 4.12; 95% CI: 1.93 to 8.81). A recent systematic review and meta-analysis of prospective trials (10 studies) also reported an increased risk of late pregnancy loss among FVL carriers (OR 1.52, 95% CI: 0.80 to 1.25). All the above reports evidence the role of FVL as a contributing factor to recurrent pregnancy loss.

1.3.2 Prothrombin G20210A mutation

Prothrombin G20210A mutation is the second most common hereditary factor follows by FVL. Prothrombin is the precursor of thrombin in the coagulation system. A transition mutation from G to A at nucleotide 20210 of the 3' untranlasted prothrombin gene; resulted in accumulation of messenger RNA (prolonged turnover) therefore increases concentration of prothrombin protein in plasma, which is, in turn, lead to the risk of thrombosis.

The prevalence of the prothrombin mutation (PTm) in Europe is approximately 1.7 to 3%. Heterozygous carriers of the 20210A allele have a 2- to 8- fold higher risk for venous thrombosis (Poort et al., 1996). Very few cases of homozygosity for this mutation have been described (Rosendaal et al., 1998). The first comprehensive meta-analysis from Rey et al. (2003) showed that a significant association between PTm and RPL below 13 weeks of pregnancy (OR 2.70, 95% CI: 1.37 to 5.34) as well as with non-recurrent fetal loss after 20 weeks of gestation.

Likewise, a meta-analysis from Kovalesky et al. (2004) reported OR 2.0 (95% CI: 1.0 to 4.0) in PTm and RPL in first 2 trimesters of pregnancy. Following that, Robertson et al. (2006) reported OR 2.49 (95% CI: 1.24 to 5.00) in early loss and OR

2.66 (95% CI: 1.28 to 5.53) in late loss. A recent systematic review and meta-analysis by Gao and Tao (2015) further confirmed a significant association with RPL, OR 1.81 (95% CI: 1.26 to 2.6). They have reported an increase risk in European rather than Middle-Eastern, suggesting the screening of PTm should be group-specific (Gao and Tao, 2015).

Individual carrying both FVL and prothrombin G20210A mutation has a 20 folds increased risk for venous thrombosis. Hence, DNA analyses of both mutations are highly recommended for patients with personal or family history of thrombosis (McGlennen and Key, 2002, Press et al., 2002).

1.3.3 Methylene tetrahydrofolate reductase C667T mutation

Another hereditary thrombophilia factor is methylentetrahydrofolate reductase (MTHFR), which is involved in methionine metabolism. Homocysteine, a non-protein-building sulfhydryl amino acid, is an intermediate product in this metabolism. Homocysteine could be demethylated or remethylated to methionine in the body to a balance methionine concentration. When body methionine is low, homocysteine will be remethylated to methionine by the addition of a methyl group from methyltetrahydrofolate via MTHFR (Rosenblatt, 1995).

A mutation found at the nucleotide 677 from C to T in the methylene tetrahydrofolate reductase gene (a thermolabile MTHFR enzyme) changes its amino acid from alanine (A) to valine (V), which increase the levels of plasma homocysteine (hyperhomocysteinemia). This mutation causes approximately 50% reduction in enzyme activity at 37 °C while increased its thermolability at 46 °C, thus lead to a mild ~ moderate hyperhomocysteinaemia (Frosst et al., 1995).

Hyperhomocysteinaemia is a risk factor for pre-eclampsia, placenta abruption and fetal neural-tube defects (van der Put et al., 1998). Initial studies suggested that homozygosity for MTHFR C677T could be related to pregnancy loss (Goodman et al., 2006, Nurk et al., 2004); however, meta-analysis from Rey et al. (2003) and Roberston et al. (2005) reported that there is a lack of significant association with neither early nor late RPL. Due to insufficient evidence to association between pregnancy complications and MTHFR polymorphisms, therefore it was not included in the guidelines of the Italian society for Haemostasis and Thrombosis [(SISET) (Lussana et al., 2009)].

1.3.4 Activated protein C resistance (APCR)

Activated protein C resistance (APCR) is another risk factor for clinical thrombosis (de Visser et al., 1999). Protein C is an anticoagulant protein that plays an important role in regulation of haemostasis balancing. It is activated when contacting with thrombin-thrombomodulin complex, termed activated protein C (APC). Later, APC binds to free protein S (cofactor) and formed a protein C/protein S complex, which degrades the activated form of factor V and factor VIII, resulted in low production of fibrin (Figure 1.2). Defects in protein C pathway molecules will lead to activated protein C resistant (APCR). The common causative factors are: **(a)** deficiencies of protein C and protein S (**Chapter 1.3.5**); **(b)** mutation in factors V gene - FVL (**Chapter 1.3.1**), factor V Cambridge (Williamson et al., 1998), Factor V Hong Kong and Factor V HR2 haplotype (de Visser et al., 2000, Bernardi et al., 1997); **(c)** elevated factor VIII levels in plasma (Laffan and Manning, 1996).

