FLOW CYTOMETRIC ASSESSMENT OF PLATELET MICROPARTICLES CD41 AND CD62P IN E/BETA-THALASSEMIA PATIENTS IN PUBLIC HOSPITALS IN SELANGOR, MALAYSIA

BAHAA HADI JABER ALMHANAWI

FPSK(m) 2016 52
FLOWCYTOMETRIC ASSESSMENT OF PLATELET MICROPARTICLES CD41 AND CD62P IN E/BETA-THALASSEMIA PATIENTS IN PUBLIC HOSPITALS IN SELANGOR, MALAYSIA

By

BAHAA HADI JABER ALMHANAWI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

November 2016
COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia.
DEDICATION

To my Family
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

FLOWCYTOMETRIC ASSESSMENT OF PLATELET MICROPARTICLES CD41 AND CD62P IN E/BETA-THALASSEMIA PATIENTS IN PUBLIC HOSPITALS IN SELANGOR, MALAYSIA

By

BAHAA HADI JABER ALMHANAWI

November 2016

Chairman : Bahariah Binti Khalid, PhD
Faculty : Medicine and Health Science

The hypercoagulability complications and an increase in thrombosis risk have been reported in B-thalassemia patients. Despite the fact that the life expectancy of B-thalassemia has been improved, thalassemic patients still suffer from many complications including thrombotic risk. High level of platelet microparticles (PMPs) in the circulation of B-thalassemia patients is believed to be responsible for the presence of hypercoagulability state in B-thalassemia patients. The main objective of this research was to assess the level of platelet microparticles in Hb E/B-thalassemia patients and normal individuals in the Malaysian population. The specific objectives were to determine the level of platelet microparticles CD41 and CD62P in Hb E/B-thalassemia and normal individuals, determine the Annexin-5 level in both, Hb E/B-thalassemia and normal individuals, and to correlate the levels of platelet microparticles with the blood parameters. A case-control study was carried out to assess the level of platelet microparticles in Hb E/beta-thalassemia patients (cases) and normal individuals (control) in the Malaysian population. A convenience sample of 37 patients with Hb E/beta-thalassemia (12 paediatrics and 25 adults) were investigated and compared with 28 normal individuals (3 paediatrics and 25 adults) who were studied in the same period. The samples were analyzed using immunophenotyping application in flow cytometer platform. PMPs were processed and analyzed directly after labeling by BD FACS-CantoII™ flow cytometer (Becton Dickinson, USA), using FlowJo software (version 10.1r1). Platelet microparticles defined as MPs that were smaller than 1.0 µm, had a positive staining for A-5, and exposed platelet-specific markers namely, CD41 and/or CD62P. However, in this research, the mean event of CD62P Vs. A-5 were significantly higher in Hb E/B-thalassemia compared to the normal individuals in the adults group (p= 0.006) respectively. There was a strong association between the phospholipid (PS) and platelet activation markers CD41 and CD62P on the activated platelet cells. In conclusion, platelet microparticles are significantly increased in Hb E/B-thalassemia patients compared to the normal individuals, and there is a strong association between platelet microparticle and some blood parameters.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENILAIAN ALIRAN SITOMETRIK MIKROPARTIKAL PLATLET CD41 DAN CD62P DALAM KALANGAN PESAKIT HB E/B-THALASSEMA DI HOSPITAL AWAM SELANGOR, MALAYSIA

Oleh

BAHAA HADI JABER ALMHANAWI

Pengerusi : Bahariah Binti Khalid, PhD
Fakulti : Perubatan dan Sains Kesihatan

November 2016

Komplikasi hypergumpalan dan peningkatan risiko thrombosis telah dilaporkan berlaku kepada pesakit thalasemia. Meskipun terbukti bahawa jangka hayat pesakit B-thalassemia telah bertambah baik, namun pesakit thalassemia masih menhadapi banyak komplikasi lain seperti risiko thrombotic. Peningkatan jumlah mikropartikal platlet (PMPs) yang tinggi dikalangan pesakit B-thalassemia adalah dipercayai berpunca daripada kewujudan hypergumpalan pada pesakit B-thalassemia. Objektif utama kajian ini adalah untuk mengetahui tahap mikroplatlet pesakit Hb E/B-thalassemia dan individu normal dalam populasi di Malaysia. Kajian ini lebih mengfokuskan bagi mengenalpasti tahap mikropartikal platlet mikropartikal CD41 dan CD62P dalam Hb E/B-thalassemia dan individu normal, mengenalpasti tahap Annexin-5 pada Hb E/B-thalassemia dan individu normal, dan untuk mengetahui hubungan tahap mikropartikal dengan parameter klinikal dan faktor lain yang berkait.

