MATERNAL AND NEONATAL CHARACTERISTICS IN FETAL/NEONATAL ALLOIMMUNE THROMBOCYTOPENIA IN NATIONAL BLOOD CENTRE

By

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DISCLAIMER

I certify that this dissertation records the results of the study performed by me and that it is my own composition. I would also like to declare that I have no financial interest in this study

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LIST OF ABBREVIATIONS

CI	Confidence Interval
FNAIT	Foetal/Neonatal Alloimmune Thrombocytopenia
FMAIT	Feto-Maternal Alloimmune Thrombocytopaenia
GP	Glycoprotein
HLA	Human Leukocyte Antigen
HPA	Human Platelet Antigen
ICH	Intracranial haemorrhage
IgG	Immunoglobulin G
IVIG	Intravenous immunoglobulin
MAIPA	Monoclonal Antibody-Specific Immobilization of Platelet Antigen
MACE	Modified Antigen Capture Enzyme-Linked Immunosorbent Assay
NAIT	Neonatal Alloimmune Thrombocytopaenia
NBC	National Blood Centre, Kuala Lumpur
NICU	Neonatal Intensive Care Unit
NITP	Neonatal Immune Thrombocytopaenia
OR	Odds Ratio
SPSS	Statistical Package for the Social Sciences

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ABSTRAK

FNAIT *(Fetal/neonatal alloimmune thrombocytopenia*) merupakan keadaan klinikal yang jarang dan disebabkan oleh aloimunisasi ibu akibat pendedahan platelet janin sehingga menyebabkan trombositopenia terhadap janin dan bayi. Kajian ini bertujuan untuk mengetahui insiden FNAIT di Malaysia serta kaitannya dengan faktor-faktor ibu dan bayi. Kajian retrospektif ini merangkumi data kesemua kes positif FNAIT serta kontrol yang dipilih secara rawak dari tahun 2011 sehingga 2019 di Pusat Darah Negara. Kajian kes kontrol ini membandingkan faktor ibu iaitu umur, etnik, nombor kandungan dan kumpulan darah berserta faktor bayi iaitu presentasi, kiraan platelet dan kumpulan darah. Sejumlah 39 kes FNAIT ditemui. Insiden FNAIT di Malaysia adalah 0.85 setiap 100 000 kelahiran hidup. Antibodi platelet yang paling lazim ditemui adalah Anti-HPA-5b, Anti-HPA-3a dan Anti-HPA-5a. Lima puluh lapan (58%) ibu adalah berusia kurang dari 35 tahun, berketurunan Melayu dan pernah mengandung lebih dari sekali. Ibu Melayu berpotensi 4.8 kali ganda untuk dapat FNAIT berbanding etnik selain Melayu. Permulaan gejala penyakit, kiraan platelet dan kumpulan darah bayi adalah tidak signifikan berbanding kontrol. Perbandingan yang paling signifikan antara kesemua faktor adalah presentasi klinikal bayi. Bayi yang bergejala lebih cenderung untuk dapat FNAIT. Insiden FNAIT di kalangan Malaysia adalah rendah. Ibu berketurunan Melayu dan bayi yang bergejala adalah berkait rapat dengan FNAIT.

Kata kunci: FNAIT, insiden, anti-HPA

MATERNAL AND NEONATAL CHARACTERISTICS IN FETAL/ NEONATAL ALLOIMMUNE THROMBOCYTOPENIA IN NATIONAL BLOOD CENTRE

ABSTRACT

Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is an uncommon condition due to maternal alloimmunization of fetal platelet leading to thrombocytopenia in fetal and neonate. The aim of this study was to determine the incidence of FNAIT in Malaysia and the associated maternal and neonatal factors. Retrospective data analysis of matched, case control study (39 FNAIT cases and 39 randomly selected controls) from the year 2011 to 2019 at National Blood Centre, Malaysia. Maternal and neonatal characteristics were compared with controls. The incidence of FNAIT in Malaysia was 0.85 per 100 000 live births. Common anti-Human Platelet Antigen (-HPA) identified in this study were Anti-HPA-5b, Anti-HPA-3a and Anti-HPA-5a. Fifty-eight (58%) ($p=0.012$) of mothers were younger than 35 years old, of Malay ethnicity and multiparous. Malay mothers were 4.8 times more likely to develop FNAIT as compared to non-Malay. Neonatal onset of presentation, platelet count and blood group were not significantly different than in control group. The most significant factor associated with FNAIT was neonatal presentation. Symptomatic neonates were more likely to develop FNAIT. Incidence of FNAIT among Malaysians was lower than reported in the literature. Malay mothers and neonates presenting with symptoms are more likely to have FNAIT.