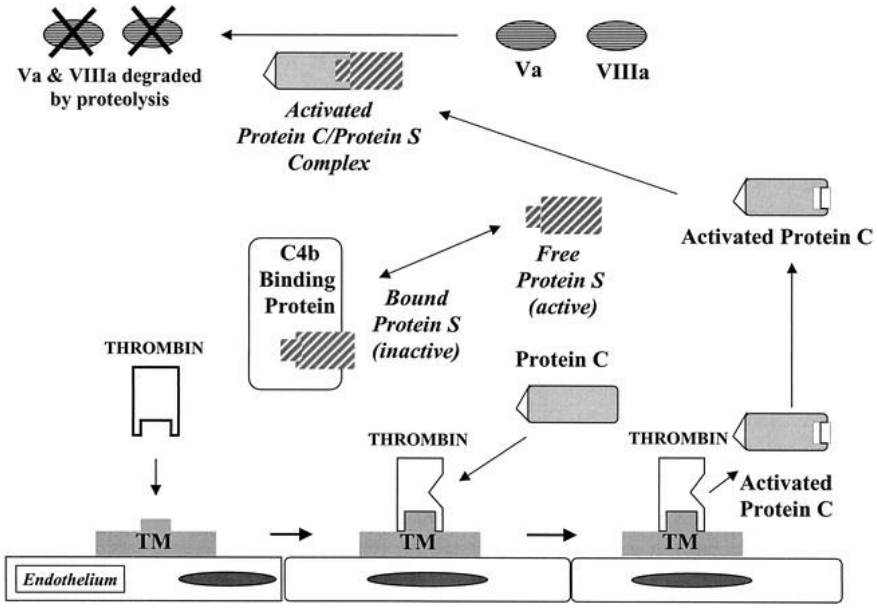

Figure 1.2 The anticoagulant pathway of protein C/protein S. Protein C was activated by thrombin-thrombomodulin (TM) complex. Then, activated protein C binds with its cofactor, free protein S, then degrades activated factors V and activated factor VIII. In addition, when thrombin binds thrombomodulin, thrombin loses its procoagulant functions (Adapted from Van Cott and Laposata, 1998).

Lindqvist et al. (2006) showed that patients with APCR have an increase prevalence of second trimester fetal loss. APCR was the most common cause of inherited thrombosis accounting for 40% to 50% of cases thus suggesting APCR as a screening test in the assessment of RPL (Rosendorff and Dorfman, 2007, Rey et al., 2003).

1.3.5 Protein C, protein S and antithrombin deficiency

Lastly, the deficiencies of protein C, protein S and antithrombin are heterogenous in nature and caused by several different genetic mutations. Their inherited traits are rarer than the genetic mutation described above yet they are contributing in clinical thrombosis (Rey et al., 2003). Protein C, protein S and antithrombin are the important

natural anticoagulants; Figure 1.3 shows that their involvement in maintaining the haemostasis system (Lipe and Ornstein, 2011).

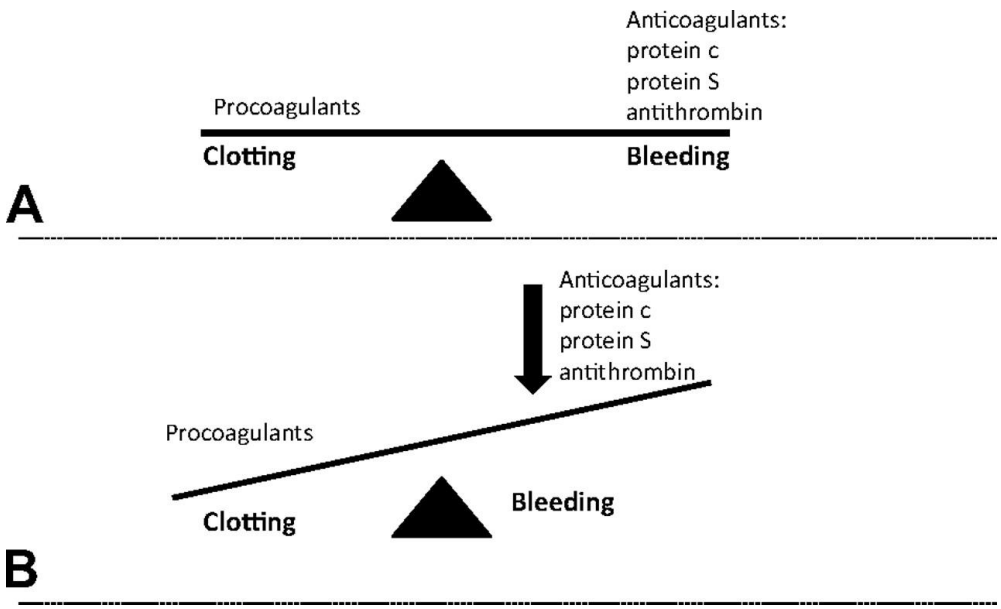

Figure 1.3 The relationship of natural anticoagulants and procoagulants between clotting and bleeding. In general, procoagulants (molecules that cause blood clots) are balanced by the natural anticoagulants (**A**). When there is a deficiency in one of the natural anticoagulants (**B)**, the individual has an increased propensity to form abnormal blood clots (Adapted from Lipe and Ornstein, 2011).