Satu kajian kes-kontrol telah dijalankan untuk mengukur tahap mikropartikal platlet pada pesakit Hb E/beta-thalassemia (kes) dan individu normal(kontrol) dalam kalangan populasi masyarakat Malaysia. Sampel yang mudah didapati diambil daripada 37 orang pesakit yang menghidap Hb E/beta-thalassemia (12 pediatrik dan 25 dewasa) dan telah dijalankan ujikaji keatas sampel tersebut, dengan membandingkannya dengan 28 individu sihat (3 pediatrik dan 25 orang dewasa) yang dikaji serentak. Analisis sampel dilakukan dengan menggunakan aplikasi immunophenotyping dalam dataran aliran sitometer. PMPs terus dianalisis selepas di tanda oleh BD FACS-CantoII™ aliran sitometer (Becton Dickinson, Amerika syarikat), menggunakan perisisan FlowJo (versi 10.1r1). Mikropartikal platlet didefinisikan sebagai MP adalah lebih kecil daripada 1.0 µm, mempunyai lekatan tanda positif untuk A-5, dan menanda platlet-spesifik terdedah dinamakan CD41 dan/atau CD62P. Meskipun begitu, min acara bagi kedua-dua CD62P dan A-5 adalah ketara lebih tinggi pada Hb E/B-thalassemia berbanding individu normal dalam kalangan golongan orang dewasa ($p= 0.006$) secara respektif. Terdapat jalinan yang kuat antara phospholipid (PS) dan mengaktifan petanda platlet CD41 dan CD62P pada sel platlet yang telah diaktifkan. Sebagai konklusi, mikropartikal platlet
meningkat secara ketara pada pesakit Hb E/B-thalasemia berbanding individu normal, dan terdapat jalinan yang kuat antara mikropartikal platlet and beberapa parameter darah.
AKNOWLEDGEMENTS

I would like to thank my family and my supervisory committee for their tremendous support.
I certify that a Thesis Examination Committee has met on 25 November 2016 to conduct the final examination of Bahaa Hadi Jaber on his thesis entitled "Flowcytometric Assessment of Platelet Microparticles CD41 and CD62P in E/Beta-Thalassemia Patients in Public Hospitals in Selangor, Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Norhafizah binti Mohtarrudin, PhD
Associate Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Chairman)

Maha binti Abdullah, PhD
Associate Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Internal Examiner)

Leong Chooi Fun, PhD
Associate Professor
Universiti Kebangsaan Malaysia
Malaysia
(External Examiner)

[Signature]

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 February 2017
This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Bahariah Binti Khalid, PhD**  
Medical Lecturer  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Chairman)

**Elizabeth George, PhD**  
Professor (Medicine)  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Member)

**Eusni Rahayu Mohd Tohit, PhD**  
Medical Lecturer  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Member)

**Zainina Seman, PhD**  
Medical Lecturer  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Member)

**Salmiah Md. Said, PhD**  
Medical Lecturer  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Member)

---

**ROBIAH BINTI YUNUS, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software

Signature: _______________________ Date: ______________________

Name and Matric No.: Bahaa Hadi Jaber Almhanawi / GS383798
Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: ____________________________
Name of Chairman of Supervisory Committee: Dr. Bahariah Binti Khalid

Signature: ____________________________
Name of Member of Supervisory Committee: Professor Dr. Elizabeth George

Signature: ____________________________
Name of Member of Supervisory Committee: Dr. Eusni Rahayu Mohd Tohit

Signature: ____________________________
Name of Member of Supervisory Committee: Dr. Zainina Seman

