SELECTED VARIATIONS OF THE ORGANIC ANION TRANSPORTING POLYPEPTIDE 2 (*OATP2*) GENE IN MALAY NEONATES WITH AND WITHOUT HYPERBILIRUBINAEMIA

by

NOOR NAMIRAH BT NAWAWI

Thesis submitted in fulfilment of the requirements

for the Degree of

Master of Science

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TABLE OF CONTENTS

	Page
Acknowledgement	ii
Table of contents	iii
List of tables	vi
List of figures	viii
List of plates	x
List of symbols and abbreviations	xi
List of appendices	xiv
Abstrak	xv
Abstract	xvii
CHAPTER 1 – INTRODUCTION	
1.1 Research background	1
1.2 Rationale of the study	5
1.3 Objectives	
1.3.1 General objective	7
1.3.2 Specific objectives	7
1.4 Characteristics of neonatal hyperbilirubinaemia	8
1.5 Classification of neonatal hyperbilirubinaemia	
1.5.1 Unconjugated hyperbilirubinaemia versus conjugated hyperbilirubinaemia	10
1.5.2 Physiological hyperbilirubinaemia versus pathological hyperbilirubinaemia	11
1.6 Bilirubin production in neonates	15
1.7 Effects of neonatal hyperbilirubinaemia	20
1.8 Management and treatment for neonatal hyperbilirubinaemia	
1.8.1 Phototherapy	23
1.8.2 Exchange transfusion	23
1.8.3 Pharmacological therapies	24
1.9 Membrane transporters	
1.9.1 General introduction	25
1.9.2 Organic anion transporting 2 (*OATP2*) gene	28
1.9.2.1 Common genetic variations of the *OATP2* gene	31
1.10 High resolution melting (HRM) analysis	
1.10.1 Introduction of HRM analysis	34
1.10.2 Principle of HRM analysis	36
CHAPTER 2 – MATERIALS AND METHODS	
2.1 Subjects and study design	37
2.2 Sample size calculation	40
2.3 Materials and methods	
2.3.1 DNA extraction from buccal cell samples	41
2.3.2 DNA extraction from blood samples	44
2.3.3 DNA quantification and qualification	45
2.3.4 Screening for genetic variations of the *OATP2* gene using HRM analysis	

2.3.4.1 Master mix for HRM analysis 46
2.3.4.2 Optimization of HRM analysis protocol 52
2.3.4.3 HRM analysis protocol 54
2.3.4.4 Validation of HRM result using DNA extracted from blood 59
2.3.4.5 Melting curve analysis 59
2.3.5 PCR amplification prior to DNA sequencing analysis
2.3.5.1 Master mix for PCR amplification 60
2.3.5.2 Optimization of PCR amplification 60
2.3.5.3 PCR amplification protocol 64
2.3.6 Preparation of agarose gel electrophoresis
2.3.6.1 Gel components 67
2.3.6.2 Preparation of 1X TBE buffer 67
2.3.6.3 Dilution of SYBR Green I 67
2.3.6.4 Preparation of 2% agarose gel 69
2.3.6.5 Protocol for agarose gel electrophoresis for PCR products 69
2.3.7 DNA sequencing analysis for confirmation of HRM results
2.3.7.1 Purification of PCR products prior to DNA sequencing analysis 70
2.4 Statistical analysis 71
2.5 Hardy-Weinberg equilibrium (HWE), pairwise linkage disequilibrium (LD) and haplotype analysis using Haploview program 72

CHAPTER 3 – RESULTS
3.1 Demographic data of the neonates enrolled in the study
3.1.1 Recruitment of subjects 73
3.1.2 Incidence of neonatal hyperbilirubinaemia development 75
3.2 DNA quantification and qualification 77
3.3 Agarose gel electrophoresis to confirm the presence of unspecific products during optimization of HRM analysis 79
3.4 Screening for genetic variations of the *OATP2* gene by HRM analysis
3.4.1 Amplification graph 81
3.4.2 HRM analysis results for genetic variations of the *OATP2* gene 83
3.4.3 Validation of HRM result using DNA extracted from blood 94
3.5 PCR amplification for promoter, exons 4 and 5 of the *OATP2* gene 97
3.6 DNA sequencing analysis to confirm the HRM result 100
3.7 Genotype, allele frequencies and risk association of the studied genetic variations
3.7.1 Common genetic variations 105
3.7.2 Other genetic variations 108
3.7.3 Undetected genetic variations 108
3.8 Association between serum bilirubin levels with the genotypes for each genetic variation 111
3.9 Association between combinations of genetic variation with hyperbilirubinaemia 113

3.10 Hardy-Weinberg Equilibrium (HWE) analysis 116
3.11 Pairwise linkage disequilibrium analysis 116
3.12 Haplotype analysis
3.12.1 Distribution of *OATP2* gene haplotypes between two groups 119
3.12.2 Distribution of c.388 A>G and c.521 T>C haplotypes and haplotype pairs 122
3.13 Association between haplotype pairs and serum bilirubin level for hyperbilirubinaemia group 125

CHAPTER 4 – DISCUSSION
4.1 Demographic data of study subjects 127
4.2 Genetic variations of the *OATP2* gene and their association with neonatal hyperbilirubinaemia
4.2.1 Common genetic variations
4.2.1.1 The c.388 A>G and their association with neonatal hyperbilirubinaemia 129
4.2.1.2 The IVS5-107_112 del CTTGTA and their association with neonatal hyperbilirubinaemia 131
4.2.1.3 The c.597 C>T and their association with neonatal hyperbilirubinaemia 132
4.2.1.4 The c.571 T>C and their association with neonatal hyperbilirubinaemia 133
4.2.1.5 The c.521 T>C and their association with neonatal hyperbilirubinaemia 134
4.2.1.6 The g.-11187 G>A and their association with neonatal hyperbilirubinaemia 136
4.2.2 Other genetic variations
4.2.2.1 The c.463 C>A and their association with neonatal hyperbilirubinaemia 137
4.2.2.2 The g.-11110 T>G and their association with neonatal hyperbilirubinaemia 138
4.2.2.3 The c.411 G>A and their association with neonatal hyperbilirubinaemia 138
4.2.3 Undetected genetic variations 139
4.3 Linkage disequilibrium and haplotypes analysis of the genetic variations and their association with neonatal hyperbilirubinaemia 140
4.4 Association between combinations of genetic variations with serum bilirubin levels 146
4.5 HRM analysis as a method for screening of genetic variations of the *OATP2* gene 147
4.6 Limitations of the study 152
4.7 Implication for future research 153