Keywords: neonatal alloimmune thrombocytopenia, incidence, human platelet antigen

CHAPTER ONE

INTRODUCTION

1.1 Overview

This chapter consists of brief introduction on platelet structure and function, platelet antigen system, fetal/neonatal alloimmune thrombocytopenia (FNAIT) pathophysiology and general management of FNAIT. This chapter also highlights the problem statement and objectives of the study.

1.2 Background of study

1.2.1 Platelet structure and its function

Platelets also known as thrombocytes are small (1-3μm), non-nucleated, discoid-shaped fragments of megakaryocytes originating from the bone marrow (1, 2). About $1x10^{11}$ platelets are released daily into the blood circulation following fragmentation where they live for 5-9 days (2).

These dynamic blood particles play important role in haemostasis. Once there is damage to the blood vessel wall, platelets response quickly by adhering to the vessel wall, undergo change in shape, aggregate with each other and cross-link with fibrinogen to form fibrin clot that prevent further blood loss (2, 3). Risk of bleeding increases when there is reduced platelet count and or functional defects of platelets (4). Besides haemostasis, platelets play vital roles in inflammation, immunity, disease pathophysiology and malignancies (2, 3).

1.2.2 Platelet antigen system and platelet antibodies

Despite its minute size, platelets have distinct internal organelles, secretory granules and complex antigenic structures on the external surface (3). There are numerous glycoproteins (GPs), blood group antigens as well as human leukocyte antigen (HLAs) present on the platelet. Human Platelet Antigens (HPAs) are epitopes on GPs specifically found on platelets. HPAs are the antigens of interest in the current study. Prior to 1990, HPAs were abbreviated with the names of patient when the antigens were first discovered (5). A standardized system was later implemented according to international consensus to avoid confusion between the abbreviations and further revised in 2003 (2, 6).

The HPAs are located on six GPs which are GPIIb, GPIIIa, GPIba, GPIbb, GPIa and CD109. Up to 2020, there are 35 HPAs being identified according to the Human Platelet Antigen Database (7). Twelve antigens are classified as biallelic groups which are HPA-1, HPA-2, HPA-3, HPA-4, HPA-5 and HPA-15. The biallelic numbering system is based on the order of discovery with ‘a’ designating a higher frequency antigen and ‘b’ designating lower frequency antigen. For instance, HPA-1a is more frequently found than HPA-1b. The designation ‘w’ is given when only antigen discovered for example HPA-8bw (2). Various studies worldwide had shown that HPA allele frequency and distributions vary across geographical locations and ethnicities (8).

Most of the HPA antigens arise from single nucleotide polymorphism except for HPA-14bw whereby there is a codon deletion (9). About 20 HPAs are found on GPIIb/IIIa and the remaining are located on other GP complexes. Below is an image representing (Figure 1) HPA antigens on GPIIb/IIIa, GPIa/IIa, GPIb/IX and CD109.

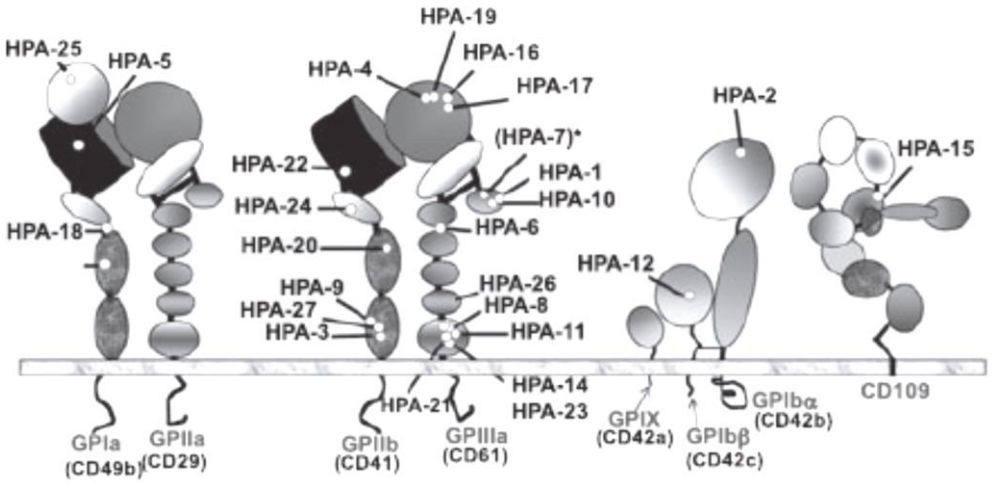

Figure 1– Platelet glycoprotein structures and HPA antigens. Adapted from (10).