Signature: ____________________________
Name of Member of Supervisory Committee: Dr. Salmiah Md. Said
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>ii</td>
</tr>
<tr>
<td>AKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>v</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction to the Chapter</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Thalassemias</td>
<td>1</td>
</tr>
<tr>
<td>1.3 β-thalassaemia</td>
<td>1</td>
</tr>
<tr>
<td>1.4 Hb E/β-thalassemia</td>
<td>1</td>
</tr>
<tr>
<td>1.5 Hb E/β-thalassemia in Malaysian Population</td>
<td>2</td>
</tr>
<tr>
<td>1.6 Hypercoagulable State in Thalassemia Patients</td>
<td>2</td>
</tr>
<tr>
<td>1.7 Platelet Microparticles and Hypercoagulability</td>
<td>2</td>
</tr>
<tr>
<td>1.8 Problem Statement</td>
<td>2</td>
</tr>
<tr>
<td>1.9 Research Significance</td>
<td>3</td>
</tr>
<tr>
<td>1.10 Research Hypothesis</td>
<td>3</td>
</tr>
<tr>
<td>1.10.1 Alternative Hypothesis</td>
<td>3</td>
</tr>
<tr>
<td>1.10.2 Null Hypothesis</td>
<td>3</td>
</tr>
<tr>
<td>1.11 Research Objectives</td>
<td>3</td>
</tr>
<tr>
<td>1.11.1 General Objective</td>
<td>3</td>
</tr>
<tr>
<td>1.11.2 Specific Objectives</td>
<td>3</td>
</tr>
<tr>
<td>1.12 Research Question</td>
<td>4</td>
</tr>
<tr>
<td>1.13 Conceptual Framework</td>
<td>4</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Thalassemia as Prothrombotic State</td>
<td>6</td>
</tr>
<tr>
<td>2.3 Hypercoagulable State in Thalassemia Patients</td>
<td>6</td>
</tr>
<tr>
<td>2.4 Hypercoagulable State in Non-Transfusion-Dependent Thalassemia</td>
<td>8</td>
</tr>
<tr>
<td>2.5 Platelet-Derived Microparticles</td>
<td>10</td>
</tr>
<tr>
<td>2.5.1 Platelet Microparticle Structure</td>
<td>11</td>
</tr>
<tr>
<td>2.5.1.1 Size</td>
<td>11</td>
</tr>
<tr>
<td>2.5.1.2 Phospholipids</td>
<td>11</td>
</tr>
<tr>
<td>2.5.1.3 Glycoproteins</td>
<td>11</td>
</tr>
<tr>
<td>2.5.2 Formation of Platelet Microparticles</td>
<td>14</td>
</tr>
<tr>
<td>2.5.2.1 Platelet Activation</td>
<td>14</td>
</tr>
<tr>
<td>2.5.2.2 Platelet activation markers</td>
<td>14</td>
</tr>
<tr>
<td>2.5.2.3 Platelet Destruction</td>
<td>17</td>
</tr>
<tr>
<td>2.6 Model of PMPs Formation</td>
<td>17</td>
</tr>
</tbody>
</table>
2.7 Clearance of PMPs from Circulation 20
2.8 Microparticle Function 20
  2.8.1 Coagulation 20
  2.8.2 Inhibition of Coagulation 20
  2.8.3 Adhesion 21
  2.8.4 Carrier Function and Cell Activation 21
  2.8.5 Clinical Disorders Associated with Microparticles 22
    2.8.5.1 Inherited Disorders 22
      2.8.5.1.1 Scott Syndrome and Castaman Syndrome 22
      2.8.5.1.2 Wiskott–Aldrich Syndrome (WAS) 22
    2.8.5.2 Acquired Disorders 22
      2.8.5.2.1 Immune Mediated 22
        2.8.5.2.1.1 Primary Immune Thrombocytopenia (ITP) 22
        2.8.5.2.1.2 Heparin-Induced Thrombocytopenia (HIT) 23
        2.8.5.2.1.3 GPIIb-IIIa Antagonist-Induced Thrombocytopenia. 23
      2.8.5.2.2 Non-Immune Mediated 23
        2.8.5.2.2.1 Paroxysmal Nocturnal Hemoglobinuria (PNH) 23
2.9 Detection of Microparticles 24
2.10 Conventional Detection Technique (Flow Cytometry) 24
2.11 Summary 25