Protein C and Protein S are vitamin-K-dependent anticoagulant proteins. Reduced levels/activities of protein C and protein S due to mutations would lead to the imbalance of haemostasis system and favour of a hypercoagulable state, hence increases the risk of thrombosis. In contrast, antithrombin is a non-vitamin K-dependent protease that inhibits thrombin (primary target) and serine protease activated factors (factor II, IX, X, XI, and XII). The activity of antithrombin is markedly exerted by the presence of heparin resulted in anticoagulation (Nguyen et al., 2016, Palta et al., 2014). Deficiency of antithrombin level predispose to thromboembolic disease.

Rey et al. (2003) in their meta-analysis confirmed the association of protein S deficiency with RPL (OR 14.72, 95% CI: 0.99 to 218.01) and late non-recurrent fetal loss (OR 7.39, 95% CI: 1.28 to 42.63). They have excluded protein C and antithrombin deficiencies as thrombophilia risk factors for pregnancy loss. Another meta-analysis failed to confirm the association of protein C, protein S and antithrombin deficiencies in late pregnancy loss. The prevalence of inherited protein C, protein S and antithrombin are extremely rare, therefore not many studies were carried out for RPL cases.

In a nutshell, protein C, protein S and antithrombin III are natural anticogualants, the deficiencies of these factors are uncommon (Seligsohn and Lubetsky 2001). Homozygosity of MTHFR (C677T) may lead to hyperhomocysteinemia but the association of RPL is relatively uncertain, even the SISET guidelines failed to recommend MTHFR as a screening test for pregnancy complications (Lussana et al., 2009). FVL and PTm are the two most common causes of inherited thrombophilia; they are very common among Caucasians, but rare in Asians and Africans (Kujovich, 2010, Rosendaal et al., 1998). Recently, a newly

discovered hereditary factor, termed *M2/ANXA5* haplotype was proposed to be a new risk factor for RPL.

1.4 Annexin A5 and its function

Annexin A5 (*ANXA5*) gene is located at ~ 9 kb of human chromosome 4 q27. It consists of an untranslated exon with 12 coding exons (Cookson et al., 1994). The regulation of *ANXA5* gene expression is yet not well known. The human *ANXA5* gene expresses several transcripts and possesses a complex promoter that is subject to intricate regulation (Carcedo et al., 2001). Mutations of *ANXA5* at -1C → T (rs11575945) variant was proposed to protect young individuals against myocardial infarction (González-Conejero et al., 2002).

Annexin A5 (ANXA5) protein was first reported in bovine aortic endothelial cells further isolated and characterized in human placenta (Funakoshi et al., 1987). ANXA5 belongs to the member of "lipocortin" or "calpactin" family. The mRNA does not have a 5'-leader sequence, which is necessary for release into the extracellular environment. However, the protein can be found both extracellularly (blood circulation) and intracellularly (kidney, cardiac, placental and so on).

Annexin A5 is a Ca^{2+} dependent anticoagulant protein and binds strongly to phosphatidylserine (PS) to prevent coagulation activation. PS is an anionic phospholipid that locates in the inner leaflets of cell plasma membrane; it will be exposed on cell membranes when trigger by **(a)** program cell death, **(b)** activated platelets and **(c)** on syncytiotrophoblasts (placenta-related-cells). Although the placenta is a foreign entity to the maternal immune system, this "natural" allograft is tolerated (Guleria and Sayegh, 2007). This is because Annexin A5 forms a highly ordered two-dimensional crystal structure over the phospholipids bilayer and blocks

the phospholipids from binding to other proteins [e.g. antiphospholipid (aPL) antibody] that could result it to be free of coagulation activation (Figure 1.4). Therefore, Annexin A5 acts as a protective shield in between the fetal and expected mother.