Other antigens such as blood group ABO(H), P and I antigens are expressed in low levels on the platelets. Number of ABO antigens on the platelets are expressed variably among individuals. Between 4 and 7% of individuals express as high as 20 times more A and/or B antigens and are known as high expresser (2, 11). As ABO blood group antigens may cause immune platelet destruction, they may lead to platelet refractoriness and FNAIT among high expresser individuals. Antibodies against P and I antigens are not associated with immune platelet disorders (2).

Human Leukocyte Antigen (HLA) class I is also identified on platelet. Variable amount of HLA Class I- A, B and C are expressed on platelet membrane individually. It is still arguable whether antibodies against HLA class I could cause FNAIT or not. Some researchers do not support Anti-HLA class I antibodies causing FNAIT (12) (13). On the contrary, others propose anti-HLA class I antibodies causing FNAIT especially if the antibody titres are high (14, 15). Panzer had extensively elaborated on the controversy of

anti-HLA-Class I in development FNAIT 20 years ago (12) and we are still in limbo up to now.

Apart from FNAIT, platelet antibodies are implicated in several clinical conditions such as post transfusion purpura, platelet transfusion refractoriness, passive alloimmune thrombocytopenia as well as transplant-associated alloimmune thrombocytopenia (16). However, among these, FNAIT is considered as the most clinically significant and probably preventable disorder (8).

1.2.3 Thrombocytopenia in neonates

At the end of first trimester, mean platelet count is around 150 x 10^9/L and increases to about 250 x 10^9/L during second trimester. Therefore term and premature neonates are expected to have at least 150 x 10^9/L platelet count (17). Neonatal thrombocytopenia is defined when the platelet count is <150 x 10^9/L. Severe neonatal thrombocytopenia is when the platelet count is <50 x 10^9/L. About 1% - 5% of neonate are thrombocytopenic at birth with severe thrombocytopenia occurring in about 0.1%- 0.5% of cases (18). Ulusoy et al reported thrombocytopenia in 3.8% of 3515 neonates with a quarter of the cases being severe thrombocytopenia. Of these severe thrombocytopenic neonates, 72% of them were born preterm (19). Thrombocytopenia is a common haematological abnormality detected in about 12% to 25% cases of neonates admitted to neonatal intensive care unit (NICU) (17, 19).

Thrombocytopenia in neonates could be due to immune or non-immune causes. Autoimmune process such as maternal immune thrombocytopenia or alloimmune condition such as FNAIT lead to platelet destruction and thrombocytopenia. Very often,

neonatal thrombocytopenia is caused by non-immune conditions such as congenital infections, aneuploidy or due to inherited diseases. Retrospective study over 23 years by Resch et al reported that some of the common causes of neonatal thrombocytopenia is due to prematurity, sepsis, necrotizing enterocolitis and asphyxia (20). Onset of neonatal thrombocytopenia could be divided as early and late. Early onset which is less than 72 hours are commonly caused by chronic fetal hypoxia or perinatal asphyxia. Late onset due to asphyxia and necrotizing enterocolitis are usually seen later than 72 hours (17).

In another large study, neonatal thrombocytopenia was due to fetal distress, maternal hypertension, drug intake, congenital abnormalities, maternal idiopathic thrombocytopenic purpura, ABO and Rh mismatch and a substantial number of cases had no identifiable causes (21).

1.2.4 FNAIT pathophysiology

Although securing haemostasis is the main function of platelet, major bleeding is unusual unless the platelet count is less than $\leq 5x10^9/L$ in clinically stable patients. Bleeding at higher platelet count may also occur in the presence of other risk factors such as disseminated intravascular coagulopathy with contributory clotting factor deficiencies, structural lesions with loss of vascular integrity and refractoriness to platelet transfusions (22).