CHAPTER 5 – CONCLUSION 155

REFERENCES 156
APPENDICES 171
LIST OF ABSTRACTS AND PRESENTATIONS 180

LIST OF TABLES

		Page
Table 1.1	Causes of unconjugated and conjugated hyperbilirubinaemia	13
Table 1.2	Causes of physiological and pathological jaundice	14
Table 1.3	Transporters, their substrates and tissue distribution in human	27
Table 2.1	Inclusion and exclusion criteria for hyperbilirubinaemia and non-hyperbilirubinaemia groups	38
Table 2.2	The studied genetic variation of the *OATP2* gene	48
Table 2.3	The list of primers used for HRM analysis	49
Table 2.4	Optimized annealing temperature and number of amplification cycle for HRM analysis for each region	53
Table 2.5	Master mix for promoter 1, exon 4-2, exon 5-1, exon 5-2, exon 5-3 regions of the *OATP2* gene	55
Table 2.6	Master mix for exon 4-1 region of the *OATP2* gene	55
Table 2.7	HRM analysis protocol for promoter 1 region of the *OATP2* gene	56
Table 2.8	HRM analysis protocol for promoter 2 and exon 5-2 regions of the *OATP2* gene	56
Table 2.9	HRM analysis protocol for exon 4-1 region of the *OATP2* gene	56
Table 2.10	HRM analysis protocol for exon 4-2 and exon 5-3 regions of the *OATP2* gene	57
Table 2.11	HRM analysis protocol for exon 5-1 region of the *OATP2* gene	57
Table 2.12	HRM analysis protocol for intron 5 region of the *OATP2* gene	57
Table 2.13	List of reagents used to prepare master mix for PCR amplification	62
Table 2.14	List of primers and annealing temperature for PCR amplification	63
Table 2.15	Preparation of master mix for PCR amplification for each region of the *OATP2* gene	65
Table 2.16	PCR amplification protocol for Promoter 1 region of the *OATP2* gene	66
Table 2.17	PCR amplification protocol for Promoter 2 region of the *OATP2* gene	66
Table 2.18	PCR amplification protocol for Exon 4 region of the *OATP2* gene	66
Table 2.19	PCR amplification protocol for Exon 5 region of the *OATP2* gene	66
Table 2.20	List of components for preparation of agarose gel	68
Table 3.1	Demographic data of the neonates enrolled in this study	74
Table 3.2	Genotype and allele frequencies of studied genetic variations of the *OATP2* gene in hyperbilirubinaemia and non-hyperbilirubinaemia group	109
Table 3.3	Serum bilirubin levels, µmol/L for each genotype for each genetic variation	112
Table 3.4	Frequency and serum bilirubin levels, µmol/L for each combination of genetic variations	114
Table 3.5	HWE analysis for each of the studied genetic variation	117

Table 3.6	Pairwise linkage disequilibrium for each of the genetic variation in the *OATP2* gene	118
Table 3.7	Haplotypes frequencies of the *OATP2* gene in hyperbilirubinaemia and non-hyperbilirubinaemia groups	120
Table 3.8	Haplotypes frequencies of c.388 A>G and c.521 T>C pairs for hyperbilirubinaemia and non-hyperbilirubinaemia groups	123
Table 3.9	Haplotype pairs frequency of the *OATP2* gene for hyperbilirubinaemia and non-hyperbilirubinaemia groups	124

LIST OF FIGURES

		Page
Figure 1.1	Classification of neonatal hyperbilirubinaemia	12
Figure 1.2	Bilirubin transportation, uptake, conjugation and excretion in the hepatocytes	18
Figure 1.3	Bilirubin metabolism pathway	19
Figure 1.4	Yellow discolouration of basal ganglia	22
Figure 1.5	Classification of transporters	26
Figure 1.6	Schematic diagram for *OATP2* gene which located in p arm of chromosome 12	30
Figure 1.7	Schematic presentation of the predicted secondary structure of the OATP2 protein with the nonsynonymous polymorphisms	33
Figure 2.1	Flow chart of the study	39
Figure 2.2	Method of buccal cell sample collection	43
Figure 2.3	96-well Piko® PCR plate used for HRM analysis	51
Figure 2.4	96-well PikoReal Real-Time PCR System	58
Figure 3.1	Graph represents the onset of hyperbilirubinaemia versus peak serum bilirubin levels of the neonates in hyperbilirubinaemia group	76
Figure 3.2	Amplification graph from HRM analysis	82
Figure 3.3	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for g.-11187 G>A of the *OATP2* gene	85
Figure 3.4	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for g.-11110 T>G of the *OATP2* gene	86
Figure 3.5	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.388 A>G of the *OATP2* gene	87
Figure 3.6	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.411 G>A of the *OATP2* gene	88
Figure 3.7	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.463 C>A of the *OATP2* gene	89
Figure 3.8	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for IVS5-107_112 del CTTGTA of the *OATP2* gene	90
Figure 3.9	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.521 T>C of the *OATP2* gene	91
Figure 3.10	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.571 T>C of the *OATP2* gene	92

Figure 3.11	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.597 C>T of the *OATP2* gene	93
Figure 3.12	Amplification graph from HRM analysis of DNA samples extracted from blood	95
Figure 3.13	Normalized and shifted melting curve, derivative melting plot, normalized and temperature-shifted different plot and different plot of the normalized data for c.388 A>G of the *OATP2* gene of DNA samples extracted from blood	96
Figure 3.14	Electropherogram from DNA sequencing analysis of g.-11187 G>A of the *OATP2* gene	101
Figure 3.15	Electropherogram from DNA sequencing analysis of g.-11110 T>G of the *OATP2* gene	101
Figure 3.16	Electropherogram from DNA sequencing analysis of c.388 A>G of the *OATP2* gene	101
Figure 3.17	Electropherogram from DNA sequencing analysis of c.411 G>A of the *OATP2* gene	102
Figure 3.18	Electropherogram from DNA sequencing analysis of c.463 C>A of the *OATP2* gene	102
Figure 3.19	Electropherogram from DNA sequencing analysis of IVS5-107_112 del CTTGTA of the *OATP2* gene	103
Figure 3.20	Electropherogram from DNA sequencing analysis of c.521 T>C of the *OATP2* gene	103
Figure 3.21	Electropherogram from DNA sequencing analysis of c.571 T>C of the *OATP2* gene	104
Figure 3.22	Electropherogram from DNA sequencing analysis of c.597 C>T of the *OATP2* gene	104
Figure 3.23	Serum bilirubin level (μmol/L) for each haplotype pairs	126

LIST OF PLATES

		Page
Plate 3.1	Gel electrophoresis representative of genomic DNA extracted from buccal cell samples	78
Plate 3.2	Gel electrophoresis representative of HRM product of exon 4-1 region of the *OATP2* gene	80
Plate 3.3	Gel electrophoresis picture for PCR product of promoter 1 with size of 384 bp	98
Plate 3.4	Gel electrophoresis picture for PCR product of promoter 2 with size of 384 bp	98
Plate 3.5	Gel electrophoresis picture for PCR product of exon 4 with size of 485 bp	99
Plate 3.6	Gel electrophoresis picture for PCR product of exon 5 with size of 609 bp	99