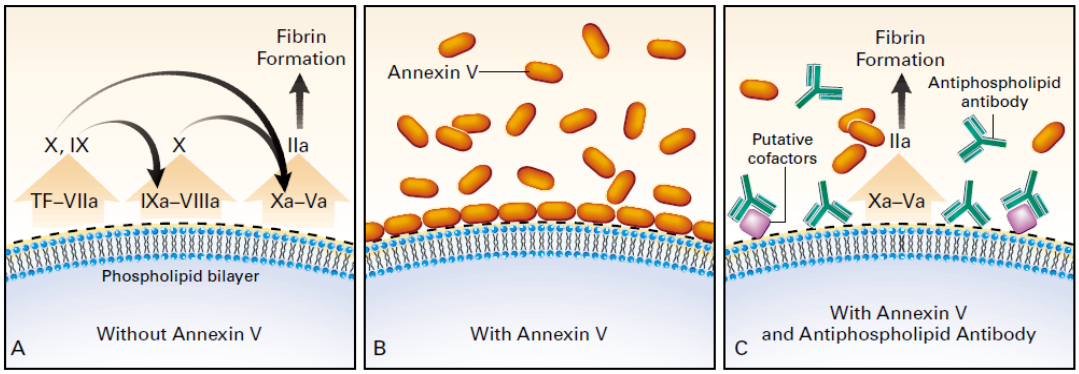

Figure 1.4. Model of protective shield of ANXA5 using trophoblast cells and endothelial cells. **(A)** anionic phospholipids (minus signs) on the surface of the cell-membrane creates a phenomenon to trigger coagulation pathway; **(B)** Annexin A5 forms a highly ordered two-dimensional crystal structure over the anionic phospholipids and block the assembly of the phospholipid-dependent coagulation complexes, thereby inhibiting coagulation while absent of aPLs antibodies; **(C)** aPLs antibodies disrupt the ability of ANXA5 to cover on the phospholipid surface. This action reduces the binding affinity of ANXA5 and permits more anionic phospholipid to be available to form complexes with coagulation proteins. As a result, coagulation is accelerated and the development of thrombosis is promoted. TF denotes tissue factor (Adapted from Rand et al., 1997).

1.4.1 *ANXA5* gene promoter variants and their activities

Although Rand et al. (1997) had proposed the model of protective shield model, why the aPLs antibody disrupts the ANXA5 protein shield was unclear. Hence, Bogdanova et al. (2007) initiated to perform a systematic screening of the coding and core promoter region of *ANXA5* gene in 70 RPL patients.

Figure 1.5 describes structure of the *ANXA5* gene core promoter region. The nucleotide 1 is at the first transcription start point of the gene, tsp 1 (Carcedo et al., 2001), which corresponded to nucleotide 4956 of the gene (NG_032042.1); whereas the 5'- UTR began at -1 and proceeded in the negative direction (upstream from nucleotide 1). The determined SNPs were numbered according to the position of tsp 1.

Figure 1.5 Sequence of the *ANXA5* gene core promoter region. Red circle is the tsp1. Bold capital letters are the nucleotides changed in *M2/ANXA5* haplotype.

They identified a constellation of 4 single-nucleotide polymorphisms (SNPs), -19 G →A, rs112782763; 1 A → C, rs28717001; 27 T → C, rs28651243; and 76 G → A, rs113588187 in the proximal core promoter region of the *ANXA5* gene. From here, two forms of haplotypes were determined: **(a)** *M1/ANXA5* haplotype: two SNPs (1 A → C, rs28717001 and 27 T → C, rs28651243); **(b)** *M2/ANXA5* haplotype: four SNPs

(-19 G →A, rs112782763; 1 A → C, rs28717001; 27 T → C, rs28651243; and 76 G → A, rs113588187).

All substitutions were at the position of transcription factor consensus that may impair the *ANXA5* promoter activity. To assess the promoter activities, *M2/ANXA5* haplotype and wild type *ANXA5* promoter sequence were cloned and analyzed using luciferase Gene Reporter Assay kits. Bogdanova et al. (2007) showed a drastic reduction of the *M2/ANXA5* haplotype activity (37 to 42% activity compared with wild type; Figure 1.6); whereas a less pronounced for *M1/ANXA5* haplotype (57 to 62% activity compared with wild type).

By comparing 70 RPL patients to controls subjects, they proposed the association of *M2/ANXA5* haplotype with RPL, whereas *M1/ANXA5* haplotype is not a predisposing factor. This was further confirmed by other studies later (Rogenhofer et al., 2012, Tüttelmann et al., 2013, Demetriou et al., 2015).

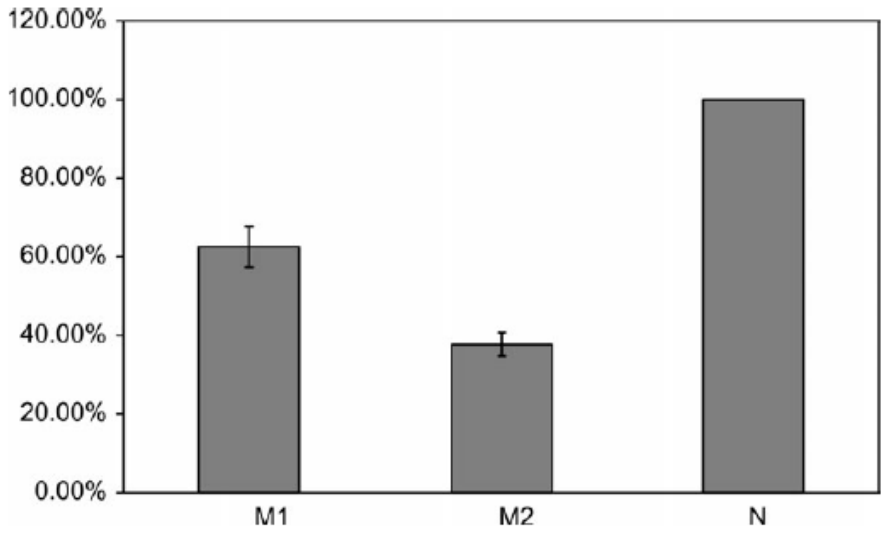

Figure 1.6 Activity of *ANXA5* gene promoter variants in luciferase reporter gene assay. N (wild-type) 100% activity. M1 (*M1/ANXA5* haplotype) contains 57 - 62% activity; M2 (*M2/ANXA5* haplotype) contains 37 - 42% activity (Adapted from Bogdanova et al., 2007).