Early-onset severe neonatal thrombocytopenia without an apparent precipitating factor often causes concern among clinicians. The most common cause of isolated severe neonatal thrombocytopenia in otherwise healthy neonates is due to FNAIT (21). FNAIT was initially reported in 1950's and it is also known with various abbreviations such as

NAIT (neonatal alloimmune thrombocytopenia), FMAIT (feto-maternal alloimmune thrombocytopenia), NITP (neonatal immune thrombocytopenia) (18).

FNAIT is a clinical condition resulting from incompatibility between maternal and fetal HPA (23). Fetal platelet antigens are expressed around 16-18 weeks of gestation (24). Under normal circumstances, there is little or no feto-maternal blood exchange. However, fetal blood had been detected in almost all mothers towards the end of third trimester (23). Following sensitization of the fetal antigens, maternal immunoglobulin G (IgG) are produced and these IgG molecules are able to pass through the placenta and subsequently bind to the fetal platelets. The antibody-coated platelets are then removed in the fetal circulation by the reticuloendothelial system and subsequently lead to fetal thrombocytopenia (23, 25, 26).

It is estimated that FNAIT occurs in 1 among 800 to 2000 pregnancies (27). The proportion of individuals having a particular antigen type varies according to the race (25). When an individual lacks a specific HPA antigen, alloimmunization may occur when these individual are exposed to antigen positive platelets through transfusion, pregnancy or transplantation (8).

1.2.5 Alloantibodies in FNAIT

The commonest alloantigen is HPA-1a but other platelet-specific alloantigen may also be targets (28). HPA-1a antibodies are the main cause of FNAIT among Western populations accounting up to 80% of the cases The anti HPA-1a is associated with the most severe outcome. Alloimmunization with other platelet-specific antigen occurs less frequently. Platelet-specific alloantibodies are only detected in about 25-30% of serum from mothers with neonates with suspected FNAIT (12).

Kamphuis et al had reported that among HPA-Ia negative women, rate of sensitization was low. Only 10% produces HPA-antibodies despite carrying an HPA-Ia positive foetus (25). These antibodies are associated with presence of HLA DRB3*0101.

FNAIT due to HPA-1a antibodies among East Asian are extremely rare and was due to low prevalence of HPA-1b homozygosity. The HPA-4b is more common in East Asian population and this antibody accounts for 72% of FNAIT in Japan. Other antibodies implicated in FNAIT among Asians are HPA-2b, HPA-5b and HPA-6b. Uniquely, HPA-21bw antibody is also found more among Asian accounting to about 1.1% in Japanese and Chinese as compared to <0.3% in Caucasian. In African countries such as Nigeria, HPA-5a and HPA-5b antibodies are the only antibody specificity identified in parous women there (8).

1.2.6 Clinical manifestation of FNAIT

Neonates born with FNAIT could be asymptomatic, have purpura or hematoma or other bleeding manifestations. FNAIT may occur as early as second trimester and excepted to resolve naturally within 1 to 3 weeks after delivery (18). In a large Brazilian study by Castro et al involving 9332 neonates, 1.5% (142) had platelet counts of <100 $x10^9$/L with about 90%(128) having count of less than <50 $x10^9$/L. Among the 142 thrombocytopenic neonates, 17 or 12% had bleeding manifestation with no intracranial haemorrhage observed, whereas 13 had subcutaneous bleeding due to birth trauma (21).

The most devastating presentation of FNAIT is intracranial haemorrhage (ICH). ICH occurs in about 10% to 30% of severe thrombocytopenia in FNAIT. Although death occurs in only 1% to 7% of neonates with ICH, the remaining ICH survivors often have severe neurological sequelae such as cerebral palsy, mental retardation, seizures and

cortical blindness. Around 25% to 50% of ICH cases related to FNAIT occur in-utero mainly between 30 weeks and 35 weeks of gestation (18). ICH due to FNAIT likely also to recur in subsequent pregnancies (29). For these reasons, FNAIT is considered to have high morbidity and mortality (21). Hence, suspecting FNAIT is important as this condition is potentially preventable (8, 21).

1.2.7 Severity of FNAIT

Severe FNAIT defined when the platelet count is less than 50×10^9/L. It could be difficult to predict severity of FNAIT. Factors leading to severe FNAIT had been subject of interest for many years. Ahlen et al had suggested that anti-HPA-1a is correlated with maternal ABO blood group genotype in which blood group O had protective effect against severe FNAIT as compared to blood group A (30). There were suggestions by few researchers that maternal serum of HPA antibody titre were associated with severity of FNAIT or degree of thrombocytopenia (25).