LIST OF SYMBOLS AND ABBREVIATIONS

%	: Percent
°C	: Degree Celsius
°C/min	: Degree Celsius per minute
°C/sec	: Degree Celsius per second
≥	: More than and equal to
≤	: Less tan and equal to
>	: More than
<	: Less than
∞	: Infiniti
α	: Alpha
±	: Plus minus
A	: Adenine
A260/A280	: Ratio at 260 absorbance
ABCG2	: ATP-binding cassette sub-family G member 2
AE	: Elution buffer
AM	: Apical membrane
ATP	: Adenosine triphosphate
BL	: Lysis buffer
BM	: Basolateral membrane
Bp	: Base pair
BSEP	: Bile Salt Export Pump
BW	: Washing buffer
C	: Cytosine
CAR	: Constitutive androstane receptor
cAMP	: Cyclic adenosine monophosphate
cGMP	: Cyclic guanosine monophosphate
CI	: Confidence interval
CO	: Carbon monoxide
CM	: Canalicular membrane
Cq	: Quantification cycle
D'	: Standardized disequilibrium coefficient
DA	: Dalton
dH_2o	: Distilled water
DMSO	: Dimethylsulfoxide
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxynucleotide triphosphates
EB	: Elution buffer
EDTA	: Ethylenediaminetetraacetic acid
e.g	: Exempli gratia
EtBr	: Ethidium bromide
G	: Guanine
G	: Gram
g.	: Genomic
G6PD	: Glucose-6-phosphate dehydrogenase deficiency
GST	: Glutathione S-transferase
He	: Heterozygous deletion

HLA : Human leukocyte antigen
HWE : Hardy-Weinberg equilibrium
Ho : Homozygous deletion
HO-1 : Heme oxygenase-1
HRM : High resolution melting
Kg : Kilogram
LD : Linkage disequilibrium
LST-1 : Liver specific transporter-1
M : Molar
mA : Milliamperes
MAF : Minor allele frequency
MATEs : Multidrug and toxin extrusion transporters
MATE1 : Multidrug and toxin extrusion transporter 1
MATE1/2-K : Multidrug and toxin extrusion transporter 1/2-K
MDRs : Multidrug resistance transporters
MDR1 : Multidrug resistance transporter 1
MDR1/3 : Multidrug resistance transporter 1/3
Mg : Milligram
$MgCl_2$: Magnesium chloride
mg/kg/day : Milligram per kilogram per day
Ml : Millilitre
mM : Millimolar
MRPs : Multidrug resistance-associated protein
MRP1 : Multidrug resistance-associated protein 1
MRP2 : Multidrug resistance-associated protein 2
MRP1/3/4 : Multidrug resistance-associated protein 1/3/4
MRP3/4 : Multidrug resistance-associated protein 3/4
MRP2 : Multidrug resistance-associated protein 2
N : Number of subjects
ug/uL : Nanogram per microlitre
NA : Not applicable
NADPH : Nicotinamide adenine dinucleotide phosphate
NTCP : Sodium-dependent taurocholate cotransporting protein
NW : Washing buffer
OAT2 : Organic anion transporter 2
OAT1/2/3 : Organic anion transporter 1/2/3
OATP1B1 : Organic anion transporting polypeptide 1B1
OATP1B3 : Organic anion transporting polypeptide 1B3
OATP2B1 : Organic anion transporting polypeptide 2B1
OATP2 : Organic anion transporting polypeptide 2
OATP8 : Organic anion transporting polypeptide 8
OATP-C : Organic anion transporting polypeptide C
OATPs : Organic anion transporting polypeptides
OATs : Organic anion transporters
OCTs : Organic cation transporters
OCT1 : Organic cation transporter 1
OCT2 : Organic cation transporter 2
OCT3 : Organic cation transporter 3
OR : Odd ratio

PB	: Bind buffer
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
PEPTs	: Peptide transporters
PEPT1	: Peptide transporter 1
PEPT1/2	: Peptide transporter ½
PS	: Power and sample size Calculation
r^2	: Correlation coefficient
RFU	: Relative fluorescence unit
Rh	: Rhesus
Rpm	: Rotations per minutes
RT	: Room temperature
RNA	: Ribonucleic acid
SD	: Standard deviation
SNPs	: Single nucleotide polymorphisms
SPSS	: Science Package Social Software
SVD	: Spontaneous vaginal delivery
T	: Thymine
TBE	: Tris/Borate/EDTA buffer
TW	: Washing buffer
U/µL	: Unit per microlitre
UDPGT	: Uridine diphosphateglucuronosyltransferase enzyme
UGT1A1	: Uridine diphosphate glucuronosyltransferase 1A1
USA	: United state of America
UV	: Ultraviolet
V	: Volts
Wt	: Wild type
µM	: Micromolar
µL	: Microlitre
µmol/L	: Micromole per litre

LIST OF APPENDICES

		Page
Appendix A	Ethical approval from USM	182
Appendix B	Consent form	183
Appendix C	Form for baseline data	188

VARIASI YANG DIPILIH PADA GEN ORGANIK ANION TRANSPORTING POLIPEPTIDA 2 (*OATP2*) DI KALANGAN NEONAT MELAYU YANG MENGALAMI DAN TIDAK MENGALAMI HIPERBILIRUBINEMIA

ABSTRAK

Hiperbilirubinemia neonatal adalah disebabkan oleh pelbagai faktor risiko termasuklah faktor genetik. Protein OATP2 yang dikodkan oleh gen *OATP2* memainkan peranan yang penting untuk membawa bilirubin dalam darah ke dalam sel hati. Terdapat pelbagai variasi genetik telah dilaporkan dan setiap daripadanya mempunyai frekuensi yang berbeza untuk setiap populasi. Fungsi variasi genetik yang terdapat pada gen *OATP2* terhadap pembentukan hiperbilirubinemia neonatal masih kontroversi dan terdapat kekurangan kajian mengenai perkaitan antara kehadiran variasi genetik ini terhadap pembentukan hiperbilirubinemia neonatal termasuklah di kalangan orang Melayu di Malaysia. Pemilihan analisis untuk penyaringan variasi genetik juga adalah sangat penting untuk memastikan keputusan yang tepat dapat diperolehi. Objektif kajian ini adalah untuk mengenalpasti kehadiran variasi genetik yang telah dipilih dengan menggunakan analisis lebur resolusi tinggi (HRM), menentukan frekuensi genotip, alel dan haplotip di antara kumpulan yang mengalami dan tidak mengalami hiperbilirubinemia serta menentukan sama ada genotip dan haplotip memainkan peranan dalam pembentukan hiperbilirubinemia neonatal. Sampel sel pipi daripada 264 neonat telah diperoleh dan DNA diekstrak dengan menggunakan kit komersial pengekstrakan DNA. Analisis HRM digunakan untuk penyaringan variasi genetik dan sampel yang mempunyai bentuk graf yang berbeza akan dipastikan melalui analisis penjujukan DNA.

Daripada 14 variasi genetik yang telah dipilih, 9 variasi wujud dalam sampel neonat Melayu dan c.388 A>G merupakan variasi genetik yang mempunyai frekuensi yang tertinggi. Kajian ini juga menunjukkan bahawa kehadiran variasi genetik pada gen *OATP2* tidak mempunyai perkaitan yang signifikan dengan pembentukan hiperbilirubinemia neonatal di kalangan neonat Melayu kecuali untuk genotip mutan heterozigot c.597 C>T yang menunjukkan frekuensi yang tinggi dalam kumpulan yang tidak mengalami hiperbilirubinemia. Analisis perkaitan antara tahap bilirubin serum dan genotip mendapati terdapat hubungan yang signifikan antara g.-11187 G>A dan tahap bilirubin serum. Walaubagaimanapun, analisis perkaitan antara tahap bilirubin serum dan haplotip variasi genetik yang lain tidak menunjukkan perbezaan yang signifikan. Oleh itu, kajian lanjut perlu dilakukan untuk pengesahan.

