# ASSOCIATION OF GENETIC POLYMORPHISMS OF *DIPEPTIDYL PEPTIDASE- 4* WITH TYPE 2 DIABETES MELLITUS IN MALAYSIAN SUBJECTS

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**KUALA LUMPUR**

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# ABSTRACT

The *DPP4* gene encoding for dipeptidyl peptidase-IV *(*DPP-IV*)*; also known as CD26, degrades glucagon-like peptide (GLP)-1 and is widely known for its regulatory effects in glucose metabolism. Elevated levels of soluble circulating form (sCD26/DPP-IV) are associated with an increased risk of developing metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM). *DPP4* gene polymorphisms are thought to be genetic determinants of increased sCD26/DPP-IV; this may be a risk factor for T2DM. The aim of this study was to investigate the fasting serum levels of sCD26/DPP-IV and active GLP-1 in Malaysian subjects with T2DM (with and without MetS), as well as the associations between sCD26/DPP-IV levels with MetS and anti-diabetic therapy. Furthermore, this study was carried out to evaluate the association of genetic polymorphisms of *DPP4* in Malaysian subjects with T2DM and to evaluate whether they had an effect on serum levels of sCD26/DPP-IV. The sCD26/DPP-IV levels, active GLP-1 levels, body mass index (BMI), glucose, insulin, A1C, glucose homeostasis indices, and lipid profiles at fasting state were assessed in 314 T2DM subjects (227 with MetS and 76 without MetS), 71 non-diabetic subjects with MetS subjects and 164 normal subjects without diabetes or MetS. The results obtained were analyzed with ANOVA, univariate and linear regression models. 10 *DPP4* SNPs were genotyped, and their association with T2DM were evaluated by logistic regression controlled for age, gender and BMI. Fasting serum levels of sCD26/DPP-IV were significantly higher in T2DM patients with MetS ($p = 1.2\times10^{-4}$), and without MetS ($p = 0.015$) than in normal subjects. However, active GLP-1 levels were significantly lower in T2DM patients with MetS ($p = 0.020$) and without MetS ($p = 0.028$) than in normal subjects. In T2DM subjects, sCD26/DPP-IV levels were associated with significantly higher A1C levels ($p = 0.009$), but were significantly lower in patients using monotherapy with metformin ($P = 0.041$). Furthermore, sCD26/DPP-IV levels were negatively correlated with active GLP-1 levels in T2DM patients with MetS

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($p < 0.001$), and without MetS ($p < 0.001$). This study revealed that obese non-diabetic subjects had reduced serum GLP-1 levels and elevated sCD26/DPP-IV levels with positively associated with insulin resistance. In normal subjects, sCD26/DPP-IV levels were associated with increased BMI ($p = 0.023$), cholesterol ($p = 0.001$), and LDL-cholesterol (LDL-c) levels ($p = 0.001$). Association analysis data of *DPP4* polymorphisms with T2DM in Malaysian subjects, showed that rs12617656 was significantly associated with T2DM in the recessive genetic model (OR = 1.98, $p = 0.006$), dominant model (OR = 1.95, $p = 0.008$), and additive model (OR = 1.63, $p= 0.001$). This association was more pronounced among Malaysian Indians, recessive (OR = 3.21, $p = 0.019$), dominant OR = 3.72, $p = 0.003$) and additive model (OR = 2.29, $p = 0.0009$). The additive genetic model showed that *DPP4* rs4664443 and rs7633162 polymorphisms were associated with T2DM (OR = 1.53, $p = 0.039$), and (OR = 1.42, $p = 0.020$), respectively. On the other hand, the rs4664443 G>A polymorphism was associated with increased sCD26/DPP-IV levels ($p = 0.042$) in T2DM subjects.

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**ABSTRAK**

Gen *DPP4* mengekod Dipeptidyl peptidase- 4 (*DPP4*); yang juga dikenali sebagai CD26, yang merendahkan glucagon seperti peptida (GLP) -1 dan terkenal dengan kesan regulatorinya ke atas metabolisme glukosa. Peningkatan tahap peredaran yang (sCD26/DPP-IV) larut dikaitkan dengan peningkatan risiko mendapat sindrom metabolik (MetS) dan diabetes melitus jenis 2 (T2DM). Polimorfisme gen *DPP4* dihipotesiskan sebagai penentu genetik bagi peningkatan sCD26/DPP-IV dan mungkin menjadi faktor risiko untuk T2DM. Tujuan kajian ini adalah untuk mengkaji paras puasa serum sCD26/DPP-IV dan aktif GLP-1 dalam subjek-subjek T2DM Malaysia dengan dan tanpa MetS, serta perkaitan di antara paras sCD26/DPP-IV, MetS dan terapi antidiabetik. Kajian ini juga dijalankan untuk menilai perkaitan polimorfisme genetik *DPP4* subjek-subjek Malaysia dengan T2DM dan menilai sama ada ini mempunyai kesan ke atas tahap serum sCD26/DPP-IV. Paras sCD26/DPP-IV dan aktif GLP-1, indeks jisim badan (BMI), glukosa, insulin, A1C, indeks homeostasis glukosa dan profil lipid pada keadaan berpuasa dinilai pada 314 subjek T2DM (227 dengan MetS dan 76 tanpa MetS), 71 subjek bukan diabetes dengan MetS dan 164 subjek normal tanpa diabetes atau MetS. Keputusan yang diperolehi dianalisis dengan ANOVA, univariat dan regresi linear. sepuluh SNP *DPP4* telah digenotip dan perkaitan mereka dengan T2DM telah dinilai menggunakan analisis regresi logistik dengan mengawal faktor umur, jantina dan BMI. Paras puasa serum sCD26/DPP-IV adalah lebih tinggi di kalangan pesakit T2DM dengan Mets ($p = 1.2 \times 10^{-4}$) dan tanpa MetS ($p = 0.015$) berbanding subjek yang normal. Walau bagaimanapun, paras aktif GLP-1 adalah lebih rendah dalam pesakit T2DM dengan MetS ($p = 0.020$) dan tanpa MetS ($p = 0.028$) berbanding subjek yang normal. Dalam subjek T2DM, paras sCD26/DPP-IV dikaitkan dengan paras A1C yang jauh lebih tinggi ($p = 0.009$), tetapi adalah lebih rendah dalam pesakit yang menggunakan monoterapi metformin ($p = 0.041$). Tambahan pula, paras sCD26/DPP-IV berkorelasi negatif dengan paras aktif GLP-1

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dalam pesakit T2DM dengan MetS (*p* <0.001) dan tanpa MetS (*p* <0.001). Kajian ini membuktikan bahawa subjek tanpa diabetes yang obes mempunyai pengurangan serum GLP-1 dan peningkatan tahap sCD26/DPP-IV, dengan pengaitan positif bersama rintangan insulin. Dalam subjek yang normal, paras sCD26/DPP-IV telah dikaitkan dengan peningkatan BMI (*p* = 0.023), paras kolesterol total (*p* = 0.001) dan kolesterol LDL (LDL-c) (*p* = 0.001). Analisis perkaitan data polimorfisme *DPP4* dengan T2DM dalam subjek-subjek Malaysia menunjukkan bahawa rs12617656 berkait secara signifikan dengan T2DM dalam model genetik resesif (OR = 1.98, *p* = 0.006), model dominan (OR = 1.95, *p* = 0.008) dan model tambahan (OR = 1.63, *p* = 0.001). Perkaitan ini adalah lebih ketara di kalangan kaum India Malaysia, model resesif (OR = 3.21, *p* = 0.019), dominan OR = 3.72, *p* = 0.003) dan tambahan (OR = 2.29, *p* = 0.0009). Model genetik tambahan menunjukkan bahawa polimorfisme *DPP4* rs4664443 dan rs7633162 dikaitkan dengan T2DM (OR = 1.53, *p* = 0.039) dan (OR = 1.42, *p* = 0.020). Sebaliknya, allele daripada rs4664443 dikaitkan dengan peningkatan paras serum sCD26/DPP-IV (*p* = 0.042) dalam subjek-subjek T2DM.

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# TABLE OF CONTENTS

Abstract ....... iii

Abstrak ....... v

Acknowledgements ....... vii

Table of Contents ....... viii

List of Figures ....... xiv

List of Tables ....... xv

List of Symbols and Abbreviations ....... xviii

List of Appendices ....... xxiv

**CHAPTER 1: INTRODUCTION ....... 1**

1.1 BACKGROUND ....... 1

1.2 STUDY DESIGN ....... 5

1.3 OBJECTIVES ....... 6

1.4 HYPOTHESIS ....... 6

1.5 OUTLINE OF THE THESIS ....... 7

**CHAPTER 2: LITERATURE REVIEW ....... 8**

2.1 DIABETES MELLITUS ....... 8

2.1.1 Historical Perspective of Diabetes ....... 8

2.1.2 Definition and Classification of Diabetes Mellitus ....... 9

2.1.3 Diagnostic Criteria for Diabetes and Metabolic Syndrome ....... 11

2.2 GLUCOSE HOMEOSTASIS AND PHYSIOLGY OF β-CELL FUNCTION ....... 15

2.2.1 Glucose Homeostasis and Glucose-Stimulated Insulin Secretion (GSIS). 15

University of Malaya

2.2.2 Insulin Biosynthesis and Role of β-Cell Function in the Development of T2DM ........ 18

2.2.3 Insulin Action Defects and Its Role in the Pathogenesis of T2DM ........ 21

2.3 METABOLIC SYNDROME ........ 23

2.3.1 Definition and Description of MetS ........ 23

2.3.2 Metabolic Syndrome and Its Role in the Development of T2DM ........ 23

2.3.3 Obesity and Its Role in the Development of T2DM ........ 24

2.3.4 Insulin Resistance and Its Role in the Development of T2DM ........ 25

2.4 GENETICS OF TYPE 2 DIABETES ........ 27

2.4.1 Genetic Variation and Genetic Background of T2DM ........ 27

2.4.2 Linkage Association Studies ........ 28

2.4.3 Candidate Gene Association Studies ........ 29

2.4.4 Genome-Wide Association Studies (GWAS) ........ 30

2.4.4.1 GWAS in Europeans ........ 30

2.4.4.2 GWAS in Non-Europeans ........ 31

2.4.4.3 Trans-ancestry GWAS Meta-Analysis ........ 32

2.5 INCRETINS SYSTEM ........ 37

2.5.1 Definition and Physiological Importance of the Incretin Effect ........ 37

2.5.2 Physiological Effects of Glucagon-Like Peptide 1 ........ 37

2.5.3 Role of the GLP-1 in the Pathogenesis of Obesity and T2DM ........ 39

2.6 *DIPEPTIDYLE PEPTIDASE- 4 (DPP4)* ........ 42

2.6.1 Definition and Genomic Organization ........ 42

2.6.2 Biology of DPP-IV and Molecular Function ........ 43

2.6.3 DPP-IV Expression and Its Alteration in T2DM ........ 45

2.6.4 Role of the Catalytic Function of DPP-IV in Inactivation of Incretins (GLP-1) 45

University of Malaya

2.6.5 Role of the Non-catalytic Interactions of DPP-IV in the Pathogenesis of Inflammation and T2DM ... 47

2.6.6 Linkage of the DPP-IV with T2DM and MetS ... 48

2.6.7 Inhibition of DPP-IV Function in T2DM ... 49

2.6.8 GENETICS of *DPP4* ... 51

**CHAPTER 3: METHODOLOGY ... 53**

3.1 BIOCHEMICAL TEST REAGENTS ... 53

3.2 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) TEST REAGENTS 53

3.2.1 sCD26/DPP-IV Test Reagents ... 53

3.2.2 Active GLP-1 Reagents ... 53

3.3 GENOTYPING ANALYSIS REAGENTS ... 54

3.3.1 General Reagents ... 54

3.3.2 DNA Gel Electrophoresis Reagents ... 54

3.3.3 Genotyping Reagents ... 54

3.4 SUBJECTS AND DATA COLLECTION ... 54

3.5 CLINICAL MEASUREMENTS ... 55

3.6 SAMPLE PROCESSING ... 56

3.7 BIOCHEMICAL ANALYSIS ... 56

3.7.1 Measurement of Glucose (FPG) ... 56

3.7.2 Measurement of Glycosylated Haemoglobin (AIC) ... 57

3.7.3 Measurement of Lipid Profile ... 57

3.7.3.1 Measurement of Triglycerides (TG) ... 57

3.7.3.2 Measurement of Total Cholesterol (TC) ... 58

3.7.3.3 Measurement of HDLc and LDLc ... 58

3.7.4 Measurement of Insulin, Insulin Resistance and β-Cell Function ... 58

University of Malaya

3.8 ELISA ASSAY ... 59
3.8.1 Measurement of Serum sCD26/DPP-IV Levels ... 59
3.8.2 Measurements of Serum Active Glucagon-Like Peptide-1 (GLP-1) levels ... 60
3.9 GENETIC ANALYSIS ... 61
3.9.1 DNA Extraction ... 61
3.9.2 Genotyping of Single Nucleotide polymorphisms (SNPs) ... 62
3.9.2.1 Selection of Polymorphisms Markers ... 62
3.9.2.2 TaqMan SNP Genotyping Assay ... 62
3.9.3 Data processing ... 67
3.10 QUALITY CONTROL ... 69
3.11 STATISTICAL ANALYSIS ... 69

**CHAPTER 4: RESULTS ... 71**
4.1 DEMOGRAPHIC AND BIOCHEMICAL ANALYSIS ... 71
4.2 SERUM LEVELS OF SOLUBLE DEPEPTIDYLE PEPTIDASE 4 IN TYPE 2 DIABETES AND ASSOCIATION WITH METABOLIC SYNDROME AND ANTIDIABETIC AGENTS AMONG MALAYSIAN SUBJECTS ... 74
4.2.1 Prevalence of Metabolic Syndrome among Subjects with Type 2 Diabetes. ... 74
4.2.2 Comparison of Fasting Serum Levels of sCD26/DPP-IV and Active GLP-1 between Groups of Study. ... 75
4.2.3 Correlation between sCD26/DPP-IV Levels and Active GLP-1 Levels among Normal, Non-Diabetic Metabolic Syndrome, Type 2 Diabetes Mellitus Subjects with and without Metabolic Syndrome ... 77
4.2.4 Associations between sCD26/DPP-IV Levels with Metabolic Syndrome, and AIC among Type 2 Diabetes Mellitus Patients. ... 78

University of Malaya

4.2.5 Associations between sCD26/DPP-IV Levels and Antidiabetic Agents..79
4.2.6 Association of sCD26/DPP-IV Levels with Metabolic Syndrome and Type 2 Diabetes Mellitus Parameters among Normal Malaysian Subjects.......82
4.2.7 Active GLP-1 Levels and sCD26/DPP-IV in Obese Non-Diabetic Subjects and their Association with Insulin Resistance.........................................83
4.3 GENETIC ANALYSIS ..........................................................................86
4.3.1 Hardy-Weinberg Equilibrium Test for the *DPP4* SNPs Genotyped in Malaysian Subjects. ......................................................................86
4.3.2 Association of *DPP4* Polymorphism with Type 2 Diabetes Mellitus among Malaysian Subjects. ......................................................................88
4.3.3 Association of *DPP4* Polymorphism with Type 2 Diabetes Mellitus among Malaysian Ethnic Groups ......................................................................101
4.3.4 Association of *DPP4* SNPs Common Haplotypes with Type 2 Diabetes Mellitus among Malaysian Subjects......................................................111
4.3.5 Association of Genotype Groups in *DPP4* (SNPs) with CD26/DPP-IV Levels ..........................................................................................113

**CHAPTER 5: DISCUSSION ..........................................................................114**
5.1 DEMOGRAPHIC AND BIOCHEMICAL ANALYSIS....................................114
5.2 SERUM LEVELS OF SOLUBLE CD26/DEPEPTIDYLE PEPTIDASE 4 IN TYPE 2 DIABETES AND ASSOCIATION WITH METABOLIC SYNDROME AND ANTIDIABETIC AGENTS AMONG MALYSIAN SUBJECTS ............114
5.2.1 Prevalence of Metabolic Syndrome among Malaysian Subjects with Type 2 Diabetes. ..........................................................................................114
5.2.2 Association of sCD26/DPP-IV with Type 2 Diabetes Mellitus and Metabolic Syndrome ..................................................................115

University of Malaya

5.2.3 Association between Active GLP-1 Levels with Type 2 Diabetes Mellitus and Its Correlation with sCD26/DPP-IV Levels .......... 118
5.2.4 Associations between sCD26/DPP-IV Levels with Metabolic Syndrome, and AIC levels among Type 2 Diabetes Mellitus Patients .......... 120
5.2.5 Associations between sCD26/DPP-IV Levels and Antidiabetic Agents 120
5.2.6 Association of sCD26/DPP-IV Levels with Metabolic Syndrome and Type 2 Diabetes Mellitus Parameters among Normal Subjects. .......... 121
5.2.7 Active GLP-1 Levels and sCD26/DPP-IV Levels in Obese Non-Diabetic Subjects and their Association with Insulin Resistance. .......... 122
5.3 GENETIC ANALYSIS .......... 124
5.3.1 Genetic Analysis of *DPP4* .......... 124
5.3.2 Association of *DPP4* Polymorphisms with Type 2 Diabetes Mellitus among Malaysian Subjects .......... 125
5.3.3 Association of *DPP4* Polymorphisms with Type 2 Diabetes Mellitus among the Ethnic Groups .......... 128
5.3.4 Association of Haplotypes Block of *DPP4* SNPs with Type 2 Diabetes Mellitus among Malaysian Subjects .......... 129
5.4 SIGNIFICANCE OF FINDINGS .......... 129
5.5 FUTURE WORK .......... 130
5.6 LIMITATIONS OF THE STUDY .......... 130

**CHAPTER 6: CONCLUSION .......... 132**

References .......... 133
List of Publications and Papers Presented .......... 168
Appendix .......... 169

University of Malaya

## LIST OF FIGURES

Figure 2.1: Classification of diabetes based on the etiologic types and stages. .............. 11

Figure 2.2: Insulin exocytosis from β-cell ................................................................... 17

Figure 2.3: Primary structure of human preproinsulin. ................................................. 19

Figure 2.4: Signal transduction in insulin action. ......................................................... 22

Figure 2.5: Schematic representation of *DPP4* gene on chromosome 2 (2q24.2). ......... 42

Figure 2.6: Producing a soluble circulating form from membrane-bound DPP-IV ........ 44

Figure 3.1: TaqMan SNP genotyping assay protocol. .................................................. 64

Figure 3.2: TaqMan allelic discrimination output signals. ............................................ 68