1.2.8 Management of FNAIT

FNAIT is assumed when there is thrombocytopenia with established mismatched between maternal and fetal antigen and detection of maternal antibody that reacts with the fetal platelet antigen. Test methods for detection of platelet antibodies are antigen capture assays such as monoclonal antibody-specific immobilization of platelet antigen (MAIPA) and modified antigen capture enzyme-linked immunosorbent assay (MACE) (27).

Recognition of FNAIT is crucial for the affected neonate as well as for the management of subsequent pregnancies. The risk of ICH would continue to persist if no treatment received in neonates with thrombocytopenia. The antenatal goal for pregnancy with past

history of FNAIT is to minimize the risk of severe fetal or neonatal haemorrhage especially intracranial haemorrhage. There are various antenatal treatment strategies adopted for woman with FNAIT (31). In general, invasive procedure such as serial fetal blood sampling and intrauterine platelet transfusion are less favoured. Silver et al reported that intravenous immunoglobulin (IVIG) had been used in fetus to raise the platelet count, however results were inconsistent (32). In recent years, IVIG is said to have successful outcome and had been practiced. Combination of corticosteroid and IVIG were also tried and were reserved for more severe cases and had proved to be highly effective in treating FNAIT fetuses as compared to non-treated siblings (18, 31).

1.3 Literature review

Platelets play important role in haemostasis, in which they adhere to damaged blood vessels, aggregate with each other and cross-links with fibrinogen to form a fibrin clot to prevent further blood loss. In the recent years, researchers have also recognised platelet roles in inflammation, innate and adaptive immunity, diseases and malignancies (2) .

Platelets have complex antigenic structures on its surface. There are numerous glycoproteins (GPs), human blood group antigens and human leukocyte antigen (HLAs) present on the platelet. Up to recent years, 33 human platelet alloantigens (HPAs) were identified on six GPs namely GPIIb, GPIIIa, GPIba, GPIbb, GPIa and CD109. Twenty HPAs are located on GPIIb/IIIa and the remaining 13 antigens are located on other GP complexes. Twelve antigens are classified in biallelic groups which are HPA-1, HPA-2, HPA-3, HPA-4, HPA-5 and HPA-15. A standardized system is used based on the order of discovery with ‘a’ designated for a higher frequency antigen and ‘b’ designated for

lower frequency antigen (2). Increasing data on the HPA allele frequency from various studies globally had clearly shown that HPA allele distributions vary widely across regions and ethnicity (8).

Other than HPAs, blood group antigens such as ABO(H), P and I antigens are expressed in low level on the platelets. Except for ABO, antibodies against P and I antigens are not associated with immune platelet disorders (2). Another important group of antigens expressed on platelet are the HLA class I antigens. HLA Class I A, B and to a lesser degree of antigens are all expressed on platelet membranes. Platelets serve as the main source of HLA class I present in blood. However, the amount of the molecules per platelet varies among individuals. (2).

In clinically stable patients, major bleeding is unusual unless the platelet count is less than $\leq 5x10^3/\mu L$. Bleeding may otherwise occur at higher levels when there is coexisting condition such as infection, disseminated intravascular coagulation and others (22).

Thrombocytopenia is a common haematological abnormality detected in about 25% cases of neonates admitted to neonatal intensive care unit (NICU) (17). Neonatal thrombocytopenia is defined when the platelet count is $<150 \times 10^9/L$ and severe neonatal thrombocytopenia is when platelet count is $<50 \times 10^9/L$ (18). About 1% - 5% of neonate are thrombocytopenic at birth with severe thrombocytopenia occurring in about 0.1%-0.5% of cases (18).

The most common cause of isolated severe neonatal thrombocytopenia is due to fetal/neonatal alloimmune thrombocytopenia (FNAIT) (21). FNAIT was initially reported in 1950's and it is also known with various abbreviations such as FMAIT (feto-

maternal alloimmune thrombocytopenia), NAITP (Neonatal alloimmune thrombocytopenia), NITP (neonatal immune thrombocytopenia) (18).

FNAIT is an immunological process which occurs when a mother produces an antibody-mediated response against a platelet-specific antigen that she herself lacks but present on the fetal platelets, that is paternally- inherited. Fetal platelet antigens are expressed around 16-18 weeks of gestation (33). The maternal immunoglobulin G (IgG) would bind to the fetal platelet acquired through transplacental transfer. The antibody-coated platelets are then removed in the fetal circulation by the reticuloendothelial system which would lead to fetal thrombocytopenia (25).