3 MATERIALS AND METHODS 28
  3.1 Introduction to the Chapter 28
  3.2 Research Design 28
  3.3 Research Location 28
  3.4 Sampling 28
    3.4.1 Research Population 28
    3.4.2 Health Assessment of Normal Respondents 28
    3.4.3 Inclusion Criteria 29
    3.4.4 Exclusion Criteria 29
    3.4.5 Sampling Frame 29
    3.4.6 Sampling Unit 30
    3.4.7 Sample Size 30
    3.4.8 Sampling Method 33
  3.5 The Variables of the Research 34
    3.5.1 Dependent Variables 34
    3.5.2 Independent Variables 34
  3.6 Research Ethical Issues 34
3.6.1 Informed Consent
3.6.2 Process of Informed Consent
3.6.3 Respondents Confidentiality
3.6.4 Respondents Privacy
3.6.5 Respect and Responsibility

3.7 Data and Sample Collection
3.7.1 Collection of Respondents Information
3.7.2 Blood Sample Collection Technique
3.7.2.1 Blood Sample Collection from Infants and Children
3.7.2.2 Blood Sample Collection from Teens and Adults
3.7.3 Blood Sample Transportation
3.7.4 Sample Processing and PMPs Preparation
3.7.4.1 Centrifugation
3.7.4.2 Storage and Thawing Conditions

3.8 Staining and Labeling of PMPs

3.9 Instrument of the Research
3.9.1 Flow Cytometer Standardization Tools in PMPs Analysis
3.9.1.1 Size Calibration Beads
3.9.2 Antibodies Titration
3.9.3 CountBright™ Absolute Counting Beads
3.9.4 Negative Controls
3.9.4.1 Isotype controls
3.9.4.2 Fluorescent Minus One (FMO) Controls
3.9.5 Flow Cytometry Analysis