Subsequently, Chinni et al. (2009) extracted placental RNA near to umbilical cord and reported that a 2-fold reduction of ANXA5 gene expression in the *M2/ANXA5* carrying placentas. Therefore, they concluded that *M2/ANXA5* haplotype could influence *ANXA5* gene expression not only *in vitro* but also *ex-vivo* (Chinni et al., 2009). In the following year, Markoff et al. (2010) similarly reported a decrease amount of *ANXA5* mRNA in *M2/ANXA5* carrying placentas and further showed that no allelic compensation was observed when comparing the expression ratio of *ANXA5* N allele mRNA in heterozygous (N/M2) vs. 'normal homozygous' (N/N) placentas. In addition, *M2/ANXA5* mRNA is reduced in all N/M2 samples regardless of the origin of the *M2/ANXA5* allele (from paternal or maternal), indicating that the reduction was from embryonic rather than wholly maternal induced risk. Ota et al. (2013) confirmed the reduced expression of *ANXA5* mRNA in *M2/ANXA5* carrying placental; they further examined the expression of ANXA5 protein levels through immunostaining (Figure 1.7) and concluded that the protein levels were affected more by the placental genotype than the maternal genotype.

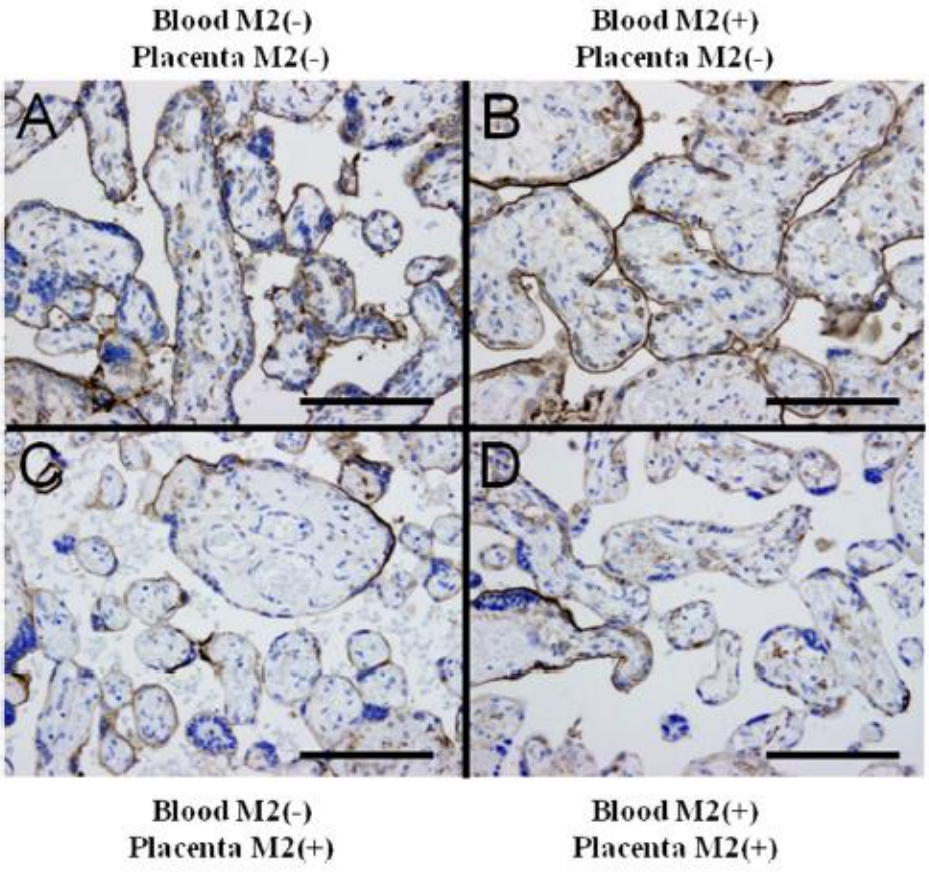

Figure 1.7 Immunostaining for ANXA5 protein in placental tissue sections. Chorionic villi were stained with a mouse anti-human ANXA5 monoclonal antibody. **(A)** Non-*M2/ANXA5* carrying placenta from a non-*M2/ANXA5* carrying mother; **(B)** Non-*M2/ANXA5* carrying placenta from an *M2/ANXA5* carrying mother; **(C)** *M2/ANXA5* carrying placenta from a non-*M2/ANXA5* carrying mother; **(D)** *M2/ANXA5* carrying placenta from a *M2/ANXA5* carrying mother. (Adapted from Ota et al., 2003).