It is estimated that FNAIT occurs in 1 of 1000 to 2000 unselected pregnancies (12). The proportion of individuals having a particular antigen type varies according to the race (25). The commonest alloantigen is HPA-1a but other platelet-specific alloantigen may also be target (28). HPA-1a antibodies are the main cause of FNAIT among Western populations accounting up to 80% of the cases (8). Akin to red cell alloimmunization, homozygous individuals who are negative for a HPA antigen are likely to develop HPA alloantibodies when exposed to platelets expressing the absent antigen either through transfusion, transplantation or pregnancy (8).

FNAIT due to HPA-1a antibodies among East Asian are extremely rare and was due to low prevalence of HPA-1b homozygosity. The HPA-4b is more common in East Asian population and this antibody accounts for 72% of FNAIT in Japan. Other antibodies implicated in FNAIT among Asians are HPA-2b, HPA-5b and HPA-6b. Uniquely, HPA-21bw antibody is also found more among Asian accounting to about 1.1% in Japanese and Chinese as compared to <0.3% in Caucasian.

FNAIT may occur as early as second trimester and resolve spontaneously within 1 to 3 weeks after delivery (18). Neonates born with FNAIT could be asymptomatic, have purpura or hematoma or other bleeding manifestations. Intracranial haemorrhage which is the most feared presentation occurs in about 10-30% of severe thrombocytopenia in FNAIT (18). Around 25% to 50% of ICH cases related to FNAIT occur in-utero mainly in the the trimester(18).

FNAIT is suspected clinically after exclusion of nonimmune pathology leading to thrombocytopenia followed by laboratory testing to confirm the presence of maternal antibody that reacts with the fetal platelet antigen.

Traditionally, invasive method of fetal blood sampling and intrauterine platelet were done which carry substantial risk for fetal death. In late 1980's non-invasive method of treatment was adopted following successful treatment with intravenous immunoglobulin (IVIG). IVIG is expensive but safe and well tolerated treatment and had been used until now. Generally, thrombocytopenia resolves in 1 to 16 weeks after birth in neonates born with thrombocytopenia (25).

1.4 Research Justification

In a multiracial population like Malaysia, very limited information is available on the incidence of FNAIT. This study would also help to identify the associated factors which may be pertinent in predicting mothers and neonates at risk for FNAIT. Literature on incidence of FNAIT have been focusing on Caucasian population and hence may not be truly representing the actual proportion in our country. The results obtained from this

study may help to highlight the incidence and improve awareness of this condition in the population.

Association of type of platelet alloantibodies with maternal characteristics such as ethnicity, age, parity and blood group are not clear. This may be useful for clinician to predict the group at risk for FNAIT.

Similarly, association of neonatal characteristics with FNAIT is not studied in our population. If there are association found, it would prove to be beneficial for the treating physician to handle the case more delicately and help prevent life-long disability or death among affected neonates.

National Blood Centre as it is the only centre performing platelet immunology investigations. Therefore, investigation for all clinically suspected FNAIT cases would be sent here. MAIPA is the standard method of testing for platelet antibodies in our centre. As this is a specialized test and technically demanding, it requires experienced and skilled personnel to do the testing.

In the current study, we hypothesize that there is association between maternal and neonatal factors in the FNAIT. We hope that findings from our study would be beneficial in providing relevant information for clinicians in predicting and diagnosing FNAIT in our population.

1.5 Research questions

1. Is the incidence of FNAIT similar as reported in other parts of the world?

2. What are the platelet alloantibodies commonly identified in our population?
3. Are there association between maternal characteristics such as age, ethnicity, parity and blood group with FNAIT cases in our population?
4. Are there association between fetal/neonatal characteristics such as disease onset, presentation, platelet count and blood group with FNAIT in our population?

CHAPTER TWO

OBJECTIVE

2.1 General Objective

To study FNAIT and its associated factors in National Blood Centre

2.2 Specific Objectives:

i. To determine the incidence of FNAIT in National Blood Centre between 2011 and 2019

ii. To identify types of HPA alloantibodies in FNAIT

iii. To determine the association between maternal characteristics (age, ethnicity, parity and blood group) with FNAIT

iv. To determine the association between neonatal characteristics (disease onset, platelet count and blood group) with FNAIT.