4 RESULTS AND DISCUSSION
4.1 Introduction to the Chapter
4.2 Response Rate in Hb E/B-thalassemia and Normal individuals
4.3 General Socio-demographics of Respondents
4.3.1 Age
4.3.2 Gender
4.3.3 Ethnicity
4.4 Demographic factors of Hb E/B-thalassemia patients
4.5 Complete Blood Count Parameters in Hb E/B-thalassemia and Normal individuals
4.6 Flow Cytometry Data (Immunophenotyping)
4.7 Expression of Studied markers (CD41, CD62P, and A-5)
4.7.1 Adults Group
4.7.2 Hb E/B-thalassemia (Paediatrics group)
4.8 Subpopulations of Platelet Microparticles
4.9 Factors Associated with PMPs
4.10 Post-hoc power test
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Surface Antigens Markers (CDs) of platelet and other circulating cells shows the common and unique surface antigens</td>
<td>15</td>
</tr>
<tr>
<td>2.2</td>
<td>Surface Antigens Markers (CDs) of platelet and other circulating cells shows the common and unique surface antigens</td>
<td>16</td>
</tr>
<tr>
<td>4.1</td>
<td>Distribution of subject’s demographics in Hb E/β-thalassemia</td>
<td>56</td>
</tr>
<tr>
<td>4.2</td>
<td>Distribution of subject’s demographics in Normal individuals</td>
<td>57</td>
</tr>
<tr>
<td>4.3</td>
<td>Mean differences of Complete blood count (CBC) parameters between Thalassemic patients and normal individuals (Adults group).</td>
<td>58</td>
</tr>
<tr>
<td>4.4</td>
<td>Mean differences in Complete blood count (CBC) parameters between Thalassemic patients and normal individuals (Paediatrics group)</td>
<td>59</td>
</tr>
<tr>
<td>4.5</td>
<td>Comparison of Platelet Activation Markers in the adults group among Hb E/B-thalassemia and normal individuals</td>
<td>63</td>
</tr>
<tr>
<td>4.6</td>
<td>Description of CD41, CD62P, and A-5 expression level in the Hb E/B-thalassemia paediatrics group</td>
<td>63</td>
</tr>
<tr>
<td>4.7</td>
<td>Comparison of Platelet Activation Markers in Hb E/B-thalassemia among paediatrics and adults groups</td>
<td>65</td>
</tr>
<tr>
<td>4.8</td>
<td>Comparison of PMPs Subpopulations in the adults group among Hb E/B-thalassemia patients and normal individuals</td>
<td>66</td>
</tr>
<tr>
<td>4.9</td>
<td>Description of Paediatrics</td>
<td>67</td>
</tr>
<tr>
<td>4.10</td>
<td>Correlation of platelet activation markers with the blood parameters in adults Hb E/B-thalassemia(N=25)</td>
<td>68</td>
</tr>
<tr>
<td>4.11</td>
<td>Correlation of platelet activation markers with the blood parameters in Paediatrics Hb E/B-thalassemia (N=12)</td>
<td>69</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Flow chart of Conceptual Framework.</td>
<td>5</td>
</tr>
<tr>
<td>2.1</td>
<td>Hypercoagulability Contributing Factors in Thalassemia. (Ali T. Taher, 2008.)</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Cells, MPs, and Exosomes. (Julie C. Williams, 2011)</td>
<td>10</td>
</tr>
<tr>
<td>2.3</td>
<td>Vesication of MPs from the Cell Membrane, transfer to recipient cell, and MPs composition (Yáñez-Mó et al., 2015)</td>
<td>13</td>
</tr>
<tr>
<td>2.4</td>
<td>Schematic Representation of the Resting Cytoskeleton. Ca++ is stored in the endoplasmic reticulum (ER). Scramblase is inactive while Translocase is active. (Piccin et al., 2007.)</td>
<td>18</td>
</tr>
<tr>
<td>2.5</td>
<td>Cellular activation. Calcium is released by ER leading to activation of calpain and gelsolin. Calpain cleaves long actin filaments. Gelsolin cleaves the actin capping proteins. The raised cytoplasmic Ca2+ also activates scramblase and inactivates translocase. Phospholipid asymmetry begins to be compromised. (Piccin et al., 2007.)</td>
<td>18</td>
</tr>
<tr>
<td>2.6</td>
<td>Cytoskeletal disruption following cellular activation. Spectrin and actin are cleaved. At this stage protein, anchorage to the cytoskeleton is disrupted allowing membrane budding. (Piccin et al., 2007.)</td>
<td>19</td>
</tr>
<tr>
<td>2.7</td>
<td>Generated microparticle is exposing increased phosphatidylserine on the external surface. (Piccin et al., 2007.)</td>
<td>19</td>
</tr>
<tr>
<td>2.8</td>
<td>Technical Approaches for microparticle detection. The combination of these methods is most frequently used for vesicle characterization. (Jean-Daniel Tissot, 2013.)</td>
<td>25</td>
</tr>
<tr>
<td>2.9</td>
<td>Thin section of normal platelet reveals intact cell membrane and some cell organelles. Magnification is 30 000x. The black arrow in the down right corner represents 0.5 μm. The platelet structure indicated by black arrows (1) alpha granules, (2) dense bodies, (3) filaments extended dense bodies. (B.H. Almhanawi et al., 2016.)</td>
<td>26</td>
</tr>
</tbody>
</table>
2.10 Thin section of platelet illustrates an advance activation stage (normal platelet-induced with EDTA) in which the cell membrane is damaged and release of PMPs and other cell organelles. Magnification is 30,000x. The black arrow in the down right corner represents 1 μm. The platelet structure indicated by black arrows (1) released platelet microparticles, (2) dense bodies and (3) alpha granules. (B.H. Almhanawi et al., 2016.)

3.1 Altman nomogram, Published by Oxford University Press on behalf of the British Journal of Anaesthesia

3.2 Work Flow of Sample collection and PMPs preparation

3.3 Workflow of antibody labeling

3.4 Flowcytometry machine (BD FACS Canto II).

3.5 Histogram plot of size calibration beads in FSC and SSC (a and b), Dot-plot of filtered (c) and unfiltered sheath fluid in (d)

3.6 An example of serial dilution of CD62P stock solution

3.7 Representative line graph shows the titration curves of selected antibodies (CD41 and CD62P) and A-5

3.8 (A) Representative flow cytometry dot plot of absolute count beads FSC vs SSC (A) linear scale, (B) log scale