SELECTED VARIATIONS OF THE ORGANIC ANION TRANSPORTING POLYPEPTIDE 2 (*OATP2*) GENE IN MALAY NEONATES WITH AND WITHOUT HYPERBILIRUBINAEMIA

ABSTRACT

Neonatal hyperbilirubinaemia is caused by many possible risk factors, including genetic factor. The OATP2 protein, which is encoded by *OATP2* gene plays a crucial role in transporting bilirubin from the circulation to the hepatocytes. There are several genetic variations that have been reported and each of them was presence at different frequencies between population to population. The role of genetic variations of the *OATP2* gene in the development of neonatal hyperbilirubinaemia is still controversial and there is lack of study investigated the association between the presence of genetic variations and neonatal hyperbilirubinaemia including in Malay population in Malaysia. The appropriate choice of screening method is also important to allow robust and accurate genotyping results. The objectives of this study is to screen for the selected genetic variations of the *OATP2* gene using high resolution melting (HRM) analysis, to determine the genotype, allele as well as haplotype frequencies between hyperbilirubinaemia and non-hyperbilirubinaemia groups and to determine the association between genotypes and haplotypes with the development of neonatal hyperbilirubinaemia. Buccal cells sample of 264 neonates were collected and DNA was extracted using commercialized DNA extraction kit. HRM analysis was performed to screen for the selected genetic variations and samples that have different pattern of melt curve were confirmed by DNA sequencing analysis. Out of 14 genetic variations that were selected, 9 were presence in Malay neonates with the most common is c.388 A>G.

This study also shows that there was no significant association between genetic variations of the *OATP2* gene with neonatal hyperbilirubinaemia in Malay neonates except for heterozygous mutant genotype of c.597 C>T that shows high frequency in non-hyperbilirubinaemia group. The analysis on the association between serum bilirubin levels and genotypes found there was a significant association of g.-11187 G>A with serum bilirubin levels. However, the analysis on the association between serum bilirubin levels and haplotypes of other genetic variations shows no significant differences. Thus, further study need to be conducted for confirmation.

CHAPTER 1

INTRODUCTION

1.1 Research background

Neonatal hyperbilirubinaemia or neonatal jaundice is common clinical condition among neonates (Akaba *et al*., 1999; Porter and Dennis, 2002). Severe hyperbilirubinaemia may lead to long-term effects including bilirubin encephalopathy and kernicterus and these cases become one of the serious problems in most of the developing countries. It caused mortality and long-term morbidity, about 10% and 70%, respectively (Blackmon *et al*., 2004; Ip *et al*., 2004).

The first primary factors that had been recognized to be associated with neonatal hyperbilirubinaemia including blood group and rhesus (Rh) incompatibilities, cephalohaematoma, breast feeding, weight loss, premature birth, polycythaemia, infants of diabetic mothers, glucose-6-phosphate dehydrogenase (G6PD) deficiency and ethnicity (Joseph *et al*., 1998; Newman and Maisels, 2000; Dennery *et al*., 2001; Harris *et al*., 2001; Huang *et al*., 2004). In addition, there were studies showed that neonatal hyperbilirubinaemia is common in male neonates as compared to female (Gale *et al*., 1990; A.A.o.P., 2004; Ip *et al*., 2004).

Even though these factors were identified to be related to the occurrence of severe hyperbilirubinaemia, however, it only contributes to 50% of neonates with non-physiological hyperbilirubinaemia (Büyükkale *et al*., 2011). Therefore, genetics is considered as one of the possible contributing factor and the

importance of genetic factors in development of neonatal hyperbilirubinaemia has been given great attention and it was recognized in many studies (Kaplan *et al*., 2002; Huang *et al*., 2004; Huang *et al*., 2005; Watchko, 2005). However, its functional role in the development of neonatal hyperbilirubinaemia remains unclear and seek for further investigation (Watchko *et al*., 2009).

Many genes have been identified that involves in the aetiology of neonatal hyperbilirubinaemia. These genes including uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*) (Laforgia *et al*., 2002; Long *et al*., 2011), glutathione S-transferase (*GST*) (Muslu *et al*., 2008), glucose-6-phosphate dehydrogenase (*G6PD*) (Valaes, 1994; Johnson *et al*., 2002), organic anion transporting polypeptide 2 (*OATP2*) (Cui *et al*., 2001; Huang *et al*., 2004; Johnson *et al*., 2009; Lin *et al*., 2009; Bielinski *et al*., 2011; Liu *et al*., 2013), heme oxygenase-1 (*HO-1*) (Bozkaya *et al*., 2010), constitutive androstane receptor (*CAR*) (Huang *et al*., 2003) and multidrug resistance-associated protein 2 (*MRP2*) (Lee *et al*., 2006). However, the frequencies and the contribution of each genetic variation in these genes were different between one population to one population across the world.

As an example, c.388 A>G variation which is located in exon 4 of the *OATP2* gene is common in Taiwanese neonates and proven to be a risk factor of developing severe neonatal hyperbilirubinaemia (Huang *et al*., 2004). In contrast, study by Watchko *et al*. (2009), shown that there was no association between c.388 A>G variation and neonatal hyperbilirubinaemia. Other than Taiwanese, this variation also been reported to be more common among African-American, Caucasian, Chinese and Malay population (Tirona *et al*., 2001; Mwinyi *et al*.,

2004; Xu *et al*., 2007; Wong *et al*., 2012). Besides that, c.571 T>C variation is more common in Finnish population (Pasanen *et al*., 2006). This indicates that there is a significant difference of genetic variations among different population and the frequencies of each genetic variation is known to be race dependent.

The OATP2 protein play an important role in the uptake of the bilirubin and its conjugates from the blood to the hepatocytes (Cui *et al*., 2001). Therefore, it is plausible that the presence of genetic variations in this gene may influence the transport activity of this protein and increase the susceptibility of the neonates to neonatal hyperbilirubinaemia. In addition, the impact the each genetic variation to the development of neonatal hyperbilirubinaemia is remained uncertain.

Few studies about the association between genetic variations of the *OATP2* gene with neonatal hyperbilirubinaemia among the Malaysian population including Malay, Chinese and Indian have been reported (Wong *et al*., 2009; Wong *et al*., 2012). Therefore, this study was carried out to further investigate the contribution of the genetic variations of the *OATP2* gene to the development of neonatal hyperbilirubinaemia among Malay population.

Other than calculating genotype and allele frequencies for each genetic variation, genetic linkage study was also conducted. In a genetic study, genetic linkage is a powerful method to determine the association between each genetic variation that lies in the same chromosome. Besides that, haplotype analysis was also included in order to determine the association between haplotypes and neonatal hyperbilirubinaemia. Many researchers have suggested that haplotype analysis is more powerful and useful method to analyse genetic variations and

their association with the disease as compared to traditional genotype-phenotype method (Niemi *et al*., 2004; Crawford and Nickerson, 2005; Jada *et al*., 2007).

In order to screen for the genetic variations, high resolution melting (HRM) analysis was used and this method is a new technology, rapid and powerful method and able to screen the genetic variations in many clinically significant genes. According to the previous studies, HRM is a useful method to detect single nucleotide polymorphisms (SNPs), insertion and deletion (Krypuy *et al*., 2006; Bastien *et al*., 2008; Millat *et al*., 2009; Temesvári *et al*., 2011; Wong *et al*., 2012).