2.3 Alternative hypothesis

i. There are significant associations between maternal characteristics such as age, ethnicity, parity, blood group and FNAIT.

ii. There are significant associations between neonatal characteristics such as disease onset, presentation, platelet count, blood group and FNAIT

2.4 Null hypotheses

H_{01}: There are no significant associations between maternal characteristics such as age, ethnicity, parity, blood group and FNAIT.

H_{02}: There are no significant associations between neonatal characteristics such as disease onset, presentation, platelet count, blood group and FNAIT.

CHAPTER THREE

METHODOLOGY

3.1 Study Background

Platelet antibody testing is done at the Platelet Serology Laboratory in National Blood Centre. The main function of National Blood Centre is for the provision of safe, secure and adequate blood supply. It also envisions to serve as premier reference centre for the field of transfusion medicine such as red cell antibody testing and crossmatching, platelet antibody testing and crossmatching, HLA typing and crossmatching as well as genotyping. This is the only centre in Malaysia performing platelet antibody testing for the diagnosis of FNAIT and platelet transfusion refractoriness. Therefore, all the samples sent for FNAIT testing could collected and analysed cumulatively. Presence of platelet antibody is confirmed by MAIPA method which had been adopted for more than 20 years here. The Platelet Serology Laboratory also participate in external quality assessment to achieve comparable results with accrediting bodies.

3.2 Study Design

To achieve objective 1 which is to determine the incidence rate and objective 2 to determine types of HPA antibodies for the duration of study period, repeated cross-sectional study design was undertaken. Matched, case control study of FNAIT and control through retrospective record review was performed to study the maternal and neonatal characteristics. Matching of cases were done based on maternal ethnicity. This involved assessment of 512 test requests for suspected FNAIT between 1st January 2011 and 31st December 2019 in National Blood Centre. Patients' demographic information, maternal

parity, neonatal onset, presentation and platelet count were acquired from the request forms written by the requesting clinicians. Results of investigation such as maternal and neonatal blood group and platelet alloantibody were obtained from the report which was attached with request form once the test was completed. Total number of requests performed for each month and year were obtained from Blood Bank Information System version 2.0 (BBIS v2.0).

3.3 Study area

The study was conducted in Platelet Serology Laboratory and archived records were traced from the Record Unit in National Blood Centre, Kuala Lumpur.

3.4 Study duration

The study was conducted from January 2019 until December 2020.

3.5 Study population

The reference population would be all suspected cases of FNAIT referred to National Blood Centre. Target population would be patients diagnosed with FNAIT between 2011 and 2019.

3.6 Subject criteria

3.6.1 Inclusion criteria

i) Neonates and infants with thrombocytopenia referred to National Blood Centre for confirmatory testing of clinically suspected FNAIT between 2011 and 2019.

ii) For cases, all confirmed FNAIT due to anti-HPA alloantibodies were included.

iii) Controls were those with no detectable platelet alloantibodies, which were randomly selected systematically using Microsoft Excel 2016.

3.6.2 Exclusion criteria

i) Cases with detectable platelet alloantibodies other than anti-HPA antibodies such as anti-HLA class I, non-specific alloantibodies such as anti-GPIIb/IIIa, -Ia/IIa, -Ib/IX, -CD 109.

ii) Controls with missing data

3.7 Sample size estimation

The sample size required for this study is taken based on largest sample calculation which is from specific objective number 3 and 4.

Objectives	Calculation
Objective 1 To determine the incidence of FNAIT in National Blood Centre between 2011 and 2019	Incidence of FNAIT was calculated based on live birth between 2011 and 2019 and represented as descriptive study. Therefore, no sample size calculation is required. Incidence calculated as: Total FNAIT cases Total live birth
Objective 2	