3.9 Shows CD41-FITC FMO Controls.

3.10 Shows A-5-PE FMO Controls.

3.11 Shows CD62P-APC FMO Controls.

3.12 Shows gating strategy of platelet microparticles.

4.1 Flow Chart of Respondent Response Rate

4.2 Flow cytometry of platelet derived microparticles. A: PMPs gate set up using size calibration beads according to the manufacturer instructions. B: Isotype control as a negative control using FITC Vs. APC. C: double fluorescent dot plot shows PMP-CD41 population. D: double fluorescent dot plot shows PMPs-CD62P population. E: double fluorescent dot plot shows PMP-CD41-CD62P population
4.3 Comparison of CD41, CD62P, and A-5 levels in the adults group between normal individuals (n=25), and Hb E/B-thalassemia (n=25). The bars represent the mean, and the small black bars represent the error bars (95% CI). The mean of CD41, CD62P, and A-5 in normal individuals were (1671, 1439, and 1609 events/µl) respectively, while in Hb E/B-thalassemia were (1940, 1802, and 1949 events/µl), respectively. Only CD62P and A-5 were significantly higher in Hb E/B-thalassemia when it compared to normal individuals. Statistics performed with independent sample Student t-test, p<0.05

4.4 Comparison of PMPs subpopulations in the adults group between normal individuals (N=25), and Hb E/B-thalassemia (N=25). The bars represent the mean, and the small black bars represent the error bars (95% CI). Statistics performed with independent sample Student t-test, p<0.05.

4.5 Altman nomogram, Shows the post hoc test for the desired power of the study. Published by Oxford University Press on behalf of the British Journal of Anaesthesia.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>A-5</td>
<td>Anxnin-5</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>APC*</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete Blood Count</td>
</tr>
<tr>
<td>Ca⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters Of Differentiation</td>
</tr>
<tr>
<td>CRF</td>
<td>Clinical Report Form</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep Venous Thrombosis</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scatter</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward Scatter</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescent Minus One</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HIT</td>
<td>Heparin-Induced Thrombocytopenia</td>
</tr>
<tr>
<td>HCT</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>ITP</td>
<td>Primary Immune Thrombocytopenia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>MPs</td>
<td>Microparticles</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal Antibodies</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean Cell Volume</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean Cell Hemoglobin</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean Cell Hemoglobin Concentration</td>
</tr>
<tr>
<td>PMPs</td>
<td>Platelets Microparticles</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary Embolism</td>
</tr>
<tr>
<td>PVT</td>
<td>Portal Vein Thrombosis</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet Rich Plasma</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet Poor Plasma</td>
</tr>
<tr>
<td>PFP</td>
<td>Platelet-Free Plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PNH</td>
<td>Paroxysmal Nocturnal Hemoglobinuria</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrins</td>
</tr>
<tr>
<td>Plt</td>
<td>Platelets</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RDW</td>
<td>Red Cell Distribution Width</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package For Social Sciences</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>TM</td>
<td>Thalassemia Major</td>
</tr>
<tr>
<td>TI</td>
<td>Thalassemia Intermedia</td>
</tr>
<tr>
<td>TEE</td>
<td>Thromboembolic Events</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>TBV</td>
<td>Total Blood Volume</td>
</tr>
<tr>
<td>TMPs</td>
<td>Total Microparticles</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous Thromboembolism</td>
</tr>
<tr>
<td>WAS</td>
<td>Wiskott-Aldrich Syndrome</td>
</tr>
<tr>
<td>WBCs</td>
<td>White Blood Cells</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Introduction to the Chapter

This research is about the platelets microparticles (PMPs) and their role in hypercoagulability status in Hb E/β-thalassemia in comparison with normal individuals. The current chapter presents the general study background, describes problem statement, research significance, hypothesis, objectives, and research questions. Moreover, the chapter summary represented in the conceptual framework of the study which is provided at the end of this chapter.

1.2 Thalassemias

Thalassemias are groups of heterogenic inherited disorders which occur as a result of, reduced or absence of globin chain synthesis, which is a protein molecule that is responsible for carrying the oxygen in the red blood cells (RBCs). There are two types of thalassemia disorder around the world: alpha (α) thalassemia and beta (β) thalassemia. Epidemiological studies show that α-thalassemia is more dominant in the Far East region while β-thalassaemia is more common in the Mediterranean region (Hoffbrand, V., & Moss, P. A. 2011).

1.3 β-thalassaemia

β-thalassemia has two main classes: β+ thalassemia, in which there is a variable reduction in the synthesis of β globin chain and β0 thalassemia, in which there is an absence of β globin chain production. β-thalassemia causes a variable anemia range from minor symptoms to life-threatening anemia. It can be classified based on the clinical symptoms into two phenotypes: thalassemia major (TM) or Cooley’s anemia in which the patient suffer from severe and life-threatening anemia that occurs within months after birth, and thalassemia intermedia (TI) which manifested less clinical severity than TM (Provan, D., & Gribben, J. Eds. 2010).