1.2 Rationale of the study

Neonatal hyperbilirubinaemia is known to cause many prolonged adverse effects in term of health, medical cost and emotional burden to neonates' parents. In term of health, neonatal hyperbilirubinaemia can cause severe brain damage if it is untreated. Even though there is standard treatment available such as phototherapy, exchange transfusion and pharmacological therapies, however, each of these methods may cause side effects to the neonates such as dehydration, infection and even death. Besides that, neonates with severe neonatal hyperbilirubinaemia may require a longer stay in hospital. Thus, it may cause an economic and emotional burden to the parents especially to whom that stay further from the hospital or come from the poor socioeconomic family.

Thus, genetic study related to neonatal hyperbilirubinaemia need to be conducted. From the study, earlier occurrence of neonatal hyperbilirubinaemia can be predicted if the neonates carried certain genetic variations. It will help the doctors to give an early and better treatment before neonatal hyperbilirubinaemia getting more severe. Other than that, genetic study is closely related to the development of personalized medicine. Getting full information about the genes and genetic variations that lead to neonatal hyperbilirubinaemia may allow us to give customized medicines and specialized for each of the population. In addition, a new therapy strategy for prevention and treatment of neonatal hyperbilirubinaemia can be done after all data regarding related genetic variations were collected.

Thus, by having an effective medicine and treatment, the requirement for phototherapy and exchange transfusion can be reduced. The cost for hospital bills and the side effects from the treatments may also lessen. Other than that, this study may also leads to a better understanding of genetic factors related to neonatal hyperbilirubinaemia and lead to the improvement of scientific knowledge regarding neonatal hyperbilirubinaemia.

1.3 Objectives

1.3.1 General objective

To study the selected genetic variations of the *OATP2* gene in Malay neonates with and without neonatal hyperbilirubinaemia.

1.3.2 Specific objectives

i. To screen for the selected genetic variations of the *OATP2* gene using HRM analysis

ii. To determine the genotype and allele frequencies of selected genetic variations of the *OATP2* gene and their association with neonatal hyperbilirubinaemia in Malay neonates

iii. To determine the haplotype frequencies of selected genetic variations of the *OATP2* gene and their association with neonatal hyperbilirubinaemia in Malay neonates

1.4 Characteristics of neonatal hyperbilirubinaemia

"Hyperbilirubinaemia" can be defined as the presence of excessive serum bilirubin in the circulation and the ranges are different between one country to other country. For example, in USA, hyperbilirubinaemia is defined as the presence of serum bilirubin levels ≥ 222 micromole per litre (µmol/L), in Australia ≥ 154 µmol/L, in India ≥ 140 µmol/L and in Hong Kong and Singapore ≥ 255 µmol/L (Lee *et al*., 1970; Palmer and Drew, 1983; Menon and Mohapatra, 1987; Ho *et al*., 1988; Maisels, 1999).

Up to 60% of full term neonates and 80% of premature neonates will develop neonatal hyperbilirubinaemia during their first day of life and usually will resolve after 7 – 8 days (Kliegman and Behrman, 1992; A.A.o.P., 1994). During their hospitalization, the maximum limit of serum bilirubin levels will reach even though there were no other diseases detected. This level is also higher as compared with adults (≥ 5 – 17 µmol/L) and it is considered as abnormal (Ho, 1992).

Visible neonatal hyperbilirubinaemia indicated that there is presence of bilirubin outside the circulation such as in the skin and other lipid fat content tissues. During the early onset of neonatal hyperbilirubinaemia, the yellowness appears in the upper part of the body (on the face). Then, it will move down to the chest, abdomen and arms. Lastly, it will appear on the legs, which indicate that neonatal hyperbilirubinaemia is getting severe. This yellowness movement is called as the rostral and caudal pattern (Thaler and Gellis, 1968; Kramer, 1969; Knudsen, 1990).

The accumulation of bilirubin is dependent on its production and excretion. If the degree of mismatch between bilirubin production and its removal is moving toward accumulation and load, this will cause the rise of serum bilirubin levels in the circulation and neonatal hyperbilirubinaemia may occur (Kaplan *et al*., 2002). According to Cohen *et al*. (2010), problem in the bilirubin clearance is normally occur to liver disease patients or it may also result by the presence of genetic variations.

The incidence of neonatal hyperbilirubinaemia is different between populations to populations. Most of the studies shown that Asian and American-Indian population have highest prevalence of neonatal hyperbilirubinaemia as compared to Caucasian population (Robinson and Lee, 1991; Akaba *et al*., 1999; Dennery *et al*., 2001; A.A.o.P., 2004; Halamek and Stevenson, 2010). In addition, neonatal hyperbilirubinaemia in Caucasian generally less severe and occur in shorter duration (Robinson and Lee, 1991).

1.5 Classification of neonatal hyperbilirubinaemia

1.5.1 Unconjugated hyperbilirubinaemia versus conjugated hyperbilirubinaemia

Hyperbilirubinaemia can be classified based on the form of bilirubin that caused it; unconjugated and conjugated hyperbilirubinaemia (Figure 1.1). Unconjugated hyperbilirubinaemia is caused by the overproduction of bilirubin due to excessive haemolysis, defects in hepatic uptake or conjugation of bilirubin. A defect in the hepatic uptake and conjugation of bilirubin is related to the reduction of enzyme activities that are responsible to transport the bilirubin from circulation to the liver and to transform the bilirubin into its conjugated form. In addition, the neonates' skin with unconjugated hyperbilirubinaemia will tend to appear bright yellow or sometimes orange.

In contrast, conjugated hyperbilirubinaemia is related to bile secretion problems. After the bilirubin was conjugated, it will be excreted into bile to be removed from the body. However, this process is disturbed if there is deficient activity of protein that plays a role to transport conjugated bilirubin to the bile. As compared to unconjugated bilirubin, conjugated bilirubin is water soluble compound. Even though, water soluble bilirubin do not cause neurotoxicity, however elevated of its concentration in the circulation may potentially lead to serious disorder (Kliegman and Behrman, 1992). Neonates' skin with conjugated hyperbilirubinaemia is tends to be greenish or muddy yellow (Kliegman and Behrman, 1992).

Table 1.1 showed the causes of unconjugated and conjugated hyperbilirubinaemia.

1.5.2 Physiological hyperbilirubinaemia versus pathological hyperbilirubinaemia

Unconjugated hyperbilirubinaemia can be further classified into physiological and pathological hyperbilirubinaemia (Figure 1.1). Physiological hyperbilirubinaemia also called as normal hyperbilirubinaemia. It is the event that occurs more common in premature neonates as compared to full term neonates. It is benign, normally appear within 3 – 4 days of life, disappear after 8 – 10 days of age (Kliegman and Behrman, 1992) and the peak of serum bilirubin levels were not exceed 290 µmol/L (Dennery *et al*., 2001). In addition, neonates with physiological hyperbilirubinaemia usually required no treatment (Robinson and Lee, 1991; Cashore, 1999).

However, pathological hyperbilirubinaemia may appear earlier, in less than 24 hours of life and occur in longer duration about more than 14 days. The peak of serum bilirubin levels was also higher (>290 µmol/L) within 24 hours of life (Dennery *et al*., 2001; Porter and Dennis, 2002). According to Halamek and Stevenson (2010), if the serum bilirubin levels is exceeded 290 µmol/L, it is not considered as physiological hyperbilirubinaemia and usually the causes of pathological hyperbilirubinaemia were identified in such neonates.