To identify types of HPA alloantibodies in FNAIT	The type and percentages of platelet alloantibodies was based on the detected cases in our population. No sample size calculation is required.
Objective 3 To determine the association between maternal characteristics (age, ethnicity, parity and blood group) with FNAIT Objective 4 To determine the association between neonatal characteristics (disease onset, platelet count and blood group) with FNAIT.	For Objective 3 and 4, sample size formula by (36) was used for this matched case-control study: $n = \frac{\left[(1/\sigma_\psi)Z_{\alpha/2} + Z_\beta\right]^2}{\delta^2}$ $\delta = \frac{\left[\sum_{k=1}^{m} \frac{kt_k\psi}{k\psi+m-k+1}\right] - 1}{\sigma(\psi)}$ $\sigma^2\left(\psi\right) = \sum_{k=1}^{m} \frac{kt_k\psi(m-k+1)}{(k\psi+m-k+1)^2}$ $t_k = p_1\binom{m}{k-1}p_{0+}^{k-1}q_{0+}^{m-k+1} + q_1\binom{m}{k}p_{0-}^{k}q_{0-}^{m-k} : k = 1,\ldots m$ $q_1 = 1 - p_1$ $q_0 = 1 = p_0$ $p_{11} = p_1p_0 + \phi\sqrt{p_1q_1p_0q_0}$ $p_{01} = q_1p_0 - \phi\sqrt{p_1q_1p_0q_0}$ $p_{0+} = \frac{p_{11}}{p_1}$ $p_{0-} = \frac{p_{01}}{q_1}$ $q_{0+} = 1 - p_{0+}$ $q_{0-} = 1 - p_{0-}$ where α = alpha, β = 1 - power, ψ = odds ratio, ϕ is the correlation coefficient for exposure between matched cases and controls, and Zp is the standard normal deviate for probability p. n is rounded up to the closest integer.

	In this study, alpha was set as 0.05, power as 80%, odds ratio as 8 (36), correlation coefficient as 0.2 (36) and prevalence of 5% (37). The sample size was calculated automatically. The final sample for each group was 39. Therefore, a total of 78 for cases and controls were recruited in this study.

3.8 Sampling method

Data were collected retrospectively from the year 2011 to 2019. All 39 FNAIT cases were included. For control, systematic random sampling of 39 cases were selected using Microsoft Excel 2016.

3.9 Research tool

Cases and controls test request forms were traced manually. Demographic and relevant maternal and neonatal information of cases and controls were recorded in the research proforma (Appendix A). Information on number of annual test request, blood group and type of platelet antibodies were traced manually from the request form and Blood Bank Information System version 2 (BBIS v2).

3.10 Statistical analysis

Descriptive statistics were estimated for cases and controls, separately. Differences between cases and controls were evaluated using chi-square test for categorical variables and t-test or Mann-Whitney test for normally and non-normally distributed variables, respectively. Statistical significance was defined as $p<0.05$. Crude odd ratios and 95%

confidence intervals were estimated from conditional logistic regression. Multivariable logistic regression was performed by including all the variables that showed a p-value of less than 0.25 in the abovementioned descriptive analysis. Variables with highest beta coefficient as well as being significant will be considered as the most influential variable in this study. Data were analysed using Statistical Package for Social Sciences (SPSS) version 26.0 for window software (SPSS, Chicago Illinois, USA).

3.11 Operational definition

i) Cases

FNAIT is diagnosed based on clinical and serologic findings. Request came from physician due to suspicion of FNAIT clinically. Laboratory diagnosis of FNAIT cases were made when there was presence of anti-HPA antibodies and incompatibility between maternal serum and paternal platelets (18) . MAIPA test was used to test all the cases and controls.

ii) Controls

Controls were cases with clinical suspicion of FNAIT but tested negative for platelet antibodies using same method of detection.

iii) MAIPA

Monoclonal antibody-specific immobilization of platelet antigen is considered as the gold standard in technique in the field of platelet

immunology (38). Platelet alloantibodies were detected using a panel of known HPAs typed platelets against paternal platelet.

iv) Parity

The condition of having given birth. The number of children borne by one woman

Primigravida - woman pregnant for the first time

Multigravida – a woman who has been pregnant one or more times previously

v) Onset

Early onset neonatal thrombocytopenia is prior to 72 hours and late onset neonatal thrombocytopenia is after 72 hours (17)

3.12 Ethical considerations

i. Subject vulnerability

This was a retrospective study thus the risk of vulnerability is low. The patients' identity was anonymised without disclosure of personal identifiable information to third-party organizations.

ii. Declaration of absence of conflict of interest

There was no conflict of interest in this study.

iii. Privacy and confidentiality

The data was recorded into data collection forms and into SPSS software. The patients involved cannot be identified directly or indirectly. Only research team members had access to the data.

iv. Community sensitivities and benefits

Not applicable.

v. Honorarium and incentives

Not applicable

vi. Ethical review board approval

Ethical board from Jawatankuasa Etika Penyelidikan Manusia (JEPeM) Universiti Sains Malaysia (Appendix B) was obtained and the study was also registered with the National Medical Research Centre (NMRR) (Appendix C and D).