Figure 4.1: Pairwise linkage disequilibrium among *DPP4* SNPs in Malaysian subjects. Values in the upper axis represent *DPP4* SNPs, while values in the left axis represent the $R^2$ value. ................................................................... 111

University of Malaya

**LIST OF TABLES**

Table 2.1: Criteria for the diagnosis of diabetes. .......... 12

Table 2.2: Categories of increased risk for diabetes (Prediabetes)* .......... 13

Table 2.3: Criteria for clinical diagnosis of the metabolic syndrome. .......... 14

Table 2.4: Summary of T2DM susceptibility gene loci for T2DM, among major ancestry groups, as confirmed by genome-wide association studies .......... 33

Table 3.1: Context sequence for primers and probe design .......... 65

Table 3.2: TaqMan genotyping mix per 10 µl reaction .......... 66

Table 3.3: PCR thermal cycler conditions .......... 66

Table 4.1: Demographic parameters among normal control, non-diabetes metabolic syndrome, type 2 diabetes mellitus with and without metabolic syndrome. 72

Table 4.2: Biochemical parameters among normal control, non-diabetes metabolic syndrome, type 2 diabetes mellitus with and without metabolic syndrome. 73

Table 4.3: Prevalence of metabolic syndrome among subjects with type 2 diabetes mellitus. .......... 74

Table 4.4: Prevalence of metabolic syndrome among Malaysian males and females. ... 74

Table 4.5: Comparison of fasting serum levels of sCD26/DPP-IV and active GLP-1 between normal, non-diabetic metabolic syndrome, type 2 diabetes mellitus subjects with and without metabolic syndrome and total type 2 diabetes mellitus. .......... 76

Table 4.6: Correlation between of fasting serum levels of sCD26/DPP-IV and active GLP-1 among normal, non-diabetic metabolic syndrome, type 2 diabetes mellitus subjects with and without metabolic syndrome. .......... 77

Table 4.7: Association of fasting serum levels of sCD26/DPP-IV with metabolic syndrome, and AIC among type 2 diabetes mellitus patients. .......... 78

Table 4.8: Comparison of fasting serum levels of sCD26/DPP-IV between combination therapy without metformin, combination therapy with metformin, monotherapy with metformin and non- treated among subjects with type 2 diabetes. .......... 80

Table 4.9: Association of fasting serum levels of sCD26/DPP-IV with antidiabetes medications groups among type 2 diabetes patients. .......... 81

University of Malaya

Table 4.10: Association of fasting serum levels of sCD26/DPP-IV with diabetic and metabolic parameters among normal subjects .......................................... 82

Table 4.11: Comparison of fasting serum levels of sCD26/DPP-IV and active GLP-1 between normal weight, overweight, and obese subjects .......................... 84

Table 4.12: Association of sCD26/DPP-IV levels with insulin resistance and other metabolic parameters among obese subjects ............................................ 85

Table 4.13: Association Hardy-Weinberg Equilibrium test for the *DPP4* SNPs genotyped in Malaysian subjects. ..................................................................... 87

Table 4.14: Association of *DPP4* rs12617656 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 89

Table 4.15: Association of *DPP4* rs4664443 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 91

Table 4.16: Association of *DPP4* rs7633162 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 93

Table 4.17: Association of *DPP4* rs1861978 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 95

Table 4.18: Association of *DPP4* rs2160927 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 96

Table 4.19: Association of *DPP4* rs1558957 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 97

Table 4.20: Association of *DPP4* rs17574 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus............................... 98

Table 4.21: Association of *DPP4* rs7608798 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............... 99

Table 4.22: Association of *DPP4* rs1014444 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus. ............. 100

Table 4.23: Association of *DPP4* rs12617656 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups ................................................ 102

Table 4.24: Association of *DPP4* rs6733162 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ............................................... 103

Table 4.25: Association of *DPP4* rs4664443 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ............................................... 104

University of Malaya

Table 4.26: Association of *DPP4* rs1861978 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ................................................ 105

Table 4.27: Association of *DPP4* rs2160927 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ................................................ 106

Table 4.28: Association of *DPP4* rs1558957 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ................................................ 107

Table 4.29: Association of *DPP4* rs17574 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ................................................ 108

Table 4.30: Association of *DPP4* rs7608798 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ................................................ 109

Table 4.31: Association of *DPP4* rs1014444 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups. ................................................ 110

Table 4.32: The estimated values of linkage equilibrium analysis between the *DPP4* SNPs in Malaysian subjects. ...................................................................... 112

Table 4.33: Association of rs4664443 polymorphism with sCD26/PP4 levels among the type 2 diabetes mellitus subjects ............................................................ 113

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| AIC | Glycosylated haemoglobin |
| AD | Anno domini |
| ADA | American diabetes association |
| ADA | Adenosine deaminase |
| AC | Adenylate Cyclase |
| ADP | Adenosine diphosphate |
| AGEs | Advanced glycation end products |
| ANOVA | Analysis of variance |
| APCs | Antigen-presenting cells |
| ApoB | Apolipoprotein B |
| ATP | Adenosine triphosphate |
| B | Regression coefficient |
| BMI | Body mass index |
| BP | Blood pressure |
| bp | Base pair |
| $Ca^{2+}$ | Calcium ion |
| CCL | Chemokine Ligand |
| CD26 | Cell surface antigen |
| CE | Cholesterol esters |
| cm | Centimeter |
| CO | Cholesterol oxidase |
| CAD | Coronary artery disease |
| CRP | C-reactive protein |
| CV | Coefficients of variation |

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| | |
|---|---|
| CVD | Cardiovascular disease |
| cAMP | Cyclic adenosine monophosphate |
| CXC-R4 | C-X-C motif receptor 4 |
| DBP | Diastolic blood pressure |
| DCCT | Diabetes control and complications trial |
| *DPP4* | Dipeptidyl peptidase- 4 gene |
| *DPP-IV* | Dipeptidyl peptidase- IV |
| DEA-HCI/AAP | Diethylaniline-HCI/4-aminoantipyrine |
| deCODE | Diabetes epidemiology: collaborative analysis of diagnostic criteria in Europe |
| DHAP | Dihydroxyacetone phosphate |
| DNA | Deoxyribonucleic acid |
| EASD | European association for the study of diabetes |
| ELISA | Enzyme Linked Immunosorbant Assay |
| EDTA | Ethylenediaminetetraacetic acid |
| FAs | Fatty acids |
| FAD | Food and Drug Administration |
| FDR | False Discovery Rate |
| FFAs | Free fatty acids |
| EGFR | Epidermal growth factor receptor |
| FPG | Fasting plasma glucose |
| G | Gram |
| G-3-P | Glycerol-3-phosphate |
| G-6-P | Glucose-6-phosphate |
| G-6-PDH | Glucose-6-phosphate dehydrogenase |
| GD | Gestational diabetes |

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| | |
|---|---|
| GHRF | Growth-hormone-releasing factor |
| GIP | Gastric inhibitory polypeptide |
| GK | Glucokinase |
| GIP | Gastric inhibitory polypeptide |
| GLM | General Linear Model |
| GSIS | Glucose-Stimulated Insulin Secretion |
| GLP-1 | Glucagon-like peptide 1 |
| GLP-1R | Glucagon-like peptide 1 receptors |
| GLUT2 | Glucose transporter 2 |
| GLUT4 | Glucose transporter 4 |
| GPO | Glycerol-3-phosphate-oxidase |
| G-CSF | Granulocyte colony stimulating factor |
| G-MSF | Granulocyte Macrophage stimulating factor |
| GWAS | Genome-wide association study |
| Hb | Haemoglobin E |
| HCL | Hydrochloric acid |
| HDL | High-density lipoprotein |
| HDLc | High-density lipoprotein cholesterol |
| HK | Hexokinase |
| HOMA-IR | Homeostasis model assessment-insulin resistance |
| HOMA-β | Homeostasis model assessment-β-cell function |
| HPO | Horseradish peroxidas |
| HWE | Hardy–Weinberg Equilibrium |
| ICA | Islet cell antibodies |
| IDF | International diabetes federation |
| IFG | Impaired fasting glucose |

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| | |
|---|---|
| indels | Insertion/deletions |
| IGF-II/M6P-R | Insulin-like growth factor II/mannose-6-phosphate receptor |
| IL- | Interleukin- |
| IRS | Insulin receptor substrates |
| kb | Kilo-base pair |
| kg | Kilogram |
| L | Liter |
| LD | Linkage disequilibrium |
| LDL | Low-density lipoprotein |
| LDLc | Low-density lipoprotein cholesterol |
| LMD | Division of laboratory medicine |
| LPL | Lipoprotein lipase |
| $m^2$ | Meter squared |
| MI | Myocardial infarction |
| MT | Melting Temperature |
| MetS | Metabolic syndrome |
| MGB | Minor groove binder |
| MODY | Maturity onset diabetes of the young |
| mRNA | Messenger ribonucleic acid |
| NADP | Nicotinamide-adenine dinucleotide phosphate |
| NADPH | Nicotinamide-adenine dinucleotide phosphate dehydrogenase |
| ng | Nano gram |
| NGSP | National glycohemoglobin standardization program |
| nm | Nano meter |
| NFQ | Non-fluorescent quencher |
| NTCs | No template controls |

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| | |
|---|---|
| NF | Nuclear factor |
| OGTT | Oral glucose tolerance test |
| OR | Odds' ratio |
| PCR | Polymerase chain reaction |
| PKA | Protein kinase A |
| POD | Peroxidase |
| PP | Pancreatic polypeptide |
| PI(3)K | Phosphatidylinositol-3'-kinase |
| RA | Rheumatoid Arthritis |
| RBC | Red blood cells |
| RLUs | Relative light units |
| RT | Room temperature |
| SGLT1 | Sodium-Glucose Transporter 1 |
| SBP | Systolic blood pressure |
| SBE | Single-base extension |
| sCD26/DPP-IV | Soluble Dipeptidyl peptidase IV |
| SNPs | Single nucleotide polymorphisms |
| SPSS | Social package of statistical science |
| SU | Sulfonylureas |
| T1DM | Type 1 diabetes Mellitus |
| T2DM | Type 2 diabetes Mellitus |
| TBE | Tris-Borate-EDTA |
| TC | Total cholesterol |
| TG | Triglycerides |
| TZD | Thiazolidinediones |
| TINIA | Turbidimetric inhibition immunoassay |

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| | |
|---|---|
| TNF- α- | Tumor necrosis factor alfa |
| UMMC | University Malaya Medical Centre |
| VAT | Visceral adipose tissue |
| VDCC | Voltage-dependent Ca2+ channels |
| VLDL | Very low-density lipoprotein |
| WBC | White blood cells |
| WHO | World health organization |
| WHR | Waist to hip ratio |
| α | Alpha |
| β | Beta |
| $r^2$ | Partial correlation coefficient |
| δ | Delta |

University of Malaya

## LIST OF APPENDICES

Appendix A: Information, Consent Form and Questionnaires for Subjects……. 172

Appendix D: Biochemical Analysis by Division of Laboratory Medicine of the UMMC ……………………………………………………………… 178

Appendix C: Genetic Analysis Reagents……………………………………… 182

Appendix D: Association of SNPs DPP4 with Metabolic Syndrome Parameters among Normal Malaysian Subjects……………………………… 185

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# CHAPTER 1: INTRODUCTION

## 1.1 BACKGROUND

Diabetes mellitus is a serious disease that is of major concern both regionally and globally, it is one of the leading causes of death in most countries (Zimmet *et al.*, 2014). Diabetes is the most prevalent metabolic disorder, estimated to affect 415 million people worldwide by the year 2015, representing of 8.5% of the world's adult population (20-79 years). This number is predicted to increase to 642 million people by the year 2040 (10% of the adult population) with the largest increase observed in middle and low-income countries (International Diabetes Federation, 2015). Type 2 diabetes mellitus (T2DM) accounts for at least 90% of global diabetes cases, the larger portion of them arising from developing countries (Rawal *et al.*, 2012). T2DM is one of the most prevalent conditions in Malaysia, has been rated as one of the Top 10 countries with the highest Prevalence (%) of diabetes in the adult populations (International Diabetes Federation, 2012). In 2011, it was estimated that 2.03 million or 11.66% of the Malaysian adult population has diabetes (International Diabetes Federation, 2011). In 2015, statistics revealed that this figure grew to 3.3 million cases, with a prevalence of 16.6% (International Diabetes Federation, 2015). The rapid increase in T2DM prevalence has been attributed to both lifestyle and environmental factors on the background of one's genetic predisposition to diabetes (Stančáková & Laakso, 2016). The two most common forms of diabetes are (i) Type 1 Diabetes Mellitus (T1DM), which is an autoimmune disorder caused by β cell destruction; accounts for only 5-10% of cases, and (ii) T2DM which is associated with obesity and accounts for roughly 90-95% of cases (American Diabetes Association, 2017). There are also less common types of diabetes, such as gestational diabetes (GD), and maturity onset diabetes of the young (MODY) (American Diabetes Association, 2016).

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T2DM is likely caused by a multitude of gene-environment and gene-gene interactions in addition to epigenetics (Franks, 2011; Kwak & Park, 2016; Stančáková & Laakso, 2016). The greatest risk factor for T2DM is obesity, to a lesser extent it can also be influenced by age, impaired glucose metabolism, unhealthy diet, physical inactivity, smoking, history of diabetes, GD, and ethnicity (Anderson *et al.*, 2003; Consortium, 2013; Forouzanfar *et al.*, 2015; Imamura *et al.*, 2015; Kollipara & Warren-Boulton, 2004; Ley *et al.*, 2014; van Tilburg *et al.*, 2001; Vazquez *et al.*, 2007; Willi *et al.*, 2007). Genetic susceptibility plays a pivotal role in the progression and/or development of T2DM (Consortium *et al.*, 2014; Morris *et al.*, 2012; Vazquez *et al.*, 2015). Many studies have observed that the incidence and prevalence of T2DM differs largely across ethnic groups (Diamond, 2003; Takeuchi *et al.*, 2009; Wong *et al.*, 2008). For example, the ethnic distribution of T2DM ranges from 50% among Arizona Pima Indians, 41% in Nauruans, 2% among European Caucasians and 1% in Chilean Mapuche Indians (Diamond, 2003). In the Asia and Pacific region, T2DM has reached epidemic levels (Chan *et al.*, 2009; Yoon *et al.*, 2006). Genetic susceptibility to T2DM appears to be greater in Asian people, with an earlier age of onset and a lesser degree of obesity (Yoon *et al.*, 2006). The South Asian population have been found to have a four-fold increased risk of T2DM than the European population (Chambers *et al.*, 2000; Ramachandran *et al.*, 2010). In Malaysia, the growth in T2DM incidence has been attributed to the consumption of a high-fat diet as well as progression towards western lifestyle habits and urbanization (Aguiree *et al.*, 2013).

An intermediate stage between diabetes and normal glucose tolerance, recognized as "impaired glucose tolerance" frequently precedes diabetes, and is associated with an increased risk for future Metabolic Syndrome (MetS), and diabetes (de Vegt *et al.*, 2001; Nathan *et al.*, 2007; Reaven, 1988; Tuomilehto *et al.*, 2001). MetS defined as a cluster of metabolic abnormalities such as abdominal obesity, elevated fasting plasma glucose

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(FPG) levels, high triglycerides (TG), low levels of high-density lipoprotein cholesterol (HDLc), and high blood pressure (BP) (Reaven, 1988). Complications associated with MetS has become a major public health challenge as it leads to a dramatic increase in the risk of cardiovascular disease (CVD), T2DM, including dyslipidemia, hypertension, hyperglycemia and obesity (Alberti *et al.*, 2009; Alberti *et al.*, 2005; Cornier *et al.*, 2008; International Diabetes Federation, 2006; Lorenzo *et al.*, 2007; Roger *et al.*, 2012). Mets confers a five-fold increase in the risk of T2DM and a two‑fold risk of CVD, over a period of 5‑10 years (Alberti *et al.*, 2009). Moreover, individuals with MetS have a 3‑ to 4‑fold risk of myocardial infarction, a 2‑ to 4‑fold augmented risk of stroke and a 2‑fold risk of mortality (Alberti *et al.*, 2005; Olijhoek *et al.*, 2004).

Most diseases are caused by complex interactions amongst multiple genes, lifestyle and environmental factors. Elucidation of these interactions will enable us to understand the genetic basis of T2DM (Langenberg *et al.*, 2014). In particular, understanding how genetic factors conferring resistance or susceptibility to T2DM affect the severity or progression of the disease. The completion of the human genome project has paved the way for a better understanding of the genetic basis of diseases and their pathogenesis. A major tool used to uncover the nature and pattern of disease-causing genes and/or single nucleotide polymorphisms (SNPs) within a gene are association studies (Lewis & Knight, 2012). SNP profiles are characteristically used to study the correlation between genetic mutation(s) and a particular disease. Association studies have provided a platform for a new assortment of analyses: candidate gene, linkage analyses and genome-wide association study (GWAS). Most disease loci have been confirmed via the GWAS approach. To date, ~ 100 susceptibility loci for T2DM have been identified and confirmed through GWAS in the human genome, with the majority of them identified in the European (Dupuis *et al.*, 2010; Mahajan *et al.*, 2014; Morris *et al.*, 2012; Saxena *et al.*, 2007; Sladek *et al.*, 2007; Unoki *et al.*, 2008; Voight *et al.*, 2010; Yamauchi *et al.*, 2010;

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Zeggini *et al.*, 2008), Asian (Cho *et al.*, 2012; Go *et al.*, 2014; Hara *et al.*, 2014; Imamura *et al.*, 2016; Kooner *et al.*, 2011; Tsai *et al.*, 2010; Unoki *et al.*, 2008; Yamauchi *et al.*, 2010), Sub-Saharan Africa (Adeyemo *et al.*, 2015), African American (M. C. Ng *et al.*, 2014; Palmer *et al.*, 2012), Arabian (Dajani *et al.*, 2015; Kifagi *et al.*, 2011), American (Below *et al.*, 2011; Waters *et al.*, 2010), and Mexican (Parra *et al.*, 2011) populations. However, the genetic basis and the mode of inheritance of the common T2DM risk loci remain unclear (Doria *et al.*, 2008; Permutt *et al.*, 2005).