Table 1.2 showed the causing factors of the physiological and pathological hyperbilirubinaemia.

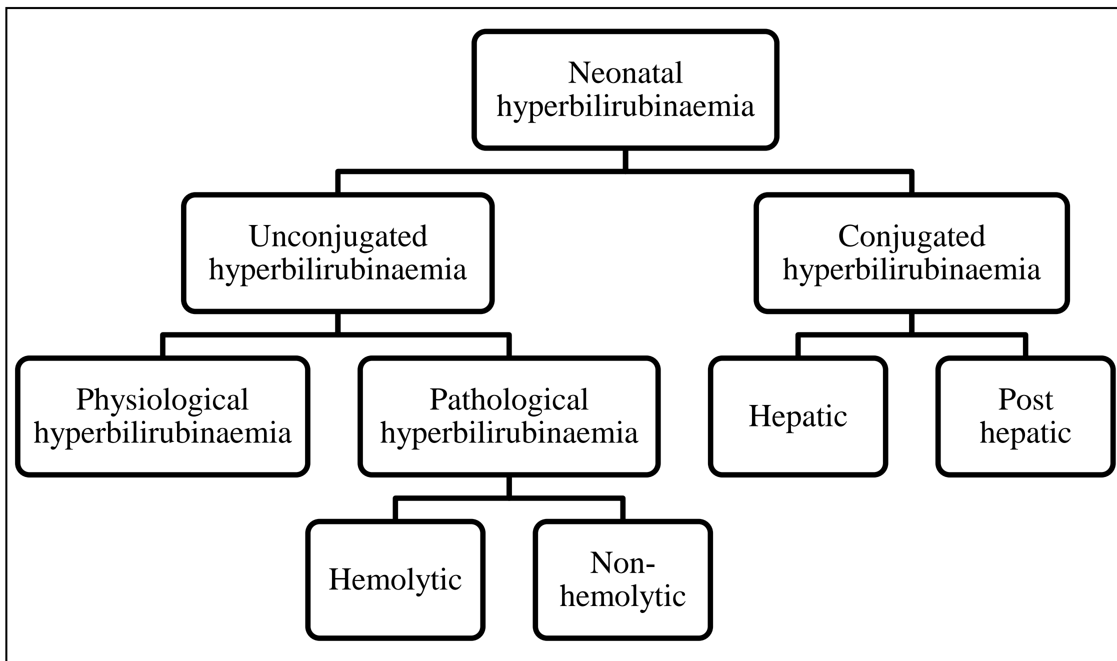

Figure 1.1: Classification of neonatal hyperbilirubinaemia

Table 1.1: Causes of unconjugated and conjugated hyperbilirubinaemia (adapted from Kliegman and Behrman (1992) and Robinson and Lee (1991))

Unconjugated hyperbilirubinaemia	Conjugated hyperbilirubinaemia
• Haemolysis factors (e.g.: blood group and Rh incompatibilities) • Structural and metabolic abnormalities of red blood cells (e.g.: G6PD deficiency and hereditary spherocytosis) • Genetic defect (reduce in enzyme activity) • Shorten of red blood cells life span • Urinary tract infection • Infant of diabetic mother • Cephalohaematoma, bruising and haemorrhage • Breast milk • Prematurity	• Intrauterine infection (e.g.: rubella, cytomegalovirus infection, toxoplasmosis and herpes simplex infection) • Viral and bacterial infection (e.g.: sepsis and neonatal hepatitis) • Metabolic disorder (e.g.: α-antitrypsin deficiency and galactosemia) • Carcinoma of the head of the pancreas • Biliary obstruction • Congenital biliary atresia • Stone in the bile duct • Pancreatic pseudocyst • Hepatocellular carcinoma • Post-asphyxia

Table 1.2: Causes of physiological and pathological hyperbilirubinaemia (adapted from Robinson and Lee, 1991; Dennery *et al*., 2001; Gartner, 2001)

Physiological hyperbilirubinaemia	Pathological hyperbilirubinaemia
• Shorten of red blood cells life span • Immaturity of hepatic uptake and conjugation process (low enzyme activities) • Increase enterohepatic circulation • Prematurity	• Haemolysis factors • Structural and metabolic abnormalities • Urinary tract infection • Bacterial infection (e.g: sepsis) • Cephalohaematoma • Bruising • Haemorrhage

1.6 Bilirubin production in neonates

Bilirubin is a yellow pigments that is produced in the liver. The production of bilirubin started when neonates are still in the womb and can be detected during 12 weeks of gestation in normal amniotic fluid. However, the levels of serum bilirubin will decreased at 36 – 37 weeks due to activation of uridine diphosphate glucuronosyltransferase (UDPGT) enzyme (Maisels, 1999).

Neonates have 2 – 3 times higher bilirubin metabolism and production rate as compared to children and adult, about 6 – 8 milligram per kilogram per day (mg/kg/day). High production of bilirubin is caused by several factors such as shorten of red blood cells life span, increase amount of haematocrit, increase of red blood cells volume and lack of hepatic function for conjugation and clearance of bilirubin (Kaplan *et al.*, 2002; Porter and Dennis, 2002; Moerschel *et al.*, 2008).

Bilirubin is a waste product of heme metabolism. Heme can be found in red blood cells and the other oxidative compound such as hepatic mitochondrial, cytochrome P450 isoenzymes, catalase and peroxides (Tukey and Strassburg, 2000; Volpe, 2008). However, about 75% of the bilirubin is from the red blood cells (Robinson and Lee, 1991). In bilirubin metabolism pathway, it involves several steps including production, transportation, hepatic uptake, conjugation, excretion and enterohepatic circulation (Volpe, 2008).

First, when the red blood cells are broken down, the heme part will be converted into the biliverdin (green pigment) and carbon monoxide by heme oxygenase. Then, biliverdin is reduced to bilirubin (yellow pigment) by biliverdin reductase in the presence of nicotinamide adenine dinucleotide

phosphate (NADPH). In this stage, bilirubin is in unconjugated and unbound form. After bilirubin reaches the blood plasma, it is tightly bound to albumin to form bilirubin-albumin complex and is transported to the liver. According to Dennery *et al*. (2001), 1 gram (g) of albumin can bind to the 8.2 milligram (mg) of bilirubin. The unconjugated bilirubin that bound to the albumin is also called as indirect bilirubin.

After bilirubin reaches the liver, uptake process is taking place in the sinusoidal cell membrane of hepatocytes. A variety of organic anion transporting polypeptide play its role in the uptake of unconjugated bilirubin from the blood to the liver and these transporters are very selective for their substrates. However, the most significant transporter for bilirubin is an organic anion transporting polypeptide 2 (OATP2) protein. When unconjugated bilirubin enters the hepatocytes, it will bind to the group of cytosolic proteins which is also known as glutathione S-transferases or ligandins to the site where the bilirubin conjugation takes place.