1.4 Hb E/β-thalassemia

The homozygote of Hb E/β-thalassemia has the phenotype of β thalassemia trait due to abnormal hemoglobin production, and thalassemia, because of the generation of the alternative splicing site by the mutation. The populations in the joint borders of Thailand, Cambodia, and Laos have the highest incidence of Hb E/β-thalassemia. Patients who inherit Hb E and β-thalassemia trait manifest TM or TI (Provan, D., & Gribben, J. Eds. 2010).
1.5 Hb E/β-thalassemia in Malaysian Population

Hb E represents the most common Hb variant in the Southeast Asia with the frequency of 50% in many different areas (Fucharoen, S., & Winichagoon, P. 1997). In Malaysia, the Hb E is quite common in Malays with 5% carrier rate and the Orang Asli of Peninsular Malaysia manifest higher rate of Hb E disorder (Traeger, J., Wood, W. G., J. B., D. J., & Wasi, P. 1980). Hb E/β-thalassemia is an extreme clinical condition that results from the interaction of Hb E with β-thalassemia. It is considered a public health problem in the Malaysian population and the most frequent type of thalassemia in Malays (George, E. 2013).

1.6 Hypercoagulable State in Thalassemia Patients

The chronic hypercoagulability state in Hb E/ Beta-thalassemia has been observed in these patients. The disturbance in the circulatory of the thalassemia patients can be manifested by peripheral arterial and venous thrombosis, transient ischemic attacks, and microcirculatory obstruction (Grisaru, D., & Rachmilewitz, E. A. 1992). The presence of PMPs in the circulation has been shown to support the procoagulant activity (Mallat, Z., Benamer, H., Hugel, B., Benessiano, J., Steg, P. G., Freyssinet, J. M., & Tedgui, A. 2000). Importantly, the procoagulant activity was corroborated by clinical research manifesting increased level of MPs in patients with risk of thromboembolic events (TEE) (VanWijk, M. J., VanBavel, E., Sturk, A., & Nieuwland, R. 2003).

1.7 Platelet Microparticles and Hypercoagulability

The exposure of PS on the PMPs surface led to binding of the coagulation factors via Ca^{2+} ions; that enable the formation of prothrombinase and tenase complex. PMPs are enriched in binding sites for activated factor Va, factor VIIIa, and factor IXa and provide the surface for thrombin formation (Sims, P. J., Faioni, E. M., Wiedmer, T., & Shattil, S. J. 1988).

1.8 Problem Statement

Increased in the level of CD41 and CD62P in thalassemic patients has been proven by recent studies in regard to thrombus formation. Studies have shown that CD41 and CD62P are increased in thrombotic patients at a significant rate. CD62P is exclusively expressed by platelet in contrast to other microparticles. CD41 has been studied on its clinical relevance in certain thrombogenic conditions. However, much mysterious about the role of these microparticles still unknown about clot formation. To our best knowledge, CD62P, and CD41 which is derived from platelet have not been studied in E/beta-thalassemic patients in Malaysia. To date, there have been efforts of risk stratification of hypercoagulable state in cancer by using CD62P that is a cell adhesion molecule found in platelet and appears to be playing a key role in response to tissue injury and inflammation and subsequently thrombus formation. However, the platelet microparticles formation will be affected by two main factors, namely serum calcium as the enzymes that responsible for the release of platelet microparticles are calcium-
dependent enzymes and the platelet number that is essential for platelet microparticles formation.

1.9 Research Significance

There were hardly any studies conducted to assess the platelet microparticles CD41 and CD62P in Hb E/β thalassemic patients and normal individuals in Southeast Asia countries, particularly in Malaysia. Very few studies have been conducted in the Middle East countries on thalassemic patients that have shown an increase in their level of platelet microparticles. It is known that thalassemia has a high prevalence in Malaysian population namely E/beta thalassemia 4.5% (George, 2001) and they are at risk to develop hypercoagulability state because they manifest a high incidence of blood clotting. Thus, this research is necessary to prove whether the platelet microparticles CD41 and CD62P are significantly increased in selected groups (cases and controls) of Malaysian patients. Moreover, to establish a new data for platelet microparticles level in thalassemic patients in Malaysia population.