One of the prominent candidate genes for T2DM is the *DPP4* gene. Located on chromosome 2q24.3, *DPP4* encodes for dipeptidyl peptidase-IV (DPP4-IV), also known as cell surface antigen CD26 (Wilson *et al.*, 2000). DPP-IV is a type II transmembrane protein; the catalytically active form of DPP-IV is liberated from the cell membrane in a process known as shedding, releasing its soluble form (sCD26/DPP-IV) as a source for the circulating DPP-IV activity in the serum/plasma (Cordero *et al.*, 2009; Lambeir *et al.*, 2003; Röhrborn *et al.*, 2015; Zilleßen *et al.*, 2016). Also, sCD26/DPP-IV in circulation originates from adipocytes and bone marrow cells (Lamers *et al.*, 2011; Wang *et al.*, 2014). DPP-IV is responsible for the catalytic degradation of N-terminal dipeptides of glucagon-like peptide (GLP-1), suggesting an important role in metabolism (Holst, 2007; Kim & Egan, 2008; Zilleßen *et al.*, 2016), and approved DPP-IV inhibitors are currently in use for the treatment of T2DM (Röhrborn *et al.*, 2015). DPP-IV also has a non-enzymatic function as a binding and signaling protein, with a role in inflammation and cardiovascular regulation (Zhong *et al.*, 2015).

The *DPP4* tagging SNPs in visceral adipose tissue (VAT) was found to be associated with DPP4 methylation and influenced mRNA abundance in subjects with severe obesity (Turcot *et al.*, 2011). A transcriptomic study discovered that *DPP4* gene expression in VAS of obese subjects with MetS was more than 1.86-fold in comparison to obese subjects without MetS, suggesting a strong association between the VAT DPP4

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overexpression and obesity-associated metabolic complications (Bouchard *et al.*, 2007). The genetic and ecological determinants of DPP4 expression are not completely understood. Nevertheless, DPP-IV appears to be multifactorial in nature, the impact of genetic factors cannot be excluded and there has been developing interest in studying the effects of genetic variation within the *DPP4* gene on sCD26/DPP-IV levels.

GWAS conducted on *DPP4* gene polymorphisms in obese individuals, for association with diabetes and obesity-related metabolic complications, described that SNPs rs7608798, rs17848915 and rs1558957 were associated with hyperglycaemia/diabetes, levels of triacylglycerol, and LDL-cholesterol in the initial stage of analysis. However, similar results failed to be replicated in a subsequent, more stringent stage of analysis (Bouchard *et al.*, 2009). Additionally, *DPP4* gene polymorphisms have been found to associated with a predisposition to myocardial infarction (MI) in European patients with atherosclerosis (Aghili *et al.*, 2012). At present, there has only been a handful of studies aiming to distinguish genetic polymorphisms of *DPP4* with T2DM (Röhrborn *et al.*, 2015). Furthermore, little is known about the associations between sCD26/DPP-IV levels, metabolic disorders and T2DM. Hence, the main focus of this study is to uncover possible associations between genetic polymorphisms within *DPP4* and sCD26/DPP-IV levels with T2DM in Malaysian subjects.

## 1.2 STUDY DESIGN

This hospital-based case-control study of 314 patients with T2DM and 315 non-diabetic control subjects was conducted at University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia.

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## 1.3 OBJECTIVES

The overall objective of this study was to investigate the possible association of genetic polymorphisms of DPP4 with T2DM and MetS in Malaysian subjects.

The specific aims of this study are:

1) To determine the fasting serum levels of sCD26/DPP-IV and active GLP-1 in Malaysian T2DM patients with and without MetS, as well as to evaluate the correlation between serum sCD26/DPP-IV levels and active GLP-1 levels.
2) To investigate the associations of sCD26/DPP-IV levels with MetS parameters in both normal and T2DM subjects, as well as with anti-diabetic agents in T2DM.
3) To determine the associations of *DPP4* SNPs with T2DM patients with and without MetS and estimated whether there had a link with the sCD26/DPP-IV levels.
4) To determine the possible haplotypes and diplotypes in the above loci and study their associations with T2DM.
5) To determine the associations of *DPP4* SNPs with T2DM among the three main Malaysian races (Malay, Chinese and Indian).

## 1.4 HYPOTHESIS

1) The serum levels of sCD26/DPP-IV and active GLP-1 are associated with T2DM with or without MetS.
2) The serum levels of sCD26/DPP-IV are associated with anti-diabetic agents, and insulin resistance and other MetS parameters which are risk factors for developing T2DM.
3) The frequency of the *DPP4* SNPs and their genetic polymorphisms are associated with T2DM, and influence the serum sCD26/DPP-IV levels.
4) The association of the above loci SNPs, and haplotypes are associated with T2DM groups.

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## 1.5 OUTLINE OF THE THESIS

This thesis is presented in the six parts. The thesis commences with the project background, study design, objectives, hypothesis, and outlines of the study. Secondly, the literature review of the study is introduced, followed by an in depth outline of methodology employed. The major findings of the study are subsequently described trailed by a discussion pertaining to the results, significance of the study, future work and limitations of the study. The conclusion of the study is summarized in the final chapter preceding the bibliography. The appendices include additional information, consent form, questionnaire for subjects, as well as supportive methodologies and results.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1 DIABETES MELLITUS

### 2.1.1 Historical Perspective of Diabetes

Diabetes is probably one of the oldest diseases; its first written reference was by ancient Egyptian physicians 3000 years ago describing how the disease leads to frequent urination (polyuria) and rapid loss of body weight (Ahmed, 2002). The term diabetes (passing through) was coined by Araetus (81-133AD), and the word mellitus (honey sweet) was incorporated by Thomas Willis in 1675. Ibn Sina (980-1037 AD) known in the West as Avicenna, attributed the sweet taste of diabetic urine to the passage of absorbed water and nutrients unchanged into urine (Ahmed, 2002; Eknoyan & Nagy, 2005). In 1776, existence of excess sugar in blood and urine was first substantiated as the reason for their sweetness (Ahmed, 2002).

In 1889, Minkowski and Mering identified a crucial role of the pancreas in the pathogenesis of diabetes. Subsequently, the isolation of insulin by Frederick Banting and Charles Best in 1921 and its clinical application in dogs with diabetes to reduce glucosuria substantiated the theory. In 1936, based on the action and sensitivity of insulin, Harold Himsworth made a distinction between types of diabetes: Type 2 Diabetes 'non-insulin sensitive' and Type 1 Diabetes 'insulin sensitive'(Himsworth, 1936; Nwaneri, 2015). In 1955, the first orally hypoglycemic agent was advertised following successful trials (Ahmed, 2002; Rosenfeld, 2002).

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### 2.1.2 Definition and Classification of Diabetes Mellitus

Diabetes is a progressive disease that is characterised by hyperglycaemia, which occurs either when the body cannot efficiently utilize the insulin it produces, or when there is inadequate insulin production by the pancreas (World Health Organization, 1999, 2016). Uncontrolled diabetes causes serious damage to the kidneys, blood vessels, heart, eyes and nerves (World Health Organization, 2016). These complication occurs more frequently in diabetic patients with chronic hyperglycemia, resulting in failure and/or dysfunction of blood vessels, eyes, heart, nerves and kidneys (American Diabetes Association, 2014).

Since 1965, the World Health organization (WHO) has implemented rules for the classification and diagnosis of diabetes. In 2005, a joint WHO and International diabetes federation (IDF) Technical Advisory Group met in Geneva to review and upgrade the present WHO guidelines (World Health Organization, 2006). The revised classification was based on the aetiology versus insulin requirement and degree of glycaemia (Figure 2.1) (American Diabetes Association, 2014; World Health Organization, 1999).

T1D results from an absolute deficiency of insulin caused by β-cell destruction (American Diabetes Association, 2017). In fact, this type of diabetes previously known as insulin-dependent diabetes or juvenile-onset diabetes, develops in children and young adults, has a strong genetic component, and transpires primarily through the HLA complex that result in an autoimmune reaction against β-cells. However, the mechanism that initiates the onset of the disease remains generally unclear (Daneman, 2006; Noble *et al.*, 2010). Two forms of T1D are recognized: type 1A, caused by a cell-intervened immune system assault on β cells (Devendra *et al.*, 2004; Lambert *et al.*, 2004; Redondo *et al.*, 2000), and type 1B, far less common, has no known cause, and generally affects people of African and Asian descent (Abiru *et al.*, 2002).

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T2DM is a metabolic disorder characterized by hyperglycemia and modified lipid metabolism, attributed to the failure of β-cells to secrete adequate insulin on the background of insulin resistance caused by obesity/overweight, inactivity and over-nutrition. Metabolic defects that contribute to the development of T2DM involve disability of β-cells to compensate for excessive glucose levels that are associated with reduced incretin response, increased glucagon secretion, excess food intake, inflammation of adipose tissue, increased endogenous glucose production, the improvement of peripheral insulin resistance and hypoadiponectinaemia (Nolan *et al.*, 2011; Wu *et al.*, 2015). Chronic fuel excess is the essential pathogenic status that drives the progress of T2DM in epigenetically and genetically susceptible individuals (DeFronzo, 2009; Prentki & Nolan, 2006). This form of diabetes, referred to as adult-onset diabetes or non-insulin-dependent diabetes, accounts for ~90–95% of all diabetes (American Diabetes Association, 2017).

There are other types of diabetes with dissimilar origins e.g., diseases of the exocrine pancreas (such as cystic fibrosis), monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]) and drug- or chemical-induced diabetes (such as in the treatment of HIV/AIDS or after organ transplantation) (American Diabetes Association, 2016). GD is characterised by any degree of glucose intolerance that is not clearly overt diabetes, occurring before pregnancy or continued post-pregnancy (American Diabetes Association, 2017).

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| Stages / Types | Normoglycemia | Hyperglycemia | | | |
|---|---|---|---|---|---|
| | | | Diabetes Mellitus | | |
| | Normal glucose tolerance | Impaired glucose tolerance or impaired fasting glucose (Prediabetes) | Insulin independent | Insulin required for management | Insulin required for survival |
| Type 1 Diabetes | | | | | |
| Type 2 Diabetes* | | | | | |
| Other specific types** | | | | | |
| Gestational Diabetes** | | | | | |

**Figure 2.1: Classification of diabetes based on the etiologic types and stages.**
*Even after presenting in ketoacidosis, these patients can briefly return to normoglycemia without requiring continuous therapy (i.e., "honeymoon" remission); **in rare instances, patients in these categories (e.g., Vacor toxicity, type 1 diabetes presenting in pregnancy) may require insulin for survival. Source: Adapted from American Diabetes Association (2014).

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### 2.1.3 Diagnostic Criteria for Diabetes and Metabolic Syndrome

For a long time, the diagnosis of diabetes was acknowledged based on plasma glucose, either FPG or 2-h plasma glucose (2-h PG) value in the 75g oral glucose tolerance test (OGTT) (American Diabetes Association, 2010). In 2008, an International Expert Committee with members appointed by the IDF, European Association for the Study of Diabetes (EASD) and American Diabetes Association (ADA) recommended the use of the glycosylated haemoglobin (A1C) test to diagnose diabetes, with a threshold of 6.5% (American Diabetes Association, 2010; The International Expert Committee, 2009). Under their recommendation, diabetes may be diagnosed based on specific diagnostic

criteria listed in (Table 2.1) (American Diabetes Association, 2017; The International Expert Committee, 2009).

**Table 2.1: Criteria for the diagnosis of diabetes.**

| Parameter | Unit |
|---|---|
| FPG | ≥ 126 mg/dl (7.0 mmol/l): Fasting is defined as no caloric intake for at least 8 h. |
| | or |
| 2–h plasma glucose | ≥ 200 mg/dl (11.1mmol/l) during an OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.* |
| | or |
| AIC | ≥ 6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.* |
| | or |
| Random plasma glucose | ≥ 200 mg/dl (11.1 mmol/l). In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasmaglucose. |

*In the absence of unequivocal hyperglycemia, results should be confirmed by repeat testing.
Adapted from American Diabetes Association (2017).

In 1997 and 2003, the Expert Committee on the Diagnosis and Classification of DM (Gavin III *et al.*, 1997; Selvin *et al.*, 2013) recognized a group of individuals whose glucose levels were too high to be considered normal but did not meet the criteria for diagnosis of diabetes. These individuals were described as 'Pre-diabetes', a term used to describe individuals with impaired fasting glucose (IFG) (FPG levels 5.6 – 6.9 mmol/l [100 –125 mg/dl]) and/or impaired (IGT) (2-h PG values in the OGTT of 7.8 –11.0 mmol/l [140 –199 mg/dl]), taking into consideration the IFG cut off at 110mg/dl (6.1mmol/l) according to WHO classification (Table 2.2) Notably, IGT and IFG in their

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own right should not be sighted as clinical entities but rather risk factors for diabetes and CVD (American Diabetes Association, 2017; The International Expert Committee, 2009).

**Table 2.2: Categories of increased risk for diabetes (Prediabetes)***

| **Parameter** | **Unit** |
|---|---|
| FPG | 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l) (IFG) |
| | or |
| 2–h plasma glucose | 140 mg/dl (7.8 mmol/L) to 199 mg/dl (11.0 mmol/l) (IGT) |
| | or |
| AIC | 5.7–6.4% (39–46 mmol/mol) |

*For all three tests, risk is continuous, extending below the lower limit of the range and becoming disproportionately greater at the higher ends of the range.
Adapted from American Diabetes Association (2017).

IFG and IGT are well recognised to be associated with obesity (particularly visceral or abdominal obesity), hypertension and dyslipidemia with extreme TG and/or low HDL cholesterol (American Diabetes Association, 2016), and these features are collectively recognised as MetS.

Based on these arguments, the criteria for clinical diagnosis of MetS is based on the existence of at least three of the following factors: elevated blood pressure, elevated FG, elevated TG, low HDL-c and central/abdominal obesity, as illustrated in Table 2.3 (Alberti *et al.*, 2009).

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**Table 2.3: Criteria for clinical diagnosis of the metabolic syndrome.**

| Measure | Categorical Cut Points |
|---|---|
| Elevated waist circumference* | Population-specific and Country-specific definitions |
| Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator†) | ≥150 mg/dL (1.7 mmol/L) |
| Reduced HDLc (drug treatment for reduced HDLc is an alternate indicator†) | Males:<40 mg/dL (1.0 mmol/L) Females: <50 mg/dL (1.3 mmol/L) |
| Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator) | Systolic ≥130 and/or<br>Diastolic ≥85 mm Hg |
| Elevated fasting glucose‡ (drug treatment of elevated glucose is an alternate indicator) | ≥100 mg/dL (5.6 mmol/L) |

HDLc indicates high-density lipoprotein cholesterol.
*It is recommended that the IDF cut points be used for non-Europeans and either the IDF or AHA/NHLBI cut points used for people of European origin until more data are available.
†The most commonly used drugs for elevated triglycerides and reduced HDLc are fibrates and nicotinic acid. A patient taking 1 of these drugs can be presumed to have high triglycerides and low HDLc. High-dose ω-3 fatty acids presumes high triglycerides.
‡Most patients with type 2 diabetes mellitus will have the metabolic syndrome by the proposed criteria.
Adapted from Alberti *et al.* (2009).

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## 2.2 GLUCOSE HOMEOSTASIS AND PHYSIOLGY OF β-CELL FUNCTION

### 2.2.1 Glucose Homeostasis and Glucose-Stimulated Insulin Secretion (GSIS).

Glucose homeostasis reflects a balance between glucose production from liver and glucose uptake by adipose tissue, liver, muscles (Kahn, 1992), and the central nervous system (Aronoff *et al.*, 2004). In physiologically healthy individuals, blood glucose levels is tightly controlled by interactions between gut (incretin hormones) and pancreatic hormones (insulin, glucagon, amylin) (Kruger *et al.*, 2016).

Pancreatic islets are comprised of several types of cells: (i) β-cells, which produce insulin (a hormone that promotes glucose disappearance from the circulation) and amylin hormone in lower levels, (ii) α-cells, which release glucagon (a hormone that increases glucose levels), (iii) pancreatic polypeptide (PP) cells, which secrete pancreatic polypeptide, and (iv) δ-cells, which produce somatostatin (Seino & Bell, 2008).

Glucose is the potent regulator of insulin secretion by pancreatic β-cells, although gastrointestinal peptides, amino acids, free fatty acid (FFA), ketones bodies, various nutrients and parasympathetic neural input, also exert effect on insulin secretion. Glucose levels >70 mg/dl (3.9 mmol/l) induce insulin synthesis, mainly by enhancing protein processing and translation. Glucose-stimulated insulin secretion from β- cells is regulated by two signalling pathways.

**(a)** The KATP channel dependent pathway "triggering pathway".

Begins with glucose entering the β-cell through the Glucose transporter 2 (GLUT2) across the plasma membrane and then rapidly phosphorylated by glucokinase (GK) (Buchs *et al.*, 1995). This is the rate-limiting step in insulin secretion (Baron *et al.*, 1988) and is subjected to glycolysis with further metabolism by which pyruvate is formed in the cytosol of the cell and passes into the mitochondria. The previous reaction ultimately generates adenosine

triphosphate (ATP), which is considered to be the main driver for insulin secretion, because it inhibits the activity of ATP-sensitive K+ channels. Closure of the K+ channels depolarizes the cell membrane, leading to the opening of voltage-dependent $Ca^{2+}$ channels (VDCC). $Ca^{2+}$ influx and escalated $Ca^{2+}$ concentration rapidly increases the rate of insulin exocytosis from the cell (Fauci *et al.*, 2008; Inagaki *et al.*, 1997) ( Figure 2.2).