The conjugation process is conducted by UDPGT enzyme in the liver. It is a process of esterification of bilirubin's propionic acid with glucuronic acid. This process allows the unconjugated bilirubin to become conjugated bilirubin (direct bilirubin), a water soluble compound. Eighty percent (80%) of bilirubin is in the diglucuronide form and another 20% is in the monoglucuronide form (McCandless, 2011). After conjugation takes place, the conjugated bilirubin will be diffused through the cytosol to be excreted to the bile. This step is mediated by the adenosine triphosphate (ATP) dependent transport protein, multidrug

resistance-associated protein 2 (MRP2) (Büchler *et al*., 1996). Figure 1.2 shows how the bilirubin is taken up to the liver until it was excreted to the bile.

From the bile, the bilirubin is excreted to the small intestine. Bilirubin in the small intestine and colon is reduced to urobilinogen (colourless) by bacterial dehydrogenase and later will be reduced again to urobilin (orange-yellow). Urobilin is excreted to the feces and giving it a light tan colour. However, small amounts of urobilinogen will be reabsorbed by the small intestine and colon to the liver and will be excreted in the urine. Higher concentration of the urobilinogen in the urine gives it dark yellow brown colour. Overall process of bilirubin metabolism pathway is shown in Figure 1.3.

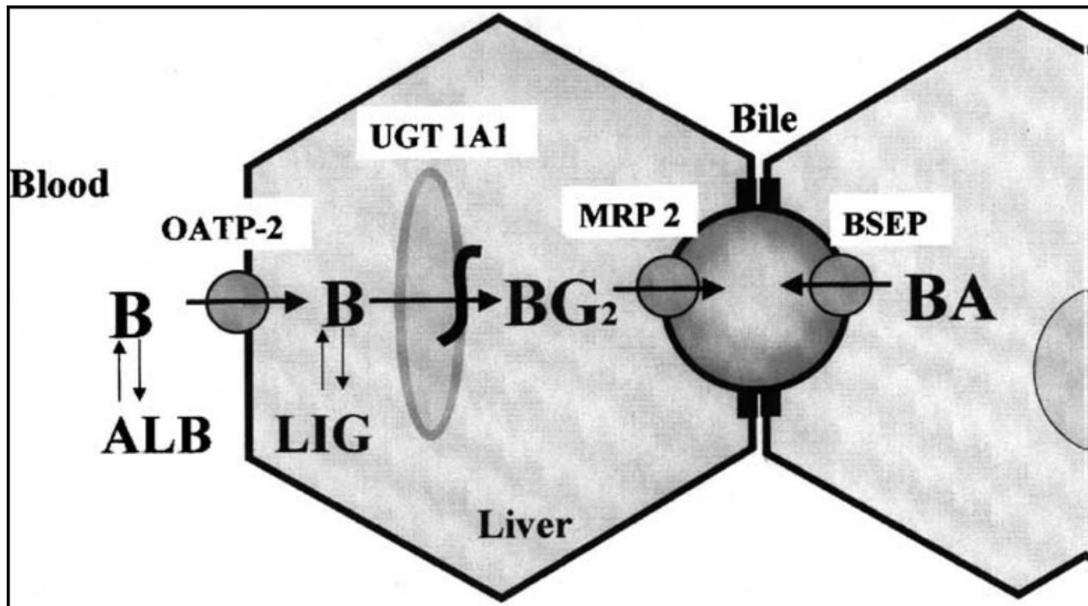

Figure 1.2: Bilirubin transportation, uptake, conjugation and excretion in the hepatocytes (adapted from Jansen and Bittar, 2004)

Note: B = Bilirubin; ALB = Albumin; LIG = Ligandin; BG_2 = Bilirubin diglucuronide; BA = Bile acid; OATP2 = Organic anion transporting polypeptide 2; UGT1A1 = Uridine diphosphate glucuronosyltransferase 1A1; MRP2 = Multidrug resistance-associated protein 2; BSEP = Bile Salt Export Pump