1.4.2 The prevalence of *M2/ANXA5* haplotype

Generally, the *M2/ANXA5* carrier rates in European and Asian (Japanese) control populations ranged from 11% to 17% (Demetriou et al., 2015, Tüttelmann et al., 2013, Rogenhofer et al., 2012, Miyamura et al., 2011, Tiscia et al., 2009, Bogdanova et al., 2007). When estimating the association between *M2/ANXA5* haplotype and RPL, the odds ratios were from 1.5 to 2 when comparing to the general population of German (Tüttelmann et al., 2013, Bogdanova et al., 2007), UK (Demetriou et al., 2015), and Bulgarian (Tüttelmann et al., 2013) ethnic backgrounds; accordingly, elevated odds

ratios (1.8 to 3.0) were documented when comparing to parous controls (negative history for infertility or miscarriage) in German (Tüttelmann et al., 2013, Bogdanova et al., 2007), UK (Demetriou et al., 2015), Italian (Tiscia et al., 2009), and Japanese (Miyamura et al., 2011) cohorts. In accordance with the proposed physiological role of *M2/ANXA5* haplotype in embryonic anticoagulation, several studies have showed similar paternal (father) risk in RPL couples (Demetriou et al., 2015, Tüttelmann et al., 2013).

1.4.3 Revised ANXA5 protective shield model

Gathering all information above (**from Chapter 1.4**), it was affirmed that *M2/ANXA5* carrying placental would have reduced *ANXA5* mRNA expression with lowered ANXA5 protein on syncytiotrophoblast resulting a predisposing factor for various thrombophilia related obstetric complications.

Previously, Rand et al. (1997) proposed that the aPLs disrupt the ability of ANXA5 to form two-dimensional structure, resulting in a lower affinity for this protein, which consequently favours the binding of coagulation-factor complexes. However, the authors did not explain why aPLs disrupt the ability of ANXA5. Recently, Bogdanova and her co-researchers reported an increased *M2/ANXA5* carrier rate in obstetric APS patients, hence this prompted them to propose a 'genetic' model for disrupted ANXA5 shielding (Bogdanova et al., 2012). This revised model could explain the emerge of aPLs in the first instance (Figure 1.8 B 'genetic' versus Figure 1.8 A 'immunologic model').

In brief, their hypothesis narrated a predisposing factor, *M2/ANXA5* haplotype, leads to attenuated coverage of exposed anionic phospholipid surfaces with clusters of ANXA5. Because of the inefficient shielding: **(a)** coagulation factors can compete for

phospholipid binding; and **(b)** there would be an enhanced exposure of phospholipid antigenic determinants that would lead to aPLs development then would further disrupt the ANXA5 protective shield.

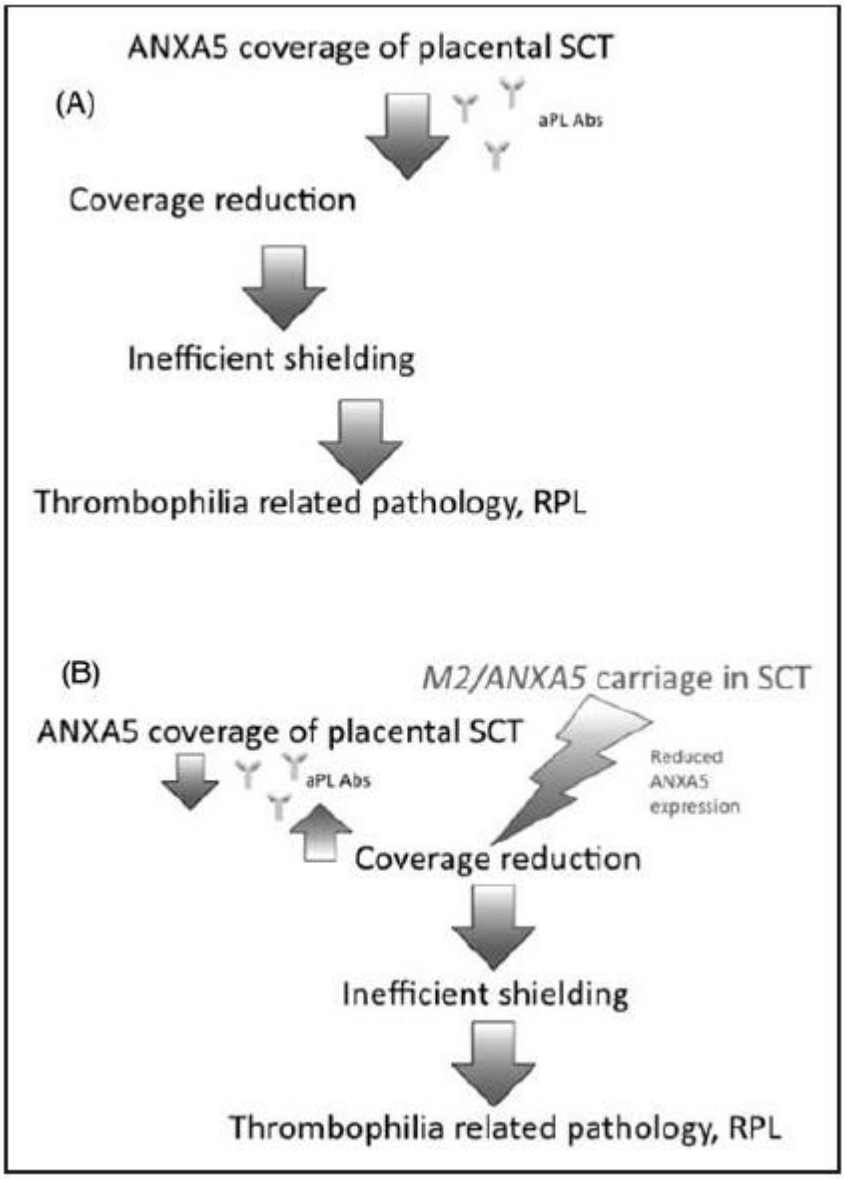

Figure 1.8 The ANXA5 protective shield model. **(A)** 'immunologic' versus **(B)** 'genetic' revised model.