**(b)** The KATP channel independent pathway "amplifying pathway"

Mediated by many factors including hormones [such as, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP)], non-glucose nutrients, and neural inputs (Komatsu *et al.*, 2013). GLP-1 and GIP (incretins), and glucagon enhance insulin secretion by activating secondary messenger pathways (Hui *et al.*, 2005; Vilsbøll & Holst, 2004). The secretory response increases under this pathway in the presence of elevated $Ca^{2+}$ concentrations (Henquin *et al.*, 2002) through increased cyclic adenosine monophosphate (cAMP) formation by activation of G-protein-coupled adenylate cyclase (AC) on the β-cell surface (Komatsu *et al.*, 2013). This pathway has been reported to influence exocytotic process and releases insulin from cells (Ozaki *et al.*, 2000) (Figure 2.2)

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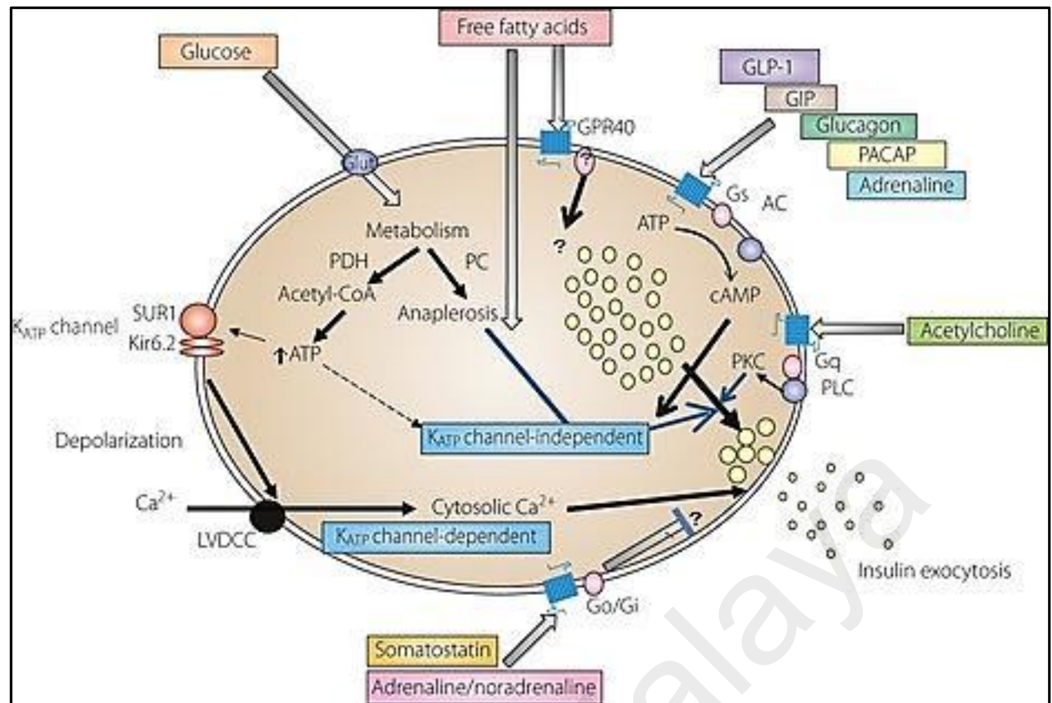


**Figure 2.2: Insulin exocytosis from β-cell**
ATP, adenosine triphosphate; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; GLP-1, glucagon-like polypeptide-1; GIP, glucose-dependent insulinotropic peptide; Glut, glucose transporter; KATP channel, K+ channel 6.2 subunits; adenosine triphosphate-sensitive K+ channel; Kir6.2, PACAP, pituitary adenylate cyclase activating peptide; LVDCC, L-type voltage-dependent calcium channel, PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PKC, protein kinase C; SUR1, sulfonylurea receptor 1, PLC, phospholipase C.
Adapted from Komatsu et al. (2013).

Insulin secretion is elevated within minutes of food ingestion due to the simultaneous release of gut peptide hormones, recognized as incretins (eg.GLP-1). Incretin plays a significant role in the maintenance of glucose homeostasis by regulating glucose levels within a relatively narrow range (Campbell & Drucker, 2013; Doyle & Egan, 2007), through its effect on the glucose-dependent insulin secretion, delayed gastric empting, and inhibiting glucagon release (Baggio & Drucker, 2007). It has been reported that incretin is responsible for at least 50% of insulin secretion after meal intake (Röhrborn *et al.*, 2015) and 60- 70% after oral glucose consumption in healthy individuals (Nauck *et al.*, 1986). Glucose stimulates GLP-1 secretion through cell depolarization via the sodium-glucose transporter 1 (SGLT1), which transports one glucose molecule together with two sodium ions during intracellular metabolism to ATP. This causes an influx of extracellular

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calcium from voltage-gated calcium channels (V-type) and activation of the exocytotic machinery (Gorboulev *et al.*, 2012; Parker *et al.*, 2012). For GIP, glucose seems to stimulate secretion by similar pathways (Gorboulev *et al.*, 2012).

Insulin is a key anabolic hormone which is considered to be the main regulator of both glucose absence and presence in the circulation. Insulin is constitutively secreted in response to raised levels of glucose and amino acids after food consumption and acts through insulin receptors present in target tissues including liver, fat, and muscle cells, primarily skeletal muscle. On the other hand, glucagon is a key catabolic hormone that plays a crucial role in glucose homeostasis through glycogenolysis and gluconeogenesis, and stimulates glucose production in liver. Amylin, a hormone that bind to amylin receptors, acts to complement the effects of insulin and suppresses glucagon secretion, which in turn influences the rate of glucose homeostasis (Aronoff *et al.*, 2004). There are also other hormones, such as catecholamines, growth hormone and cortisol, that counteract the effects of insulin (Mitrakou *et al.*, 1992).

### 2.2.2 Insulin Biosynthesis and Role of β-Cell Function in the Development of T2DM

The normal β-cells of the pancreatic islets of Langerhans are responsible for maintaining a narrow range of blood glucose levels in a strictly controlled manner via insulin secretion. It is secreted throughout the day at a basal rate and in response to stimulation by glucose, fatty acids and other nutrients. Insulin secreting β-cells constitute most part of islets cells (60–80%) in a normal pancreas, followed by α-cells (20–30%), δ-cells (5–15%) and PP cells. In addition to proinsulin and insulin, β-cells also secrete C-peptide (connecting peptide) and amylin (Gastaldelli, 2011; Poitout *et al.*, 2015).

Insulin consists of two polypeptide α and β chains, connected through disulfide bonds and the C-peptide. The biosynthesis of insulin begins from the precursor molecule

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preproinsulin and the final post-translational modification occurs when the C-peptide is cleaved from the proinsulin to produce insulin by proteolytic enzymes, exoprotease carboxypeptidase E and prohormone convertases (PC1 and PC2) (Figure 2.3) (Poitout *et al.*, 2015). The mature insulin and C-peptide are stored in secretory vesicles in the β-cells which are subsequently released into the portal vein upon metabolic demand in a pulsatile manner (Fu *et al.*, 2013).

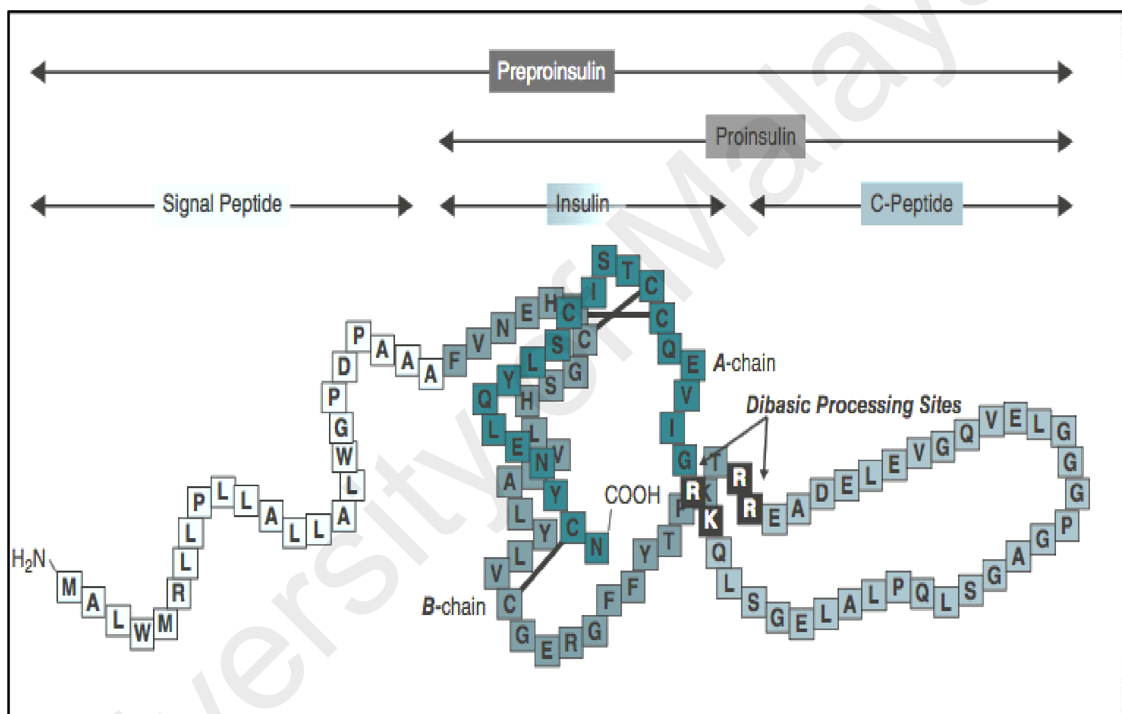


**Figure 2.3: Primary structure of human preproinsulin.**
Adapted from Poitout et al. (2015).

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In the fasting state, the rate of insulin secretion increases with the level of insulin resistance. As glucose tolerance deteriorates and T2DM develops, basal insulin secretion increases as well. T2DM develops when the β-cell secretory capacity is not sufficient to overcome the insulin resistance of the tissues (Gastaldelli, 2011).

β-cell dysfunction plays a critical role in the transition from normal glucose tolerance to hyperglycaemia (DeFronzo, 2009). This could be attributable to insufficient glucose sensing to stimulate-insulin secretion and consequently higher glucose levels predominate. Persistent raised glucose levels above the maximum physiological range continuously causes the appearance of hyperglycaemia (Cerf, 2013), and plays a key role in the transition from normal glucose tolerance to hyperglycaemia .

It is clear that modifications in β-cell characteristics might cause defective insulin secretion and development of T2DM (Ashcroft & Rorsman, 2012). In certainty, β-cell dysfunction frequently precedes the appearance of insulin resistance (Turner *et al.*, 1988). The exact mechanisms responsible for β-cell impairment remains uncertain, but failure to respond to glucose, glucose uptake abnormalities, reduced β-cell mass, and defective exocytotic machinery are considered as the main mechanisms for β-cell dysfunction (Ashcroft & Rorsman, 2004; Biden *et al.*, 2004). Furthermore, β-cell mass is affected by a balance between pro-apoptotic and proliferative signals, and modulated by numerous hormones, cytokines and growth factors. It has also been shown that high levels of glucose and FFA, inflammatory cytokines and deposition of islet amyloid polypeptide are classically implicated in β-cell apoptosis (Folli *et al.*, 2011; Guardado-Mendoza *et al.*, 2013; Jurgens *et al.*, 2011; Tripathy *et al.*, 2013).

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### 2.2.3 Insulin Action Defects and Its Role in the Pathogenesis of T2DM

When insulin is secreted into the portal venous system, approximately 50% is catabolized in the liver. The non-extracted insulin subsequently enters the systemic circulation and binds to specific insulin receptors (IRS) of target tissues. This leads to auto-phosphorylation, whereby phosphorylation of cellular proteins including Shc, Cbl, and IRS family member ensues. In turn, interaction with signalling molecules containing SH2 and PH domains lead to activation of diverse series of signal transduction pathways such as of phosphatidylinositol-3'-kinase PI(3)K and downstream PtdIns(3,4,5)P3-dependent protein kinases, Cbl/CAP, ras and the MAP kinase cascade. These pathways promote both mitogenic and metabolic effects (Figure 2.4) (Saltiel & Kahn, 2001).

For instance, activated PI(3)K stimulates translocation of glucose transporters (GLUT4) to the plasma membrane (Avruch, 1998). The regulation of glucose transport is considered to be the rate-limiting step in glucose utilization and storage (Pessin & Saltiel, 2000). Likewise, activation of other insulin signalling pathways can lead to the induction of protein synthesis, lipogenesis, glycogen synthesis, gene expression, and regulation of different genes in insulin-responsive cells (Saltiel & Kahn, 2001).

Insulin is required for maintenance of glucose homeostasis through reducing hepatic glucose output (by reduced glycogenolysis and gluconeogenesis) and enhancing glucose uptake and metabolism, mainly into adipose tissues and striated muscle. In fat and muscle cells, the clearance of glucose from circulation depends on the translocation of GLUT4 to the cell surface (Pessin & Saltiel, 2000; Shulman, 2000) . Also, insulin strongly influences lipid metabolism, decreasing free fatty acids (FFAs) release from TG in muscle and fat, and increasing lipid biosynthesis in fat and liver cells. Hence, insulin resistance transpires once normal circulating levels of the insulin are inadequate to regulate these processes properly (Pessin & Saltiel, 2000).



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Deficiencies in signal transduction may occur at various points with decreasing kinase activity. Changes to PI(3)K activity, phosphorylation of IRS-1 and -2, translocation of glucose transporters, and alterations in receptor binding and expression were considered to adversely influence or inhibit insulin action (Pessin & Saltiel, 2000). However, abnormal insulin action presents in most patients before T2DM is diagnosed (Gastaldelli, 2011), and insulin resistance in skeletal muscle is the main cause of impaired insulin action (Stefan *et al.*, 2016).

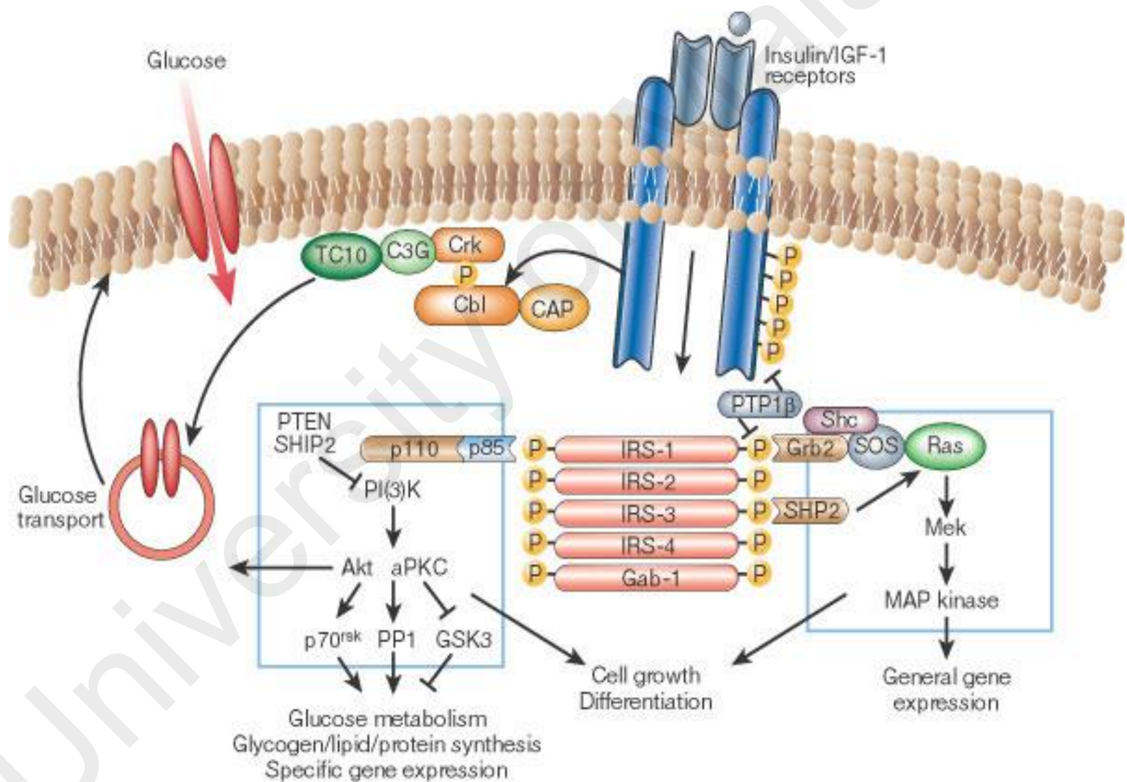


**Figure 2.4: Signal transduction in insulin action**.
Adapted from Saltiel and Kahn (2001).

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## 2.3 METABOLIC SYNDROME

### 2.3.1 Definition and Description of MetS

MetS was first described by Reaven in 1988, as "Syndrome X,"(Reaven, 1988). Obesity, in particular excess visceral adiposity, is associated with insulin resistance, dyslipidaemia, hyperglycaemia, and hypertension, which together are termed ''MetS'' (Alberti *et al.*, 2009). MetS is a growing problem worldwide and is associated with adverse health outcomes (Schlaich *et al.*, 2015). It appears that visceral adipose tissue is the most metabolically active (Tewari *et al.*, 2015). Adipose tissue dysfunction and insulin resistance are considered to be the basic mediators of MetS (Goossens, 2008; Lann & LeRoith, 2007).

Worldwide prevalence of MetS ranges from <10% to as much as 84% (Kaur, 2014). In the United States, 22.9% of adults have MetS (Beltrán-Sánchez *et al.*, 2013) while in other countries, it ranges from 65.3% Brazilian (de Carvalho Vidigal *et al.*, 2013), 22.7 % in Spain (Guallar-Castillón *et al.*, 2014), 33.5 % in Southern India (Prasad *et al.*, 2012), and in East Asia, ranging between 8% - 13% in men and 2% -18% in women (Pan *et al.*, 2008).

### 2.3.2 Metabolic Syndrome and Its Role in the Development of T2DM

The foundation of MetS is not fully comprehended, but central obesity and insulin resistance are considered to be important causative factors (Eckel *et al.*, 2005; Gallagher *et al.*, 2010). It has been suggested that the pathophysiology of MetS results from the failure of adipose tissue to buffer postprandial lipids (Laclaustra *et al.*, 2007). In addition, genetic factors play auxiliary roles in the pathogenesis of MetS through identification of variants, located generally in or near genes involved in lipid metabolism regulation (O'Neill & O'Driscoll, 2015; Stančáková & Laakso, 2014). Physical inactivity, such as sedentary lifestyle, aging, hormonal changes, and a proinflammatory state might be

serious too (Anderson *et al.*, 2001). It was demonstrated that those with MetS have a five times higher risk of developing T2DM and three times higher risk of developing CVD (O'Neill & O'Driscoll, 2015; Sookoian & Pirola, 2011).

### 2.3.3 Obesity and Its Role in the Development of T2DM

The current global prevalence of obesity has been doubled since the 1980s, and the number of overweight individuals have exceeded more than 1.9 billion adults, including ~600 million obese adults in 2014 (World Health Organization, 2015). Notably, Malaysia is considered to be one the most obese countries in Asia (Ng *et al.*, 2014). Moreover, it has been reported that the prevalence of overweight and obese women have increased from around 24.8% and 14.7%, respectively, in 2003 to 31.4% and 22.9%, respectively, in 2014. Among men, statistics reveal an increase from 28.6% and 9.7% in 2003 to 33.3% and 14.5%, respectively, in 2014 (Baharudin, 2015).

Substantial studies have revealed evidence that obesity is a main risk factor for developing T2DM (Mokdad *et al.*, 2003). The increase in obesity has been mirrored through the rise in the incidence of T2DM. It has been assessed that 60–90% of all individuals with T2DM are obese (Felber & Golay, 2002). The relative risk for an obese person to progress T2DM is 11.2-fold for men and 10-fold for women (Field *et al.*, 2001).