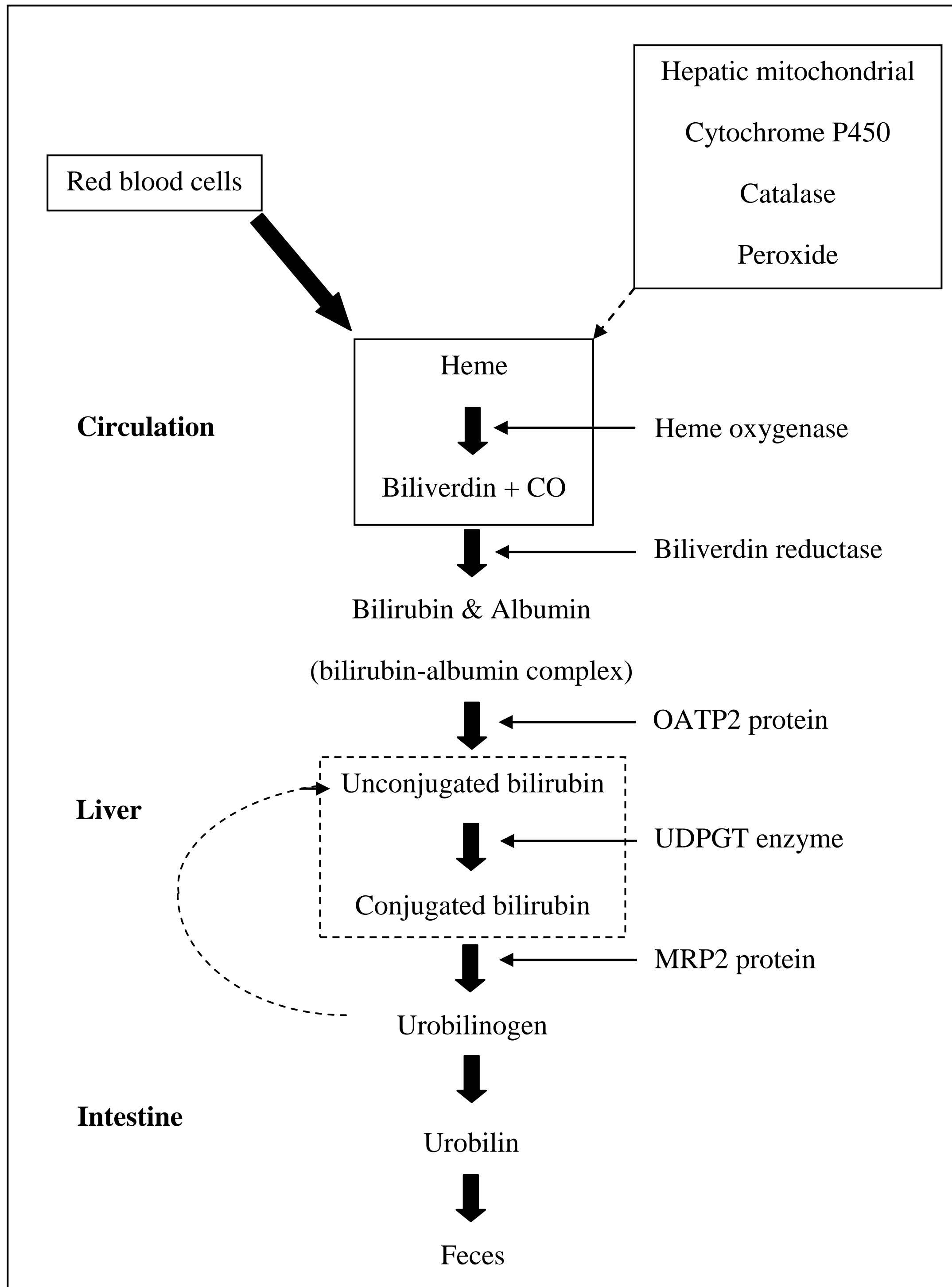

Figure 1.3: Bilirubin metabolism pathway

1.7 Effects of neonatal hyperbilirubinaemia

In the early stage, neonatal hyperbilirubinaemia normally present in a benign state and later on become more serious if it is unmonitored or untreated (A.A.o.P., 2004). Free bilirubin (unbound to albumin) is able to enter the brain tissue through several ways, including blood brain barrier or the choroid plexus and can cause kernicterus (Kaplan and Hammerman, 2005). However, the most significant and important is through the blood brain barrier (Volpe, 2008). The presence of bilirubin in the central nervous system may result in brain encephalopathy (acute bilirubin intoxication), kernicterus (the most severe form of brain damage) and sometimes leads to death (Bhutani and Johnson, 2003; Morioka *et al.*, 2013). The term "kernicterus" was described as a presence of yellow staining in basal ganglia and this term was first used by Schmorl in early 1904 (Figure 1.4).

According to Ives (2011), these effects are more likely to occur in premature neonates as compared to the full term neonates. Even though full term neonates have high serum bilirubin levels than premature neonates, however, the risk of kernicterus is low (Kliegman and Behrman, 1992). This situation is due to the immaturity of central nervous system and lower albumin level in premature neonates.

Brain encephalopathy usually occurs within the first weeks of life and it shows some clinical manifestation such as lethargy, hypertonia, irritability, fever, apnea and seizure. However, kernicterus can cause chronic and permanent clinical sequelae of bilirubin toxicity such as athetoid cerebral palsy, facial grimacing, dental-enamel dysplasia, paralysis and hearing loss (Robinson and

Lee, 1991; A.A.o.P., 2004). Other than neurotoxicity effect, increase of bilirubin in the body may cause cellular injury. Previous work indicated that high levels of bilirubin may cause disturbance in mitochondrial enzyme activity, oxidative phosphorylation, amino acid and protein metabolism, deoxyribonucleic acid (DNA) synthesis and synaptic transmission (Amato *et al*., 1994; Chuniaud *et al*., 1996).

Neonatal hyperbilirubinaemia is the common reason for hospital admission of neonates and it is the second cause of death with percentage of 14.1% (Owa and Osinaike, 1998). English *et al*. (2003) also found neonatal hyperbilirubinaemia is the major cause of death for the neonates with a mortality rate of 24%. In addition, Ugwu *et al*. (2006) had reported that the tendency for the neonates to die due to neonatal hyperbilirubinaemia is same as birth asphyxia and this is higher than sepsis.

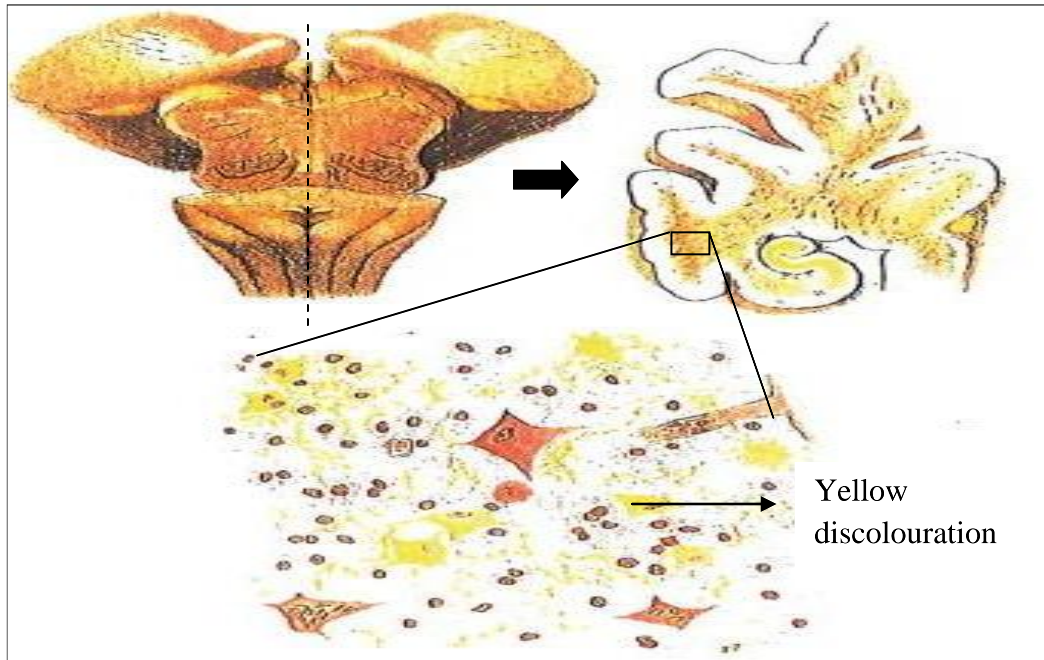

Figure 1.4: Yellow discolouration of basal ganglia (modified from Hansen (2000))

1.8 Managements and treatments for neonatal hyperbilirubinaemia

1.8.1 Phototherapy

In most hospitals, phototherapy is a standard treatment for neonatal hyperbilirubinaemia. It is involves exposure of the blue light with a wavelength of approximately 450 nm to the neonates' body (preferably naked) with eye covered. Bilirubin will absorb the blue light and will transform to the lumirubin which will be excreted more easily by the liver and kidney than other isomer (McDonagh and Lightner, 1985; Ennever *et al*., 1987). However, diarrhea, intestinal hypermotility and insensible water loss are the complications of phototherapy (Maisels and McDonagh, 2008).

1.8.2 Exchange transfusion

Exchange transfusion is a procedure involves replacement of patient's blood with an equal amount of fresh blood. Exchange transfusion is the best method of choice if the neonates suffer from hemolytic anaemia or when the phototherapy treatment is fail. Other than that, it is also suitable for the neonates who are facing rapid increasing of serum bilirubin levels probably 340 µmol/L within 48 hours or the neonates with serum bilirubin levels of 430 µmol/L with 48 – 72 hours of life (A.A.o.P., 1994; Jansen and Bittar, 2004). In 1950s, exchange transfusion was the primary treatment for neonatal hyperbilirubinaemia (Watchko, 2005). Similar to phototherapy treatment, exchange transfusion can cause some complication such as vasospasm, thrombocytopenia, electrolyte imbalance, infection and even death.

1.8.3 Pharmacological therapies

Intravenous immunoglobulin (IVIG), phenobarbital and ursodeoxycholic acid was used in order to reduce the bilirubin concentration, improve bile flow and enhance enzyme activity that involves in the conjugation and excretion of bilirubin (Stern *et al*., 1970; Dennery, 2002). As compared to all medicine, phenobarbital is the most common drug used to treat neonatal hyperbilirubinaemia. However, phenobarbital may alter the oxidation properties of bilirubin and increase the risk of occurrence of kernicterus (Hansen and Tommarello, 1998; Kaplan and Hammerman, 2002).