1.5 RPL in Malaysia

Malaysia is a multi-ethnic society with different cultural and socioeconomic backgrounds. Malay is the largest ethnic group (55%) in Malaysia. The incident of pregnancy loss in Malaysia was 23% according to Survey on Health and Family planning (Arshat et al., 1985).

Since the two most common causes of inherited thrombophilia (FVL and PTm) are virtually absent in Asians, especially in Southeast Asian individuals (Rees et al., 1996); and only two publications (Yusoff et al., 2002, Ayadurai et al., 2009) reported the prevalence of FVL and PTm (1% and 0.3%, respectively) in RPL Malay. There are no official data regarding RPL incidence among Malay to date. Based on the statistics available from the Malaysian Population and Family Survey (MPSF), 14% of early pregnancy losses are recorded from total pregnancies (Tey et al., 2012). However, this reported abortion rate could be an underestimate. Notably, a high proportion of pregnancy losses at the Sultan Abdul Halim Hospital from 2013 to 2015, 2296/2575 (89.2%), occurred in Malay women. The spontaneous abortion rate in Malay women at this hospital was 20.3% from a total of 11,306 admissions, although no records were available on RPL.

1.6 Objectives

Owing to the high pregnancy loss rate and the scarcely studied of genetic aspect of RPL in Malaysia, the aims of the present study were

1) To determine the frequency of *M2/ANXA5* haplotype in a cohort of Malays;
2) To elucidate whether *M2/ANXA5* haplotype is an independent RPL risk factor in the Malay population;
3) To estimate the power of *M2/ANXA5* haplotype in Malay population, after confirming the potential of *M2/ANXA5* haplotype as risk factor;
4) To examine the circulating ANXA5 levels in
    i) non-pregnant and pregnant women with or without history of RPL,
    ii) *M2/ANXA5* carriers vs. non *M2/ANXA5* carriers of these clinically defined groups;
5) To develop a method for the detection of Annexin A5 variants for screening tests of RPL, providing a more precise assessment of individual disease risks;
6) To determine the role of *M2/ANXA5* haplotype and RPL in published literatures via meta-analysis.

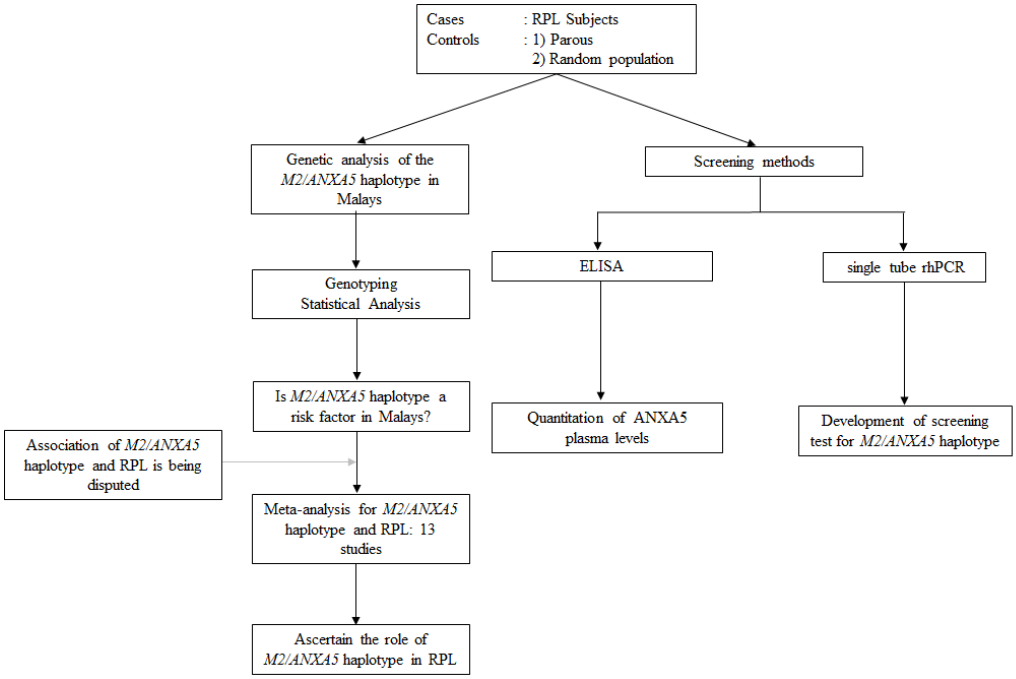

Figure 1.9 An overview of the study design of *M2/ANXA5* haplotype and RPL. Detail of each study design will be presented in each chapter.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical and reagents

All common chemicals (analytical grade) and reagents were used without further purification and purchased from Sigma-Aldrich (Missouri, United States) and Merck (New Jersey, United States) unless specified below or in the text.