The causal links between obesity and T2DM include cellular processes, insulin resistance, disrupted FFAs metabolism and pro-inflammatory cytokines (Bano, 2013). Obesity has a strong association with hyperinsulinemia and glucose intolerance, eventually leading to T2DM (Gill *et al.*, 2011). Prolonged duration of obesity along with functional defects such as impairment of insulin secretion and β-cell dysfunction, these factors ultimately lead to the development of T2DM (Golay & Ybarra, 2005; Goldstein, 2002). Another defect underlying this progression is a 50% increase in β-cell mass caused by obesity (Kahn *et al.*, 2006).

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### 2.3.4 Insulin Resistance and Its Role in the Development of T2DM

Insulin resistance precedes the pathogenesis of numerous modern diseases. Therefore, understanding its pathogenesis has become increasingly critical to guide the development of economic strategies, inform health, and future therapies (Samuel & Shulman, 2012).

Insulin resistance signifies that insulin no longer exert its normal effects within target tissues, including adipose tissue, skeletal muscle, liver and pancreas; primary tissues involved in glucose metabolism (Laakso & Kuusisto, 2014). The progression from insulin resistance alone to IGT/IFG to overt T2DM is largely dependent on the relationship between insulin secretion and insulin resistance (DeFronzo, 2009). Hyperglycemia occurs when β-cell secretion is inadequate for the level of insulin resistance (Brufani *et al.*, 2010; D'Adamo & Caprio, 2011; Prentki & Nolan, 2006). It has been postulated that raising insulin resistance worsens previously impaired β-cells (Cali *et al.*, 2009; Taha *et al.*, 2006). However, abnormal insulin action presents in most patients before T2DM is diagnosed (Gastaldelli, 2011). Notably, insulin resistance in skeletal muscle is the main cause of impaired insulin action (Stefan *et al.*, 2016). Although the exact causes of insulin resistance have not been fully understood, it may be causally linked to genetic influences, and it could be associated with obesity, higher levels of FFAs, (Gastaldelli, 2011; Samuel *et al.*, 2004), and increased inflammation (Shoelson *et al.*, 2006). It has been hypothesized that reduced glucose uptake in adipocytes accelerates lipolysis and increases FFAs levels in tissues and plasma; this can potentially contribute to both hepatic and peripheral insulin resistance (Gastaldelli, 2011). Furthermore, long-term exposure to FFA, derived from lipolysis of fat depots or from diet, suppresses GSIS and has been proposed to reduce insulin biosynthesis, impaired glucose metabolism and β-cell loss (Poitout & Robertson, 2008; Yaney & Corkey, 2003). Persistently elevated levels of saturated FFAs in circulation, results in glucose and fatty acids competing for uptake and metabolism in

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tissues and with chronic hyperglycemia, augmented FFAs can induce a glucotoxic and lipotoxic state (Cerf, 2013).

Glucotoxicity, a condition of chronic hyperglycaemia, refers to the functional and structural damage in β-cells and primary target tissues of insulin (Donath & Halban, 2004). These alterations increases oxidative stress, consequently decreasing insulin secretion and biosynthesis (Cerf, 2013), contributing to aggravates insulin resistance (Kaneto *et al.*, 2004; LeRoith, 2002). Indeed, glucotoxicity is mediated through several aberrant glucose metabolic and signalling pathways (Kawahito *et al.*, 2009; Yan, 2014). It was found that aggravating insulin resistance by glucotoxicity is mediated in part, through down-regulation of the glucose transporter system (Kruszynska & Olefsky, 1996). Glucotoxicity is thus considered to be the key central factor in the pathophysiological events of hyperglycaemia leading to T2DM (LeRoith, 2002).

Lipotoxicity is second detrimental outgrowth of T2DM. It has numerous similarities to glucotoxicity, represent of the link between obesity and insulin resistance, and refers to the damage that results from the prolonged exposure to high FFAs levels in non-adipose tissue (Garbarino & Sturley, 2009). It has numerous similarities to glucotoxicity and similarly provides a link between obesity and insulin resistance. Elevated levels of FFAs cause insulin resistance in subjects with and without T2DM. This may occur in muscle cells owing to the suppression of insulin-stimulated glucose uptake because of disrupted phosphorylation, glucose transport, or both in cells (Boden, 2001).

Unlike genetic defects, insulin therapy can be administered to rectify these metabolic derangements (LeRoith, 2002). In addition, regular exercise and reduced energy intake through consuming a healthy diet improves insulin sensitivity (Weickert, 2012), and insulin resistance and/or obesity and β-cell dysfunction (Cerf, 2013).

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## 2.4 GENETICS OF TYPE 2 DIABETES

### 2.4.1 Genetic Variation and Genetic Background of T2DM

The term 'genetic variation' refers to both chromosomal alterations and aberrations in the DNA sequence among individuals. These variations may contribute to an individual's susceptibility to a particular disease (Bakhtadze, 2009). A variation that is present to some palpable degree within more than 1% in a population, it is called a polymorphism. In the human genome, the most common variations are known as single nucleotide polymorphisms (SNPs), which is a result of insertion/deletion or substitution of one or more single nucleotide base (A, C, G, and T) at a specific position in a DNA sequence. SNPs can contribute to a disorder by affecting a gene's function (Barreiro *et al.*, 2008; Lander *et al.*, 2001). The number of SNPs in the human genome is estimated to be ~10 million SNPs (Frazer *et al.*, 2007; Gabriel *et al.*, 2002). A haplotype is a cluster of SNPs on one chromosome is most likely to be inherited together. This knowledge is helpful in understanding the genetic origins of common diseases (Gibbs *et al.*, 2003; The International HapMap Consortium, 2005).

Identifying the genetic components of T2DM is pivotal to diabetes research because elucidation of diabetes-related genes (alleles) will improve our understanding of the disease, including the associated complications, possible treatments and preventative measures (Parchwani *et al.*, 2013). The genetic loci that have been identified represent approximately only 10% of T2DM heritability (Herder & Roden, 2011; McCarthy, 2010; Parchwani *et al.*, 2013). Uncovering the 'missing heritability' is fundamental to the development of comprehensive T2DM genetic studies and its translation into clinical practice (Parchwani *et al.*, 2013).

Evidence supporting the contribution of genetic factors in the development of T2DM has been well established (Herder & Roden, 2011). The evidence includes familial aggregation; considered as the main substantiation for genetic contribution in T2DM.

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This is so because the concordance rate in family and twin studies have shown to range from 26% to 69 % for T2DM (Almgren *et al.*, 2011; Poulsen *et al.*, 1999). There are also considerable differences in the prevalence of diabetes between racial groups based on ethnically-specific genetic variations (Diamond, 2003; Groop & Pociot, 2014), even if they share similar environments and familial aggregations for diabetes. Interestingly, it was reported that individuals with T2DM-affected siblings have a two to fourfold increased risk of developing T2DM compared to the general population (Hemminki *et al.*, 2010; Rich, 1990), and there is also a ~30-40% increased risk of T2DM in an offspring when one parent has diabetes, this is nearly doubled to 70% if both parents have diabetes (Meigs *et al.*, 2000).

Due to complex patterns of inheritance and environmental interactions, identification of genes implicated in T2DM is challenging. Monumental progress has been made through multiple approaches, including candidate gene association studies, linkage association studies and genome wide association studies. They have been applied to better understand the genetic architecture of complex diseases, including T2DM (Parchwani *et al.*, 2013; Stančáková & Laakso, 2016).

### 2.4.2 Linkage Association Studies

The linkage studies identifies genetic marker that strongly segregate with the disease within families (sibling pairs, parents- offspring) (Stančáková & Laakso, 2016). Linkage studies has been extremely successful in recognizing causative variants for monogenic disorders such as MODY (Vaxillaire & Froguel, 2006). Only two candidate genes for T2DM were reliably identified by linkage analysis, CAPN10 (calpain10) and TCF7L2. CAPN10, which is first T2DM gene identified by linkage analysis in a Mexican-American population (Horikawa *et al.*, 2000), was not confirmed in any GWAS to be related with T2DM. The second T2DM susceptibility locus, TCF7L2, was mapped on

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chromosome 10q in an Icelandic population (Grant *et al.*, 2006), and later confirmed in multiple GWAS in different ethnic groups, making it the best-replicated genetic association with T2DM to date (Tong *et al.*, 2009).

### 2.4.3 Candidate Gene Association Studies

The candidate gene approach has been the basis of a revolution in complex genetics (Ahlqvist & Groop, 2015). If there is a prior strong candidate gene for the disease based on their inferred physiological role, the best approach is to seek for association between SNPs in the gene and the disease. The objective in a case-control genetic association study is to clarify whether genetic variants occur more or less common in cases than controls (Parikh & Groop, 2004). Gene frequencies may be higher or lower regardless of how the allele is expressed, and it can change depending on the conditions that ensue. The Hardy-Weinberg Equilibrium (HWE) describes the frequency of genotypes at a given locus in a population, thus, frequently serves as a basis for genetic inference (Guo & Thompson, 1992). It can be used to discover the possible genotype frequencies in a population. In genetic association studies, SNP frequencies differ considerably between cases and controls if the studied SNP is implicated in the disease. A SNP associated with the disease might reside in linkage disequilibrium (LD) with other SNPs. In the genome, alleles at different loci are sometimes found together more or less. Hence, LD analysis is considered to be important in genetic association studies (Goldstein & Weale, 2001).

Although candidate gene studies have provided a large numbers of gene variants for T2DM-related traits, these findings have often not been replicated in other populations, partially attributed to the lack of statistical power (Stančáková & Laakso, 2016), small sample sizes, variation in environmental exposures, differences in T2DM susceptibility across ethnic groups and gene-environment interaction (Parchwani *et al.*, 2013).

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However, there were at least 2 gene namely, PPARG and KCNJ11,that have been discovered to be associated with T2DM across multiple studies (Stančáková & Laakso, 2016). The Pro12A1a substitution (rs1801282) of PPARG was first identified in 1997 (Yen *et al.*, 1997), found to be associated with T2DM and replicated in numerous studies involving meta-analysis of candidate studies (Altshuler *et al.*, 2000), and GWAS (Saxena *et al.*, 2007; Scott *et al.*, 2007; Zeggini *et al.*, 2007). The E23K polymorphism (rs5219, Gly23Lys) in KCNJ11 was first shown to be associated with T2DM in 1998 and was later substantiated by GWAS (Saxena *et al.*, 2007; Scott *et al.*, 2007; Zeggini *et al.*, 2007).

Several other genetic loci identified by candidate gene approach was later confirmed to be associated with T2DM by GWAS. These are IRS (Almind *et al.*, 1993), WFS1 (Minton *et al.*, 2002), HNFI and HNFIB (Bonnycastle *et al.*, 2006).

### 2.4.4 Genome-Wide Association Studies (GWAS)

#### 2.4.4.1 GWAS in Europeans

The first GWAS on T2DM was published in February 2007 and it revealed two novel T2DM-associated loci HHEX/IDE and SLC30A8 (Sladek *et al.*, 2007). A few months later, three global consortia published their own GWAS and they too reported these outcomes (Saxena *et al.*, 2007; Scott *et al.*, 2007; Zeggini *et al.*, 2007). Through cooperation, they confirmed the previously known T2DM loci of PPARG, TCF7L2, FTO and KCNJ11, and discovered SNPs in the intronic regions of IGF2BP2, and CDKAL1, as well as the intergenic region of CDKN2A/2B as novel T2DM loci. Simultaneously, other GWAS performed by DECODE researchers confirmed the CDKAL1, SLC30A8 and HHEX-IDE signals (Steinthorsdottir *et al.*, 2007).

In 2008, after the initial success of GWAS, there was a need to verify these novel genetic loci in a larger sample size. Accordingly, meta-analysis of GWAS applied on previously GWAS results, encompassing more than 10,000 subjects. They identified six

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novel loci associated with T2DM in/near NOTCH2, JAZF1, TSPAN8-LGR5, CDC123-CAMK1D, ADAMTS9, and THADA (Zeggini *et al.*, 2008). In 2009, GWAS reported association of two loci, MTNR1B and IRS1 with T2DM (Bouatia-Naji *et al.*, 2009; Lyssenko *et al.*, 2009; Rung *et al.*, 2009).

A second wave of T2DM GWAS in European populations was released in 2010; was completed by combining the data from 21 GWAS-Meta-analysis. It identified five novel susceptibility loci associated with T2DM: ADCY5, GCKR, GCK, PROX1, and DGKB-TMEM195 (Dupuis *et al.*, 2010). A large-scale meta-analysis conducted within the European population identified twelve novel association signals with T2DM (BCL11A, DUSP9, CHCHD9, KLF14, HNF1A, TP53INP1, ZBED3, KCNQ1, ZFAND6, HMGA2, CENTD2, and PRC1) (Voight *et al.*, 2010). Furthermore, a GWAS combining data from 11 independent GWAS identified a novel T2DM susceptibility locus, RBMS1, on chromosome 2q24 (Qi *et al.*, 2010). In 2012, large-scale meta-analyses of 25 studies in the European population identified a one additional T2DM locus (GATAD2A/CILP2/PBX4) (Saxena *et al.*, 2012). A meta-analysis conducted in 114,981 controls and 34,840 T2DM cases of European descent successfully identified eight novel T2DM associated loci, MC4R, ZMIZ1, ANKRD55, CILP2, TLE1, KLHDC5, ANK1, and BCAR1 (Morris *et al.*, 2012).

#### 2.4.4.2 GWAS in Non-Europeans

In 2008, the first GWAS in the East Asian population reported a variant in KCNQ1 that was associated with T2DM in Japanese, Chinese and Koreans (Unoki *et al.*, 2008; Yasuda *et al.*, 2008). In 2011, a GWAS was also performed in the South Asian population, identifying 6 novel loci for T2D in/near (GRB14, AP3S2, ST6GAL1, HMG20A, VPS26A, and HNF4A) (Kooner *et al.*, 2011). In 2012, the meta-analysis of GWAS performed on East Asians identified novel loci associated with T2DM in/ near GCC1-PAX4, GLIS3, PSMD6, PEPD, ZFAND, KCNK16, MAEA, FITM2-R3HDML-HNF4A



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(Cho *et al.*, 2012). Also, there was a reported SNP between RND3 and RBM43 as a T2DM susceptibility locus in African Americans (Palmer *et al.*, 2012). In 2013, two additional T2DM loci, TMEM163 and SGCG, were reported by two GWAS in South Asians (Saxena *et al.*, 2013; Tabassum *et al.*, 2012). 1n 2014, two new loci in CCDC63 and C12orf51 on the 12q24 region were identified in a Korean population (Go *et al.*, 2014). In addition, two novel loci, INS-IGF2 and HLA-B, were characterized in African Americans (Palmer *et al.*, 2014). In a GWAS of individuals of Latin American descent, a novel locus spanning SLC16A13 and SLC16A11 was found to be associated with T2DM (Williams *et al.*, 2014).

There are also other GWAS and meta-analysis studies that have been conducted in non-European cohorts, adding new loci to the list of genome-wide significant associations (Imamura *et al.*, 2012; Li *et al.*, 2013; Parra *et al.*, 2011; Shu *et al.*, 2010).

#### 2.4.4.3 Trans-ancestry GWAS Meta-Analysis

In 2014, to further detect novel T2DM-associated loci and to fine map the previously reported loci, a trans-ethnic GWAS meta-analysis across four major ethnic groups was conducted (European, South Asian, East Asian, and Mexican ancestry) (Mahajan *et al.*, 2014). 7 novel T2DM susceptibility loci was identified (MPHOSPH9, POU5F1/TCF19, TMEM154, SSR1/RREB1, ARL15, LPP, and FAF1), in addition to substantiating the 69 previously reported T2DM susceptibility loci (Mahajan *et al.*, 2014). These findings highlight the benefits of trans-ethnic GWAS to the discovery and description of novel T2DM susceptibility loci and to broaden insight into the pathogenesis and genetic architecture of diabetes and other complex diseases across diverse ethnic group (Mahajan *et al.*, 2014). The 83 susceptibility loci for T2DM identified by GWAS are summarized in (Table 2.4). Recently, a GWAS meta-analysis for T2DM in the Japanese population identified 7 new loci in/near CCDC85A, FAM60A, DMRTA1, ASB3, near ATP8B2, MIR4686 and INAFM2 (Imamura et al., 2016).