1.10 Research Hypothesis

1.10.1 Alternative Hypothesis

The level of platelet microparticles, namely CD41 and CD62P are significantly increased in Hb E/β-thalassemia patients.

1.10.2 Null Hypothesis

The level of platelet microparticles, namely CD41 and CD62P are not significantly increased in patients with Hb E/β-thalassemia.

1.11 Research Objectives

1.11.1 General Objective

To evaluate the level of platelet microparticles (CD41 and CD62P) in Hb E/β-thalassemia patients and normal individuals.

1.11.2 Specific Objectives

i. To determine the level of platelet microparticles CD41 and CD62P in Hb E/β-thalassemia and normal individuals in the Malaysian population.

ii. To determine the phospholipid level in both, normal and Hb E/β-thalassemic subjects.

iii. To correlate the levels of platelet microparticles CD41 and CD62P, and Annexin-5 with complete blood count parameters.
1.12 Research Question

Is there any association between CD41, CD62P, and A-5 with Hb E/β-thalassemia?

1.13 Conceptual Framework

Figure 1:1. Provides a detailed description of the conceptual framework of the research. Two groups were chosen to be investigated regarding platelet microparticles (CD41 and CD62P) level in this research. The first group is Hb E/β thalassemia patients and the second is normal individuals. Platelet-derived microparticles (CD41 and CD62P) were set as dependent variables. And the other factors that may affect the level of platelet-derived microparticle in these two groups which are demographic factors (age, gender and ethnicity), patients characteristics (splenectomy status, and Iron chelation status), clinical severity (Blood transfusion status) and other factors (Complete blood count (CBC)) were set as independent variables. Importantly, CD41 and CD62P are platelet-derived microparticles that are blebing from activated platelets. Sources provide that patients with thalassemia and thrombosis have thrombotic complication combined with a notable augmentation in the level of platelet microparticles.
Figure 1.1: Flow chart of Conceptual Framework.
REFERENCES


Traeger, J., Wood, W., Clegg, J., Weatherall, D., & Wasi, P. (1980). Defective synthesis of HbE is due to reduced levels of βE mRNA.


86
BIODATA OF STUDENT

The student was born on 21st March 1990, in Aldiwaniya, Iraq. He passed the Elementary, Secondary and high school education in 2007 in Aldiwaniya. In 2008, he pursued 4 (four) years Bachelor of laboratory investigation in faculty of science at Kufa university. In 2014, he has enrolled as a master student in the field of Haematology at pathology department, faculty of medicine and health sciences, University Putra Malaysia under the supervision of Dr. Bahariah Khalid.
UNIVERSITI PUTRA MALAYSIA

STATUS CONFIRMATION FOR THESIS / PROJECT REPORT AND COPYRIGHT

ACADEMIC SESSION: ________________________

TITLE OF THESIS / PROJECT REPORT:

FLOWCYTOMETRIC ASSESSMENT OF PLATELET MICROPARTICLES CD41 AND CD62P IN EBETA-THALASSEmia PATIENTS IN PUBLIC HOSPITALS IN SELANGOR, MALAYSIA

NAME OF STUDENT: BAHAA HADI JABER ALMHANAWI

I acknowledge that the copyright and other intellectual property in the thesis/project report belonged to Universiti Putra Malaysia and I agree to allow this thesis/project report to be placed at the library under the following terms:

1. This thesis/project report is the property of Universiti Putra Malaysia.

2. The library of Universiti Putra Malaysia has the right to make copies for educational purposes only.

3. The library of Universiti Putra Malaysia is allowed to make copies of this thesis for academic exchange.

I declare that this thesis is classified as:

*Please tick (V)

☐ CONFIDENTIAL (Contain confidential information under Official Secret Act 1972).

☐ RESTRICTED (Contains restricted information as specified by the organization/institution where research was done).

☐ OPEN ACCESS I agree that my thesis/project report to be published as hard copy or online open access.

This thesis is submitted for:

☐ PATENT Embargo from __________ until __________

(date) (date)

Approved by:

(Signature of Student) (Signature of Chairman of Supervisory Committee)
New IC No/ Passport No: Name:

Date: Date:

[Note: If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization/institution with period and reasons for confidentially or restricted.]