2.2 Ethical Approval

The present study was approved by the Human Ethics Research Committee of the Universiti Sains Malaysia (USMKK/PPP/JEPeM [245.3.(2)]) and the National Institutes of Health, Ministry of Health, Malaysia (NMRR-11-1044-9519), Appendix A. The study was carried out in accordance with The Code of Ethics of the World Health Organization (Declaration of Helsinki). The controls and subjects who agreed to participate in this study have signed an informed consent before collection of peripheral blood samples.

2.3 DNA Extraction and genotyping

Blood samples were collected in a 3 ml of BD Vacutainer™ plastic blood collection tubes (New Jersey, United States) contained K_2 Ethylenediaminetetraacetic acid (EDTA). The sample collected was then centrifuged at 1,600 × g for 15 minutes (min) at room temperature. Buffy coat was aliquoted and stored at -80 °C until further used for DNA extraction.

Genomic DNA extraction was carried out using commercially available kit (Omega Bio-tek Inc., Norcross, USA) according to the manufacturer's protocol. The extracted DNA was carried out by PCR prior to DNA sequencing. The PCR reaction was carried out as described in Bogdanova et al. (2007) with minor modification as following. Optimized PCR reactions were performed on 100 ng of genomic DNA in a total reaction volume of 25 µL. The PCR mixture contained: 1 × PCR reaction buffer (Biotools, Spain); 1 mM $MgCl_2$ (Biotools, Spain); 5% dimethyl sulfoxide (DMSO); 0.2 µM of each (forward: ANXA5.P + ex1.Fwd and reverse: ANXA5.P + ex1.Rv) primer; 200 µM dNTPs; 100 - 200 ng genomic DNA and 1 U *Taq* polymerase (Biotools, Spain). The primers used are listed in Table 2.1. Cycling conditions were as follows: initial denaturation at 95 °C for 5 min; followed by 25 cycles of amplification; 95 °C for 30 s; 63 °C for 30 s; 72 °C for 30 s. The final extension was performed at 72 °C for 5 min.

Table 2.1 Primers for genotyping of *M2/ANXA5* haplotype.

Primer Name	Primer Sequence	Size	Tm	Amplicon Size
ANX5.P +ex1.Fwd	5'- CCGAGCCCTGGACAGCTCCCCA-3'	22 bps	67.9 °C	439 bps
ANX5.P+ex1.Rv	5'- GCCCCGCGACCACGCTCTCCTCT- 3'	23 bps	69.5 °C	

The PCR products were purified using MultiScreen^{TM} PCR plates (Millipore, Massachusetts, United States). Purified amplicons were directly sequenced in 96-well plates using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI/Perkin-Elmer, Massachusetts, United States); sequencing reactions were analyzed on an ABI PRISM*w* 3700 DNA Analyzer (ABI/Perkin-Elmer,

Massachusetts, United States). DNA sequences were analyzed on FinchTV version 1.4 (Geospiza Finch Suite, Perkin Elmer, Massachusetts, United States). Genotypes were scored in table format and four digits coded for further processing (Table 2.2).

Table 2.2 Codes for *M2/ANXA5* genotyping.

Genotyping	Codes
N/N	0101
N/M2	0103
M2/M2	0303

2.4 Allele-Specific PCR

The developed allele-specific PCR is based on the principle of nested PCR. The first PCR reaction was carried out as described in **Chapter 2.3**. Second PCR comprises of two independently parallel allele-specific reactions (wild-type and *M2/ANXA5* haplotype, respectively). The primers sequence used are listed in Table 2.3. The final optimized protocol included 1 µL of 10 × diluted first PCR product as template, 1 × PCR reaction buffer (Biotools, Spain), 0.5 mM $MgCl_2$ (Biotools, Spain), 0.2 µM of each primer, 5% dimethyl sulphoxide (DMSO) and 1.25 U *Taq* polymerase. The PCR cycling condition was the same as first PCR reaction. Then, the second PCR product was analyzed on 2% agarose gel in 1 × TAE at 100 V for 25 min. The amplicon size is 139 bps.

Table 2.3 List of Allele-Specific Primers.

Primer Name	Primer Sequence	Size	Tm	Amplicon size
AS Normal-Fwd	5'-TGGCGCGGCCGGCCTGCGGTTGG- 3'	23 bps	75.6 °C	139 bps
AS Normal-Rv	5'-GAGATGCAGACGCTGAAGGATCC- 3'	23 bps	59.6 °C	
AS M2-Fwd	5'-TGGCGCGGCCGGCCTGCGGTTGA- 3'	23 bps	74.7 °C	139 bps
AS M2-Rv	5'- GAGATGCAGACGCTGAAGGATCT-3'	23 bps	58.7 °C	