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**Table 2.4**: Summary of T2DM susceptibility gene loci for T2DM, among major ancestry groups, as confirmed by genome-wide association studies

| SNP | Genes | NCBI 38 Position | Chromosome | Risk allele | RAF | OR | P-Value | Reference |
|---|---|---|---|---|---|---|---|---|
| European | | | | | | | | |
| rs340874 | PROX1 | 213,985,913 | 1 | C | 0.50 | 1.07 | $4 \times 10^{-9}$ | (Dupuis *et al.*, 2010) |
| rs10923931 | NOTCH2 | 119,975,336 | 1 | T | 0.11 | 1.13 | $4 \times 10^{-8}$ | (Zeggini *et al.*, 2008) |
| rs7578597 | THADA | 43,505,684 | 2 | T | 0.90 | 1.15 | $1 \times 10^{-9}$ | (Zeggini *et al.*, 2008) |
| rs780094 | GCKR | 27,518,370 | 2 | C | 0.62 | 1.06 | $1 \times 10^{-9}$ | (Dupuis *et al.*, 2010) |
| rs2943641 | IRS1 | 226,155,937 | 2 | C | 0.61 | 1.19 | $9 \times 10^{-12}$ | (Rung *et al.*, 2009) |
| rs243021 | BCL11A | 60,357,684 | 2 | A | 0.46 | 1.08 | $3 \times 10^{-15}$ | (Voight *et al.*, 2010) |
| rs7593730 | RBMS1 | 160,314,943 | 2 | C | 0.83 | 1.11 | $4 \times 10^{-8}$ | (Qi *et al.*, 2010) |
| rs1801282 | PPARG | 12,351,626 | 3 | C | 0.92 | 1.14 | $6 \times 10^{-10}$ | (Saxena *et al.*, 2007) |
| rs4402960 | IGF2BP2 | 185,793,899 | 3 | T | 0.29 | 1.14 | $2 \times 10^{-9}$ | (Saxena *et al.*, 2007) |
| rs11708067 | ADCY5 | 123,363,551 | 3 | A | 0.78 | 1.12 | $1 \times 10^{-20}$ | (Dupuis *et al.*, 2010) |
| rs4607103 | ADAMTS9 | 64,726,228 | 3 | C | 0.81 | 1.09 | $1 \times 10^{-8}$ | (Zeggini *et al.*, 2008) |
| rs459193 | ANKRD55 | 56,510,924 | 5 | G | 0.70 | 1.08 | $6 \times 10^{-9}$ | (Morris *et al.*, 2012) |
| rs4457053 | ZBED3 | 77,129,124 | 5 | G | 0.26 | 1.08 | $3 \times 10^{-12}$ | (Voight *et al.*, 2010) |
| rs7754840 | CDKAL1 | 20,661,019 | 6 | C | 0.31 | 1.12 | $4 \times 10^{-11}$ | (Saxena *et al.*, 2007) |
| rs972283 | KLF14 | 130,782,095 | 7 | G | 0.55 | 1.07 | $2 \times 10^{-10}$ | (Voight *et al.*, 2010) |
| rs864745 | JAZF1 | 28,140,937 | 7 | T | 0.52 | 1.10 | $5 \times 10^{-14}$ | (Zeggini *et al.*, 2008) |
| rs2191349 | DGKB/TMEM195 | 15,024,684 | 7 | T | 0.47 | 1.06 | $1 \times 10^{-8}$ | (Dupuis *et al.*, 2010) |
| rs4607517 | GCK | 44,196,069 | 7 | A | 0.20 | 1.07 | $5 \times 10^{-8}$ | (Dupuis *et al.*, 2010) |
| rs896854 | TP53INP1 | 94,948,283 | 8 | T | 0.48 | 1.06 | $1 \times 10^{-9}$ | (Voight *et al.*, 2010) |

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*Table 2.4, continued.*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| rs13266634 | SLC30A8 | 117,172,544 | 8 | C | 0.75 | 1.12 | $5x10^{-8}$ | (Saxena *et al.*, 2007) |
| rs13292136 | TLE4 (CHCHD9) | 79,337,213 | 9 | C | 0.93 | 1.11 | $3x10^{-8}$ | (Voight *et al.*, 2010) |
| rs10811661 | CDKN2A/B | 22,134,095 | 9 | T | 0.79 | 1.20 | $8x10^{-15}$ | (Saxena *et al.*, 2007) |
| rs2796441 | TLE1 | 101,927,502 | 9 | G | 0.57 | 1.07 | $5x10^{-9}$ | (Morris *et al.*, 2012) |
| rs1111875 | HHEX/IDE | 92,703,125 | 10 | C | 0.56 | 1.13 | $6x10^{-10}$ | (Saxena *et al.*, 2007) |
| rs7903146 | TCF7L2 | 112,998,590 | 10 | T | 0.25 | 1.37 | $2x10^{-34}$ | (Saxena *et al.*, 2007) |
| rs12571751 | ZMIZ1 | 79,182,874 | 10 | A | 0.52 | 1.08 | $1x10^{-10}$ | (Morris *et al.*, 2012) |
| rs12779790 | CDC123/CAMK1D | 12,286,011 | 10 | G | 0.23 | 1.11 | $1x10^{-10}$ | (Zeggini *et al.*, 2008) |
| rs1552224 | CENTD2(ARAP1) | 72,722,053 | 11 | A | 0.88 | 1.14 | $1x10^{-22}$ | (Voight *et al.*, 2010) |
| rs5219 | KCNJ11 | 17,387,083 | 11 | T | 0.50 | 1.14 | $7x10^{-11}$ | (Saxena *et al.*, 2007) |
| rs10830963 | MTNR1B | 92,940,662 | 11 | G | 0.30 | 1.09 | $2x10^{-3}$ | (Dupuis *et al.*, 2010) |
| rs7961581 | TSPAN8/LGR5 | 71,269,322 | 12 | C | 0.23 | 1.09 | $1x10^{-9}$ | (Zeggini *et al.*, 2008) |
| rs7957197 | HNF1A | 121,022,883 | 12 | T | 0.85 | 1.07 | $2x10^{-8}$ | (Voight *et al.*, 2010) |
| rs1531343 | HMGA2 | 65,781,114 | 12 | C | 0.10 | 1.10 | $4x10^{-9}$ | (Voight *et al.*, 2010) |
| rs11063069 | CCND2 | 4,265,207 | 12 | G | 0.21 | 1.10 | $2x10^{-8}$ | (Mahajan *et al.*, 2014) |
| rs10842994 | KLHDC5 | 105,369,709 | 12 | C | 0.80 | 1.10 | $6x10^{-10}$ | (Morris *et al.*, 2012) |
| rs8042680 | PRC1 | 90,978,107 | 15 | A | 0.22 | 1.07 | $2x10^{-10}$ | (Voight *et al.*, 2010) |
| rs11634397 | ZFAND6 | 80,139,880 | 15 | G | 0.56 | 1.06 | $2x10^{-9}$ | (Voight *et al.*, 2010) |
| rs7202877 | BCAR1 | 75,213,347 | 16 | T | 0.89 | 1.12 | $4x10^{-8}$ | (Morris *et al.*, 2012) |
| rs8050136 | FTO | 53,782,363 | 16 | A | 0.45 | 1.17 | $1x10^{-12}$ | (Scott *et al.*, 2007) |
| rs4430796 | *HNF1B (TCF2)* | 37,738,049 | 17 | G | 0.53 | 1.10 | $2x10^{-11}$ | (Gudmundsson *et al.*, 2007) |
| rs12970134 | *MC4R* | 60,217,517 | 18 | A | 0.27 | 1.08 | $3x10^{-8}$ | (Morris *et al.*, 2012) |
| rs3794991 | *GATAD2A/CILP2/PBX4* | 19,499,787 | 19 | T | 0.08 | 1.12 | $6x10^{-9}$ | (Saxena *et al.*, 2012) |

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*Table 2.4, continued.*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| rs5945326 | *DUSP9* | 153,634,467 | X | A | 0.79 | 1.27 | $3x10^{-10}$ | (Voight *et al.*, 2010) |
| *East Asian* | | | | | | | | |
| rs6780569 | UBE2E2 | 23,294,959 | 3 | G | 0.83 | 1.17 | $7x10^{-9}$ | (Yamauchi *et al.*, 2010) |
| rs83157 | PSMD6 | 64,062,621 | 3 | C | 0.61 | 1.09 | $8x10^{-11}$ | (Cho *et al.*, 2012) |
| rs6815464 | MAEA | 1,316,113 | 4 | C | 0.58 | 1.13 | $2x10^{-20}$ | (Cho *et al.*, 2012) |
| rs1535500 | KCNK16 | 39,316,274 | 6 | T | 0.42 | 1.08 | $2x10^{-8}$ | (Cho *et al.*, 2012) |
| rs9470794 | ZFAND3 | 38,139,068 | 6 | C | 0.27 | 1.12 | $2x10^{-10}$ | (Cho *et al.*, 2012) |
| rs6467136 | GCC1/ PAX4 | 127,524,904 | 7 | G | 0.79 | 1.11 | $5x10^{-11}$ | (Cho *et al.*, 2012) |
| rs791595 | MIR129-LEP | 128,222,749 | 7 | A | 0.08 | 1.17 | $5x10^{-13}$ | (Hara *et al.*, 2014) |
| rs515071 | ANK1 | 41,661,944 | 8 | C | 0.81 | 1.18 | $1x10^{-8}$ | (Imamura *et al.*, 2012) |
| rs7041847 | GLIS3 | 4,287,466 | 9 | A | 0.41 | 1.10 | $2x10^{-14}$ | (Cho *et al.*, 2012) |
| rs17584499 | PTPRD | 8,879,118 | 9 | T | 0.07 | 1.57 | $9x10^{-10}$ | (Tsai *et al.*, 2010) |
| rs11787792 | GPSM1 | 136,357,696 | 9 | A | 0.87 | 1.15 | $2x10^{-10}$ | (Hara *et al.*, 2014) |
| rs10886471 | GRK5 | 119,389,891 | 10 | C | 0.79 | 1.12 | $7x10^{-9}$ | (Li *et al.*, 2013) |
| rs2237892 | KCNQ1 | 2,818,521 | 11 | C | 0.61 | 1.42 | $2x10^{-24}$ | (Yasuda *et al.*, 2008) |
| rs1359790 | SPRY2 | 80,143,021 | 13 | G | 0.71 | 1.15 | $6x10^{-9}$ | (Shu *et al.*, 2010) |
| rs7403531 | RASGRP1 | 38,530,704 | 15 | T | 0.33 | 1.10 | $4x10^{-9}$ | (Li *et al.*, 2013) |
| rs7172432 | C2CD4A/B | 62,104,190 | 15 | A | 0.59 | 1.12 | $4x10^{-14}$ | (Yamauchi *et al.*, 2010) |
| rs312457 | SLC16A13 | 7,037,074 | 17 | G | 0.08 | 1.20 | $8x10^{-11}$ | (Hara *et al.*, 2014) |
| rs391300 | SRR | 2,312,964 | 17 | G | 0.63 | 1.28 | $3x10^{-9}$ | (Tsai *et al.*, 2010) |
| rs3786897 | PEPD | 33,402,102 | 19 | A | 0.56 | 1.10 | $1x10^{-8}$ | (Cho *et al.*, 2012) |
| South Asian | | | | | | | | |
| rs6723108 | TMEM163 | 105,373,629 | 2 | T | 0.86 | 1.31 | $3x10^{-9}$ | (Tabassum *et al.*, 2012) |

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*Table 2.4, continued.*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| rs3923113 | GRB14 | 164,645,339 | 2 | C | 0.74 | 1.09 | 1x10$^{-8}$ | (Kooner *et al.*, 2011) |
| rs16861329 | ST64GAL1 | 186,948,673 | 3 | G | 0.75 | 1.09 | 3x10$^{-8}$ | (Kooner *et al.*, 2011) |
| rs1802295 | VPS26A | 69,171,718 | 10 | A | 0.26 | 1.08 | 4x10$^{-8}$ | (Kooner *et al.*, 2011) |
| rs9552911 | SGCG | 23,290,518 | 13 | G | 0.92 | 1.49 | 2x10$^{-8}$ | (Kooner *et al.*, 2011) |
| rs2028299 | AP3S2 | 89,831,025 | 15 | C | 0.31 | 1.10 | 2x10$^{-11}$ | (Kooner *et al.*, 2011) |
| rs7178572 | HMG20A | 77,454,848 | 15 | G | 0.52 | 1.09 | 7x10$^{-11}$ | (Kooner *et al.*, 2011) |
| rs4812829 | HNF4A | 44,360,627 | 20 | A | 0.29 | 1.09 | 3x10$^{-10}$ | (Kooner *et al.*, 2011) |
| African American | | | | | | | | |
| rs7560163 | RND3–RBM43 | 150,781,422 | 2 | C | 0.86 | 1.33 | 7x10$^{-9}$ | (Palmer *et al.*, 2012) |
| Hispanic/Latino | | | | | | | | |
| rs13342692 | SLC16A11 | 7,042,968 | 17 | C | 0.22 | 1.25 | 4x10$^{-13}$ | (Williams *et al.*, 2014) |
| Trans-ancestry | | | | | | | | |
| rs12454712 | BCL2 | 63,178,651 | 18 | T | 0.63 | 1.09 | 3x10$^{-6}$ | (Saxena *et al.*, 2012) |
| rs17106184 | FAF1 | 50,444,313 | 1 | G | 0.92 | 1.11 | 5x10$^{-4}$ | (Mahajan *et al.*, 2014) |
| rs6808574 | LPP | 188,022,735 | 3 | C | 0.79 | 1.08 | 6x10$^{-9}$ | (Mahajan *et al.*, 2014) |
| rs6813195 | TMEM154 | 152,599,323 | 4 | C | 0.59 | 1.08 | 3x10$^{-14}$ | (Mahajan *et al.*, 2014) |
| rs702634 | ARL15 | 53,975,590 | 5 | A | 0.76 | 1.08 | 7x10$^{-9}$ | (Mahajan *et al.*, 2014) |
| rs3130501 | POU5F1/TCF19 | 31,168,937 | 6 | G | 0.74 | 1.07 | 7x10$^{-10}$ | (Mahajan *et al.*, 2014) |
| rs9505118 | SSR1/RREB1 | 7,258,384 | 6 | A | 0.61 | 1.06 | 2x10$^{-4}$ | (Mahajan *et al.*, 2014) |
| *rs4275659* | MPHOSPH9 | 123,156,306 | 12 | C | 0.50 | 1.06 | 4×10$^{-4}$ | (Mahajan *et al.*, 2014) |

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## 2.5 INCRETINS SYSTEM

### 2.5.1 Definition and Physiological Importance of the Incretin Effect

The term “incretin” was coined in 1932 by La Barre to express a putative substance that is released from the upper gut mucosa, leading to glucose reduction without activating exocrine secretions (Creutzfeldt, 2005; Labarre, 1932). However, the notion that gut hormones lead to glucose regulation was only confirmed in the 1960s (Creutzfeldt, 2005). Eventually, in 1985 GLP-1 and its truncated form GLP-1(7-36) were identified as true incretins (Creutzfeldt, 2005).

The incretin effect refers to the phenomenon whereby oral glucose leads to higher insulin secretory responses compared with does intravenous glucose, in spite of inducing comparable levels of glycaemia, in normal subjects (Nauck & Meier, 2016). This effect, is caused by nutrient-stimulated (eg, glucose-stimulated) and mediated release of incretin hormones and their insulinotropic action on β-cells (Creutzfeldt, 1979; Drucker & Nauck, 2006). The incretin effect is carried out by GLP-1 and GIP (Holst, 2007; Vilsbøll & Holst, 2004), which contribute to at least 60% of insulin secretion in healthy individuals after glucose intake. GLP-1 implicated in delays gastric emptying and suppressing glucagon secretion (Holst, 2007). Indeed, it is clearly established influence in the maintenance of glucose homoeostasis, and incretin-based therapies may be considered as greatest promising new therapies for T2DM (Nauck & Meier, 2016).

### 2.5.2 Physiological Effects of Glucagon-Like Peptide 1

GLP-1 is a major member of the incretin hormone family, and secreted by L-cells from the epithelial layer of the intestinal mucosa within minutes of food ingestion. The two major biologically active form including GLP-1 (737) and GLP-1 (736 amide) (Baggio & Drucker, 2007; Campbell & Drucker, 2013). GLP-1 receptors (GLP-1R), composed of 463 amino acids (~63 kDa), are found in pancreatic islet β and α cells, heart, brain and

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lung. The widespread locations of GLP-1R reflect the many physiological roles of GLP-1 (Baggio & Drucker, 2007; Campbell & Drucker, 2013; Drucker, 2013; Lund *et al.*, 2011).

The primary function of GLP-1 is to regulate tissue sensitivity to/and manage secretion of insulin. Its effects have been attributed to increased glucose uptake in, liver, muscle and adipose tissue (Drucker & Nauck, 2006; Holst & Deacon, 2005; Mayo *et al.*, 2003). GLP-1 also suppresses glucagon secretion and/or activity as well as reducing postprandial glucose levels, appetite reduction, and delaying gastric emptying (Cho *et al.*, 2014; Madsbad, 2014; Wang *et al.*, 2015). It also aids in enhancing β-cell proliferation and survivability, and inhibits β-cell apoptosis (Buteau *et al.*, 2003; Doyle & Egan, 2007; Wang *et al.*, 2015). Recently, Meier and Nauck (2015) suggested that GLP-1 instigates changes in the composition of gut microbiome and bile acids.

Overall, the most recognized effect of GLP-1 is its effects on insulin secretion. β-cells express GLP-1R, which is linked to cAMP) via AC. cAMP stimulates either protein kinase A (PKA) or cAMP-regulated guanine nucleotide exchange factor II (Epac2), which in turn raises intracellular $Ca^{2+}$ levels. In addition, GLP-1 suppresses β-cell repolarization by diminishing $K^+$ channel flows in a cAMP/PKA-dependent pathway. These mechanisms augment glucose-induced insulin secretion initiated by the closure of $K^+$/ATP channels (Figure 2.2), (illustrated previously in insulin secretion part).

GLP-1 augments insulin biosynthesis, insulin gene transcription, inhibits β-cell apoptosis and promotes β-cell proliferation by phosphatidylinositol 3-kinase (PI3K). On the other hand, GLP-1 decreases glucagon secretion from α-cells, perhaps indirectly by switch-off signals from β-cells such as zinc and insulin (Cho *et al.*, 2014; Ding *et al.*, 2011; Gorboulev *et al.*, 2012; Jung *et al.*, 2014; Parker *et al.*, 2012).

GIP is synthesized and secreted from intestinal K cells, which is located mainly in the upper jejunum and duodenum (Campbell & Drucker, 2013; Mentis *et al.*, 2011). GIP

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receptors, expressed in the pancreas, the brain and on adipocytes, exert their activities via coupling to G-proteins (Campbell & Drucker, 2013; Meier *et al.*, 2002). GIP is secreted in lower levels and is physiologically less pertinent to humans than GLP-1 (Maranhão *et al.*, 2014). GLP-1, but not GIP, controls glycemia by extra activities, such as glucagon secretion, food intake, and suppression of gastric emptying (Drucker, 2006).

Overall, there seems have not been main or physiologically for GIP secretory defect for GIP in T2DM patients. (Holst *et al.*, 2011). Therefore, GIP is not expected to be used as an anti-diabetic drug (Nauck *et al.*, 1993; Peters, 2010) primarily because it increases glucagon secretion and stimulates lipogenesis (Peters, 2010). Moreover, GIP does not improve anti-diabetic effects of GLP-1 in T2DM patients (Mentis *et al.*, 2011)

### 2.5.3 Role of the GLP-1 in the Pathogenesis of Obesity and T2DM

The impaired release or effects of GLP-1 have many negative consequences on diabetes, obesity and insulin resistant-related diseases (Wang *et al.*, 2015). The metabolic disorders observed in obesity might be mediated partially due to the functional failure of hormones included in glucose and energy metabolism. Particularly, those released from adipose tissue or gastric tract, such as GIP, GLP-1, ghrelin, leptin, amylin, pancreatic polypeptide cholecystokinin and adiponectin (Bloom *et al.*, 2008; Moran & Dailey, 2009). These are all possible candidates for envelopment, either because of their impact on insulin secretion from the β-cells, or their impact on food intake as hunger signals or satiety (Madsbad, 2014).

There is growing evidence that the release or action of GLP-1 may be impaired in obese subjects (Drucker, 2006; Madsbad, 2014; Stouwe *et al.*, 2015; Wikarek *et al.*, 2014), even in those with normal glucose tolerance (Madsbad, 2014), which may lead to failure of glucose homeostasis (Maranhão *et al.*, 2014). Moreover, GLP-1 levels are intensely and independently associated with body fat mass, mainly in young and healthy

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adult men. It is therefore likely that GLP-1 might be involved in the regulation of body fat mass (Stouwe *et al.*, 2015). Impaired release or dysfunction of GLP-1 in obese individuals causes excessive insulin secretion, and plays a pivotal role in the development and/or progression of T2DM in subjects with MetS (Krentz *et al.*, 2008; Meyer & Stahl, 2009). This may also help explain the reduced satiety signalling and augmented gastric emptying observed in obesity. Hence, it is possible that GLP-1 impairment may be part of the pathophysiological bridge between obesity and T2DM (Madsbad, 2014). Furthermore, it was established that the low GLP-1 levels ware an important risk factor for T2DM (Lastya *et al.*, 2014).

Several studies examining GLP-1 levels between healthy subjects and diabetic patients have found that subjects with T2DM displayed compromised GLP-1 levels (Bagger *et al.*, 2011; Knop *et al.*, 2012; Knop *et al.*, 2007; Madsbad, 2014; Muscelli *et al.*, 2008; Nauck *et al.*, 2011; Vilsbøll *et al.*, 2001). A large cross-sectional study has showed a moderate decrease in postprandial GLP-1 levels in T2DM patients compared to normal subjects. In addition, there was also minor decrease in GLP-1 levels in IGT subjects (Toft-Nielsen *et al.*, 2001). The emerging pattern appears to be based upon the degree of hyperglycaemia (Meier & Nauck, 2010).

However, there is some controversy regarding the pathophysiology of GLP-1 impairment in different stages of the disease. Several studies have found that prolonged periods of elevated glucagon levels and increasing severity of T2DM have been associated with reduction in GLP-1 levels (Meier & Nauck, 2008; Nauck *et al.*, 2011), while other studies have found no such effect (Knop *et al.*, 2012; Ryskjær *et al.*, 2006; Smushkin *et al.*, 2011; Vollmer *et al.*, 2008). A meta-analysis study proposed that there is no decrease in GLP-1 levels in subjects with T2DM compared with normal glucose tolerance (Nauck *et al.*, 2011), and no differences in GLP-1 levels between patients with



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and without T2DM (Nauck *et al.*, 2011; Vollmer *et al.*, 2008). On the other hand, a meta-analysis by Calanna *et al.* (2013) reported augmented GLP-1 levels in T2DM subjects.

The conflicting results observed in T2DM studies might be attributed to the incapability of the pancreas to respond to GLP-1 (Kjems *et al.*, 2003; Vilsbøll *et al.*, 2003; Vilsbøll *et al.*, 2002), which may be caused by hyperglycaemia; thus it may be reversible (Nauck & Meier, 2016). Altogether, these studies have provided better evidence to the notion that diminished GLP-1 action in T2DM patients progresses secondary to chronic hyperglycaemia and is complemented by impaired β-cell function in these subjects (Meier & Nauck, 2006; Nauck *et al.*, 2004; Singh, 2015). In addition, impaired release or action of GLP-1 might be attributed to restored expression of GLP-1 R, which may be reduced in T2DM (Xu *et al.*, 2007). Recently, evidence for GLP-1 resistance of β-cell in T2DM patients have emerged. Differences in GLP-1R signal transduction and expression may result in impairing incretin signalling in β-cells (Roussel *et al.*, 2016). A study recently suggested that lipotoxicity and glucotoxicity plays a pivotal role in reducing GLP-1R expression and it may contribute to the deterioration of β-cell function in T2DM (Cho *et al.*, 2014). Furthermore, ethnic differences in GLP-1 levels and GLP-1 responsiveness has been suggested among East-Asians, in particular, the Japanese and Koreans (Singh, 2015). In humans, GLP-1 and GIP certainly undergo very rapid degradation in circulation, facilitated by dipeptidyl-peptidase-IV (DPP-IV) (Deacon, 2004).

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## 2.6 *DIPEPTIDYLE PEPTIDASE- 4 (DPP4)*

### 2.6.1 Definition and Genomic Organization

The protein, DPP-IV was first recognised in 1966 as a glycylproline naphthylamidase (Hopsu-Havu & Glenner, 1966), and was consequently purified from rat liver (Hopsu-Havu & Sarimo, 1967), and pig kidney (Hopsu-Havu *et al.*, 1968), prior to detection in numerous different tissues (Hopsu-Havu & Ekfors, 1969). It was later determined to be identical to the T-cell activation antigen cluster of differentiation (CD)-26 (Ulmer *et al.*, 1990; Vivier *et al.*, 1991).

The 70-kb human *DPP4* gene was first identified in 1992 (Abbott et al., 1994; Misumi et al., 1992; Tanaka et al., 1992). It is located on the long arm of chromosome 2 (2q24.3; Figure 2.5) and comprises of 26 exons that encode a 766- amino acid single-pass type II integral transmembrane glycoprotein (DPP-IV), also known as CD26 (Drucker, 2003). This protein belongs to S9b DPP family, which also include fibroblast activation protein (FAP), quiescent cell proline dipeptidase (Lambeir et al., 2003; Mulvihill & Drucker, 2014; Richard et al., 2000). The protein's catalytic site is encoded by two exons, exons 21 and 22. Of interest, the human *DPP4* gene is located adjacent to the GLP-1encoding gene.

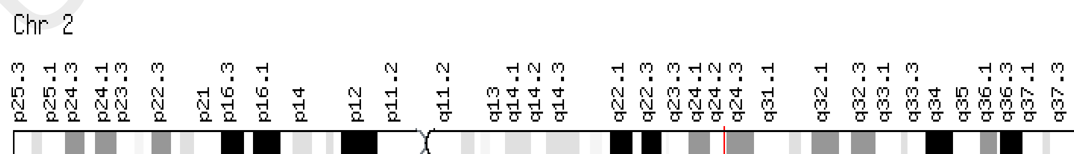


Figure 2.5: Schematic representation of *DPP4* gene on chromosome 2 (2q24.2).
Adapted from GeneCards

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### 2.6.2 Biology of DPP-IV and Molecular Function

DPP-IV has a transmembrane domain (TMD) (residues 7–28), a short cytoplasmic domain (1–6), a flexible stalk segment (29–39) and an extracellular domain (40–766). The extracellular domain is responsible for the catalytic function of DPP-IV, which can be further separated into a highly glycosylated region, the catalytic region, and the cysteine-rich region (Lambeir *et al.*, 2003; Röhrborn *et al.*, 2015). The catalytic pocket located terminal of the extracellular domain cleaves many substrates; it is about 8Å and confined within a 15Å wide opening (Rasmussen *et al.*, 2003). Residues 340-343 and residue 294 inside the cysteine-rich segment is fundamental for adenosine deaminase (ADA) and matrix proteins binding (Bauvois *et al.*, 2000; Richard *et al.*, 2000).

DPP-IV exists as a monomer, homodimer or homotetramer on the cell surface. The homodimer is the primary catalytically active form of the protein (Mulvihill & Drucker, 2014). It is subjected to post-translational modification through N-terminal sialytion and glycosylation, both of which have been proposed in the regulation of its catalytic activity (Engel *et al.*, 2003; Mulvihill & Drucker, 2014).

Catalytically active DPP-IV is liberated from the membrane, producing the soluble form sCD26/DPP-IV (727a.a) found in many biological fluids (Figure 2.6) (Mulvihill & Drucker, 2014), which lacks the transmembrane and cytoplasmic domains (Durinx *et al.*, 2000; Morimoto & Schlossman, 1998; Mulvihill & Drucker, 2014). These two domains are cleaved and released into the circulation in a process called shedding through the action of matrix metalloproteases (MMPs) (Lambeir *et al.,* 2003; Röhrborn *et al.,* 2014). sCD26/DPP-IV is also released from adipocytes, immune cells and bone marrow cells (Lamers *et al.*, 2011; Matteucci & Giampietro, 2009; Wang *et al.*, 2014), and accounts for a considerable and essential proportion of DPP-IV activity in human serum (Cordero *et al.*, 2009; Zilleßen *et al.*, 2016).

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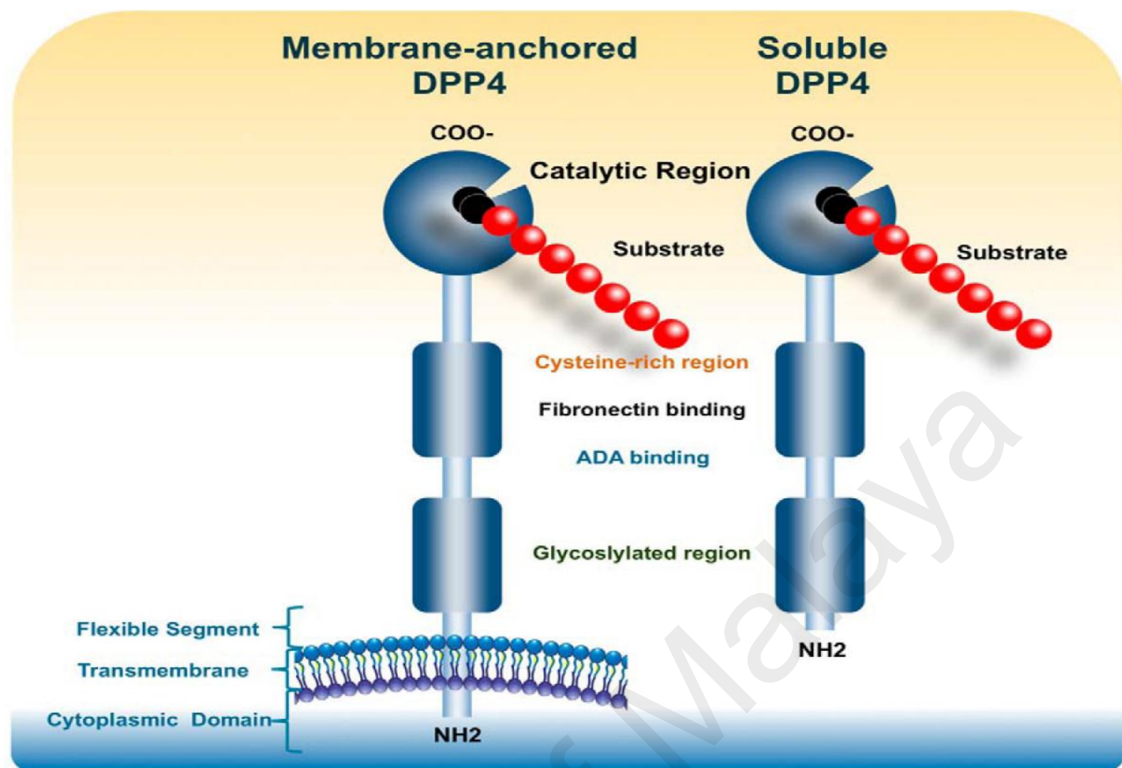


**Figure 2.6: Producing a soluble circulating form from membrane-bound DPP-IV.**
Adapted from Mulvihill and Drucker (2014)

The catalytic activity of sCD26/DPP-IV in circulation plays a pivotal role in triggering intracellular signalling pathways and augments the proliferation of human lymphocytes, independently by binding to ADA (Yu *et al.*, 2011), or catalytic activity (Yu *et al.*, 2011). Also, sCD26/DPP-IV play a key role in impairing insulin-mediated activation of actin in human adipocytes, smooth muscle cells and skeletal muscles, *in vitro* (Lamers *et al.*, 2011). Intriguingly, sCD26/DPP-IV has been implicated in T-cell migration regulation and up-regulation upon co-stimulation with molecules such as CD86 (Ikushima *et al.*, 2002; Ohnuma *et al.*, 2001). The mechanisms of signal transduction by sCD26/DPP-IV is probably via interaction with mannose 6-phosphate/insulin-like growth factor II receptor (Ikushima *et al.*, 2002), or via other molecular interactions (Zhong, Rao*, et al.*, 2013) that are not fully clear and still remain debatable.

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### 2.6.3 DPP-IV Expression and Its Alteration in T2DM

DPP-IV is expressed in several diverse cell types including leukocyte, fibroblasts and epithelial cells. Molecular mechanisms that mediate *DPP4* gene transcription and enzymatic activity are not completely understood thus far, and may be dependent on the microenvironment and cell type (Gong *et al.*, 2015; Röhrborn *et al.*, 2015).

The DPP-IV promoter region comprises of consensus sites for various transcription factors like SP-1 (specificity protein 1), nuclear factor (NF)-κB, AP-1 factor (activator protein 1), EGFR (epidermal growth factor receptor) (Böhm *et al.*, 1995). It has been shown that DPP-IV expression is increased in diabetes. Exposure of human glomerular endothelial cells to high levels of glucose in vitro resulted in higher expression of DPP-IV (Pala et al., 2003; Yu et al., 2006). Likewise, DPP-IV expression was augmented in HepG2 cells in response to high glucose (Miyazaki *et al.*, 2012). DPP-IV expression is increased in T cells of T2DM patients (Lee *et al.*, 2013), and correlating with insulin resistance (Lee *et al.*, 2013; Miyazaki *et al.*, 2012). A recent study reported that high DPP-IV expression in human adipocytes contributes to the adipocyte differentiation process (Zilleßen *et al.*, 2016), and positively correlates with BMI (Lamers *et al.*, 2011; Sell *et al.*, 2013). DPP-IV expression has also been positive correlated with the amount of adipocyte size, visceral adipose tissue, AIC, and inflammation (Sell *et al.*, 2013).

In addition, to diabetes, changed DPP-IV expression has also been described in several further diseases such as systemic lupus erythematosus (Stancikova *et al.*, 1991), and Rheumatoid Arthritis (RA) (Hildebrandt *et al.*, 2000).

### 2.6.4 Role of the Catalytic Function of DPP-IV in Inactivation of Incretins (GLP-1)

DPP-IV exerts its peptidase function through its ability to remove X-proline or X-alanine dipeptides from the N-terminus of a wide range of proteins including GLP−1,

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GIP, Chemokine Ligand (CCL)-5, Chemokine Ligand 9 (CXCL-9), and neuropeptide Y (NPY) (Gong *et al.*, 2015; Klemann *et al.*, 2016; Lambeir *et al.*, 2003). DPP-IV substrates can be grouped into regulatory peptides, neuropeptides, cytokines and chomokines (Lambeir *et al.*, 2003; Ryskjær *et al.*, 2006). Incretins hormones (GLP-1, GIP), and growth-hormone-releasing factor (GHRF) are the main regulatory peptides that have been shown to be cleaved by DPP-IV. In 1990s, Mentlein *et al.* (1993) was the first study that confirmed the role of DPP-IV in the degradation of GLP-1 and GIP. Subsequent studies reported the metabolism of GLP-1 and GIP, induced via DPP-IV in vivo (Deacon *et al.*, 1995; Kieffer *et al.*, 1995).

In line with this, DPP-IV inhibitors soon appeared as a novel curative approach for T2DM treatment (Yazbeck *et al.*, 2009), and was accepted worldwide due to their modest influences on A1C, as well as the absence of severe side effects and their ease of management (Scirica *et al.*, 2013; White *et al.*, 2013).

DPP-IV reduces the half-life of GLP-1 and GIP to less than 2 minutes and converts active GLP-1(7–36) to the inactive form, GLP-1(9–36) amide. Likewise, GIP (1–42) is rapidly inactivated and converted to GIP (3–42). The binding affinity of kinactive GLP-1(9-36) for GLP-1 receptor is approximately 100-fold lesser compared to the active GLP-1(7–36) amide (Deacon *et al.*, 2002). Similarly, affinity of GIP(3-42) is about 4-fold lower than that of GIP(1-42) (Deacon *et al.*, 2006). GHRF (1–29), is rapidly inactivated by DPP-IV and converted to GHRF (3–29). Also, DPP-IV cleaves the N-terminal dipeptides, consequently inactivating the chemotactic activities of chemokines such as CCL5, CCL22, CXCL9, CXCL10, CCL11 and CXCL12 (Gong *et al.*, 2015). DPP-IV has also been implicated in the degradation of a number of cytokines such as Granulocyte colony stimulating factor (G-CSF), granulocyte macrophage stimulating factor (G-CSF), IL-3 and Epo (Broxmeyer *et al.*, 2012). Development of obesity and improved glucose



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tolerance, and hyperinsulinemia are refractory in mice lacking *DPP4* gene (Broxmeyer *et al.*, 2012; Marguet *et al.*, 2000).

### 2.6.5 Role of the Non-catalytic Interactions of DPP-IV in the Pathogenesis of Inflammation and T2DM

Recently, there have been increasing interest with regards to inflammation, considered to be a major contributor to insulin resistance and T2DM (Després & Lemieux, 2006; Horng & Hotamisligil, 2011; Hotamisligil, 2006). Adaptive and innate immunity together are responsible for the development of metaflammation (a metabolic inflammatory state) (Després & Lemieux, 2006; Horng & Hotamisligil, 2011; Hotamisligil, 2006; Sell *et al.*, 2012).

DPP-IV has non-catalytic functions; it is able to interact with a wide range of ligands such as fibronectin, ADA, C-X-C motif receptor 4 (CXC-R4), and caveolin-1. These non-catalytic interactions suggest that DPP-IV plays important roles in a variety of processes, which includes immune regulation, functional modulation of antigen-presenting cells (APCs), and enhancing T-cell activation (Gong *et al.*, 2015; Klemann *et al.*, 2016; Wagner *et al.*, 2016).

The mechanism responsible for potentiating inflammation in T2DM via the action of DPP-IV includes both its non-catalytic and catalytic function. There is growing evidence regarding the role of DPP-IV's non-catalytic function in the regulation of T-cell activation (Klemann *et al.*, 2016; Ohnuma *et al.*, 2001).

It is well established that sCD26/DPP-IV on the cell surface of T cells to provides co-stimulatory signal via binding to ADA (Pacheco *et al.*, 2005). The engagement of ADA by DPP-IV promotes T-cell activation by activating CD45 (Wagner *et al.*, 2016). ADA-DPP-IV interaction might also promote T cell activation through clearing peri-cellular adenosine. Extracellular adenosine is created from ATP by cell surface CD73 and CD39



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(Deaglio *et al.*, 2007). Furthermore, it has been recently demonstrated that sCD26/DPP-IV expressed on APCs (involving dendritic and macrophage cell) also leads to enhanced metaflammation through interacting with ADA (ZhongRao, *et al.*, 2013).

The sCD26/DPP-IV on T cells might also interact with caveolin-1 existing on APCs causing in their activation (Ohnuma *et al.*, 2001; Ohnuma *et al.*, 2004), this interaction has also been identified to be implicated in the pathogenesis of arthritis (Ohnuma *et al.*, 2006).

Adipose tissue inflammatory cells have been higher levels of sCD26/DPP-IV when compared with their counterparts in the circulation. In visceral adipose tissue, augmented sCD26/DPP-IV expression was observed in obese subjects, with a positive association reported between sCD26/DPP-IV levels and degree of insulin resistance (ZhongRao, *et al.*, 2013). These outcomes suggest that sCD26/DPP-IV activities on both APCs and T-cells may promote adipose inflammation, supporting the view that sCD26/DPP-IV contributes to insulin resistance and T2DM through its non-catalytic function.

### 2.6.6 Linkage of the DPP-IV with T2DM and MetS

It is well known that a soluble form of (sCD26/DPP-IV) considered as source for the circulating DPP-IV activity in the serum/plasma and constitute about 90–95% of the overall DPP-IV-like activity (Busso *et al.*, 2005; Durinx *et al.*, 2000; Friedrich *et al.*, 2007; Kobayashi *et al.*, 2002; Lee *et al.*, 2013). It is well established that subjects with T2DM display impaired incretin effect, suggesting that DPP-IV plays critical role in the degradation of incretin. DPP-IV activity in T2DM patients also correlates with the degree of glucose homeostasis and A1C. Mannucci *et al.* (2005) revealed that DPP-IV activity is elevated in T2DM patients compared to controls or those with IGT. In fact, it has been reported that the increase of DPP-IV activity in individuals with diabetes results in a decrease of GLP-1 levels (in response to foods and in fasting) (Lugari *et al.*, 2004).

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There is a wide range of evidence supporting augmented sCD26/DPP-IV levels in circulation and are that it is associated with hallmarks of T2DM and obesity, such as insulin resistance, elevated adipocyte hypertrophy and BMI (Röhrborn *et al.*, 2015).

Despite the clear clinical associations between the DPP-IV and the severity, onset of diabetes and/or obesity. Findings regarding DPP-IV activity in T2DM have been inconsistent. Some studies have reported increased DPP-IV activity (Fadini *et al.*, 2012; Ryskjær *et al.*, 2006), whilst other studies have found decreasing DPP-IV activity in T2DM patients (Feron *et al.*, 2009; Firneisz *et al.*, 2010; McKillop *et al.*, 2008). Discrepancy in the results of these studies may be partially explained by different patient characteristics.

Furthermore, it was suggested that sCD26/DPP-IV levels are associated with factors contributing to insulin resistance and aspects of MetS (Lamers *et al.*, 2011). And increased of DPP-IV activity is a critical predictor of the onset of insulin resistance and MetS in healthy subjects (Yang et al., 2014). Recent data suggest that DPP-IV activity is significantly associated with human heart failure dysfunction (dos Santos *et al.*, 2013).

### 2.6.7 Inhibition of DPP-IV Function in T2DM

DPP-IV degrades incretin (e.g. GLP1) and is extensively recognized for its regulatory roles in glucose homeostasis (Drucker, 2007; Drucker & Nauck, 2006). Indeed, mice lacking the DPP-IV enzyme results in improved glycemic control and decreased fat mass (Conarello *et al.*, 2003; Marguet *et al.*, 2000; Topham & Hewitt, 2009). Accordingly, DPP-IV inhibitors are promising candidates for treating T2DM because they inhibit the cleavage of incretin, which results in elevated endogenous incretin levels leading to glucose reduction in the postprandial state (Drucker & Nauck, 2006; Topham & Hewitt, 2009). More specifically, it has been suggested that DPP-IV inhibitors play critical roles in improving β-cell function, measured via the homeostasis model assessment of β-cell

function (HOMA-β), and enhancing the proinsulin: insulin ratio (Raz *et al.*, 2006). However, DPP-IV inhibitors are only moderately effective as sole glucose- reducing drugs, with a 0.6–0.8% lowering of A1C in patients with a baseline level around 8% (Inzucchi & McGuire, 2008).

Furthermore, it has been observed that T-cell activation can be blocked by DPP-IV inhibition (Flentke *et al.*, 1991; Schön *et al.*, 1987). Suppression of DPP-IV activity causes a decrease in cytokines production, such as interferon (IFN) -γ, interleukin (IL)-12, IL-10, and IL-2, via T-cells and mononuclear cells (Reinhold *et al.*, 1997; Reinhold *et al.*, 1993; Schön *et al.*, 1987).

DPP-IV inhibitors are a relatively new class of oral anti-diabetic drug and are often used as monotherapy or in combination with other oral medication such as metformin, sulfonylureas (SU) or thiazolidinediones (TZD) (Chen *et al.*, 2015; Drucker & Nauck, 2006). They have been increasingly used in in clinics primarily due to its safety, with very low incidence of side effects (Lambeir *et al.*, 2008; Raz *et al.*, 2006). There are many DPP-IV inhibitors that have been approved for use by the Food and Drug Administration (FDA). Examples include sitagliptin, saxagliptin, linagliptin, and alogliptin, approved in 2006, 2009, 2011, and 2013 respectively. Vildagliptin was approved for use in Europe in 2007, whilst anagliptin and teneligliptin was approved in Japan in 2012 (Pattzi *et al.*, 2010).

In Malaysia, three DPP-IV inhibitors: sitagliptin, vildagliptin, and saxagliptin are currently available for use. The two DPP-IV inhibitors used to treat T2DM patients at UMMC are vildagliptin, and sitagliptin. However, their use is not widespread due to the lack of specific guidelines in Malaysia concerning the appropriate use of these drugs (Zaman Huri *et al.*, 2014).

University of Malaya

### 2.6.8 GENETICS of *DPP4*

Although a considerable amount of studies have unambiguously confirmed the importance of DPP-IV in the development of diabetes and T2DM-associated diseases through its involvement in the degradation of incretin, few studies have attempted to identify the possible genetic variants within the *DPP4* gene and their association with T2DM (Röhrborn *et al.*, 2015).

The *DPP4* gene may be a candidate gene for MetS development. In a multi-stage study with a comprehensive genetic analysis of the *DPP4* gene, three SNPs, rs7608798, rs17848915 and rs1558957, were found to be associated with hyperglycaemia/diabetes, cholesterol/LDL-cholesterol levels and triglyceride levels in the first stage of analysis. However, the same results failed to be replicated in the second stage of analysis. The study also reported that obese men with MetS exhibited higher expression of the *DPP4* gene (Bouchard *et al.*, 2009). Turcot *et al.* (2011) examined the methylation rate of the *DPP4*-promoter CpG islands of different *DPP4* polymorphisms by extracting DNA from VAT of subjects with severe obesity. The study found that methylation levels of the *DPP4* gene differ significantly between three *DPP4* SNPs, rs17848915, rs13015258, and c.1926. Intriguingly, methylation levels were negatively-associated with *DPP4* mRNA abundance but positively-associated with plasma lipid profiles, which may also be a marker of VAT inflammation. After two years, the same group conducted a follow-up study analysing *DPP4* gene methylation levels in obese subjects with and without MetS in VAT. They reported that there were no difference in the methylation levels of the CpGs within or near the second exon of the *DPP4* gene between obese subjects with MetS or without MetS. However, they observed an association between cholesterol levels and the methylation levels (Turcot *et al.*, 2013).

This further supports a link between epigenetic modification of the *DPP4* gene and lipid metabolism. A GWAS examining 875 European participants with coronary artery

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disease (CAD) were divided into two groups: participants with MI and participants without MI. They observed that *DPP4* polymorphisms increased the risk of MI and development of atherosclerosis in patients with CAD. Particularly, one SNP (rs3788979) was identified, in both the additive and dominant inheritance modes, to be significantly associated with low levels of *DPP4* and may increase the risk of MI in CAD patients (Aghili *et al.*, 2012).

Dyslipidemia, characterized by elevation of lipid levels in blood, is a common feature of T2DM. This disorder can be identified by the quantity of apolipoprotein B (ApoB) in blood. Recently, it has been reported that DPP4 SNP rs4664443, was associated with ApoB levels in South Asians (Bailey *et al.,* 2014).

Despite the body of research in this area, little is known about the associations between fasting serum sCD26/DPP-IV levels, metabolic disorders, and T2DM. In addition, the link between serum sCD26/DPP-IV levels in T2DM patients, with or without MetS, is still unclear. Review of scientific literature has revealed that the genetic polymorphisms of *DPP4* and their association with T2DM have rarely been investigated (Röhrborn *et al.*, 2015), and that association of the *DPP4* genetic polymorphisms gene with T2DM among Asian populations has yet to be published. To the best of our knowledge, no such study has been initiated in Malaysia.

University of Malaya

# CHAPTER 3: METHODOLOGY

## 3.1 BIOCHEMICAL TEST REAGENTS

Dimension® clinical chemistry system Flex® reagent cartridge kits produced by Siemens Healthcare Diagnostics Inc., Deerfield, IL USA were used for the measurements of FPG, AIC, TC, HDL-c and TG (reference numbers; DF39A, DF105A, DF69A, DF27 and DF48 respectively). ADVIA Centaur assay (reference number 128323) supplied by Siemens Healthcare Diagnostics Inc., Deerfield, IL USA was used for measurement of insulin.

## 3.2 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) TEST REAGENTS

### 3.2.1 sCD26/DPP-IV Test Reagents

Human CD26 (DPP-IV) ELISA Kit from RayBiotech, Inc., USA (reference number ELH-CD26-001/96) was used for the detection of sCD26/DPP-IV levels in serum.

### 3.2.2 Active GLP-1 Reagents

Active GLP-1 ELISA Kit from EMD, Millipore Inc., USA (reference number EGLP-35K/96) was used for the detection of active GLP-1 levels in serum.

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## 3.3 GENOTYPING ANALYSIS REAGENTS

### 3.3.1 General Reagents

Sodium hydroxide, tris (hydroxymethyl)-aminomethane (Tris), hydrochloric acid (HCl) (Merck KGaA, Germany), boric acids and sodium acetate (Sigma-Aldrich Co. LLC, USA), ethylenediaminetetraacetic acid (EDTA), were used for preparation of buffers. Other chemicals such as ethanol and isopropanol (Thermo Fisher Scientific Inc., USA) were used for DNA extraction from whole blood.

### 3.3.2 DNA Gel Electrophoresis Reagents

Agarose, 100bp and 50bp ladder with loading buffer (Promega Corporation, Madison, WI, USA), ethidium bromide (Gibco Brl Life Technologies, Japan), were used for DNA electrophoresis.

### 3.3.3 Genotyping Reagents

Pre-designed TaqMan® genotyping assay (available from (Applied Biosystems of Life Technologies. (Foster city, CA, U.S.A.) (reference numbers 4351379 and 4381656/4371357 respectively) were used for SNP genotyping analysis.

## 3.4 SUBJECTS AND DATA COLLECTION

This hospital-based case-control study was conducted at the UMMC, Kuala Lumpur. Patients previously diagnosed with T2DM (FPG ≥7.0 mmol/L) based on the ADA definition who were in the follow-up treatment at the UMMC, and were invited to participate in this study (case group). Subjects who were enrolled for a routine medical check-up (FPG <6.1mmol/l) at UMMC were approached to participate in this study as

University of Malaya

constituents of the control group. All participants included in the current study were Malaysian aged 31–67 years in which non-probability sampling was used. Subjects were excluded from the study if they were taking DPP-IV inhibitors, had any acute or chronic infections and with severe medical conditions (malignancy, liver cirrhosis, renal failure, and chronic congestive heart failure). In addition, all study participants provided written informed consent before commencement of the study (Appendix A. 1 and Appendix A. 2). This study was reviewed and approved by the Medical Ethics Committee of the UMMC (Reference No. 387.15).

## 3.5 CLINICAL MEASUREMENTS

Blood pressure (BP) measurements were taken from each subject's right arm in the seated position by using an Omron IntelliSense Automatic Blood Pressure Monitor after 10minutes of rest in a quiet room in the morning. Two to three successive BP readings were obtained at 5minutes intervals and averaged. The subjects' height (in meter), and weight (in Kg) were measured using Seca scale (Seca gmbh & co. kg. Germany) and body mass index (BMI), defined as weight (kg)/height squared ($m^2$), was calculated. Waist circumference was measured half-way between the lower rib margin and the anterior superior iliac spine. Hip circumference was measured at the point of maximum extension of the buttocks. The circumferences were assessed while subjects were in a standing position. Waist to hip ratio (WHR) was calculated by dividing the waist to hip circumferences (Al-Hamodi *et al.*, 2012). The therapy regimens of the anti-diabetic and anti-lipid medications for diabetic patients were obtained from patient records at UMMC. In addition, smoking history and diabetes history were recorded for all participants.

University of Malaya

## 3.6 SAMPLE PROCESSING

Venous blood samples (10ml) were collected under sterile conditions from every subject after signing the consent form and recording the questionnaires (volunteer subjects) (Appendix A.3a and Appendix A.3 b). The subjects were previously informed that they have to fast 12hours. Blood samples were collected within a 2hour window (8:00 to 10:00 AM). The blood samples were immediately transferred into three labelled Vacutainer® tubes (BD, New Jersey, USA); the first one contained sodium fluoride for FPG measurement, the second plain tube contained $K_2$EDTA for DNA extraction and AIC determination, and the last tube without any anticoagulants for insulin, sCD26/DPP-IV, active GLP-1 and lipids profile (TG, TC and HDL-c). The collected blood samples were immediately kept in an ice box during collection. The genomic DNA was extracted on the same day, from each whole EDTA blood sample. Moreover, the serum/plasma was immediately separated gently by using Allegra® X-12R centrifuge (Beckman Coulter. Inc USA) for a 15min at 2500-3000×g at 4°C. Then the plasma/serum and extracted DNA were separated into corresponding micro tubes and immediately kept at –80 °C pending further investigations.

## 3.7 BIOCHEMICAL ANALYSIS

FPG, insulin, AIC, TG, TC, HDL-c and low density lipoprotein cholesterol (LDL-c) levels were determined on the day of blood collection at Division of Laboratory Medicine (LMC) of the UMMC, Kuala Lumpur.

### 3.7.1 Measurement of Glucose (FPG)

FPG was measured by an automated analyzer Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics Inc., Deerfield, IL USA) in the LMC, UMMC (Appendix B. 1). The method used was adapted from Kunst *et al.* (1983)

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using the hexokinase-glucose-6-phosphate dehydrogenase method. The analysis was performed using the Glucose Flex® reagent cartridge, from Siemens Healthcare Diagnostics Inc., Deerfield, IL USA. The intra-assay and inter-assay coefficients of variation (CV) were 0.6% and 1.6%, respectively with a sensitivity of 0mmol/dL (1mg/dL) for the glucose assayed.

### 3.7.2 Measurement of Glycosylated Haemoglobin (AIC)

AIC in whole blood was measured by turbidimetric inhibition immunoassay (TINIA) using automated analyzer Dimension® RxL Max® Integrated Chemistry System produced by Siemens Healthcare Diagnostics Inc., Deerfield, IL USA in the LMC, UMMC (Appendix B. 2).

### 3.7.3 Measurement of Lipid Profile

Lipids profiling, which involved of TG, TC, HDL-c and LDL-c were measured by an automated analyzer Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics Inc. Deerfield, IL USA) in the serum. The procedure was done by the LMC, UMMC.

#### 3.7.3.1 Measurement of Triglycerides (TG)

Serum TG level was determined enzymatically by using Flex® reagent cartridge and measured by an automated analyzer Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics Inc. Deerfield, IL USA) (Appendix B.3a). The inter-assay CV was 1.3% and intra-assay was 0.4% with a sensitivity of 0.17 mmol/L (15mg/dL) for the TG assayed.

University of Malaya

#### 3.7.3.2 Measurement of Total Cholesterol (TC)

Serum levels of TC were determined by using Flex® reagent cartridge and measured by an automated analyzer Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics Inc. Deerfield, IL USA), which was adapted from Rautela and Liedtke (1978), (Appendix B.3 b). The intra-assay and inter-assay CV were 0.84% and 1.3%, respectively with a sensitivity of 1.3mmol/L (50mg/dL) for the TC assayed.

#### 3.7.3.3 Measurement of HDLc and LDLc

HDL-c levels were enzymatically measured directly without prior centrifugation steps using the AHDL Cholesterol Flex® reagent cartridge and measured by an automated analyzer Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics Inc. Deerfield, IL USA) (Appendix B.3 c). The intra-assay and inter-assay coefficients of variation were 1.57% and 2.00%, respectively with a sensitivity of 0.26mmol/L (10mg/dL) for the HDL-c assayed.

The LDL-c level was calculated from the TC, HDL-c and TG concentrations according to Friedewald equation (Friedewald *et al.*, 1972), since all TG levels measured were below 4.5mmol/l. LDL-c concentration (mmol/l) = TC – HDL-c – (TG/2.2).

### 3.7.4 Measurement of Insulin, Insulin Resistance and β-Cell Function

Insulin levels were measured by ADVIA Centaur assay XP Immunoassay System (Siemens Healthcare Diagnostics Inc. Deerfield, IL USA) with a sensitivity of 0.5mU/L, and intra-assay and inter-assay CV of 4.6 and 5.9%, respectively. The procedure was done by the LMC, UMMC (Appendix B.4).

Insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were assessed by the Homeostasis Model Assessment (HOMA2) Calculator v2.2, which is available online



University of Malaya

from Diabetes Trials Unit (University of Oxfordwww.dtu.ox.ac.uk) according to Matthews *et al.* (1985). This program used fasting insulin and plasma glucose measurement to calculate the HOMA-IR and HOMA-β.

## 3.8 ELISA ASSAY

### 3.8.1 Measurement of Serum sCD26/DPP-IV Levels

sCD26/DPP-IV levels were analysed by Human CD26 ELISA kit (RayBiotech, Inc., USA). This assay is quantitative determination of circulating sCD26/DPP-IV in serum. The measurement is based on a double antibody sandwich ELISA. CD26 antibody Microplat coated onto ELISA plate wells will be reacted with complexed and free CD26 present in serum. After incubation for 2.5 hours and followed by washing appropriate of the plate. Biotinylated anti-human CD26 antibody was added in a second incubation step to act divalently, a bridge was formed between CD26 immobilized on the plate and CD26 Biotin. After discard the solution and repeat washing. The amount of CD26 Biotin bound was detected in a third incubation step by addition of Streptavidin Peroxidase, which binds specifically to Biotin. Unbound Streptavidin Peroxidase and excess antibody were washed away, addition of 3,3',5,5'–tetramethylbenzidine (TMB) in the dark with gentle shaking resulted in formation of a blue colour in proporation to the amount of CD26 bound. This reaction was stopped by addition of stop solution causing the well contents to turn yellow. Absorbance of the yellow reaction mixture in 96-well plates was read at 450 nm immediately with a microplate reader (Hydroflex Elisa, Chemopharm, Austria). The intra-assay CV was $< 10\%$ while inter-assay CVs were $< 12\%$. The minimum detectable dose of sCD26/DPP-IV is typically less than 25pg/ml.

University of Malaya

### 3.8.2 Measurements of Serum Active Glucagon-Like Peptide-1 (GLP-1) levels

Active GLP-1 levels were analyzed by ELISA kit (EMD, Millipore Inc., USA), which is quantification of active forms of GLP-1 [(7-36 amide) and GLP-1 (7-37)] in human serum, without cross-reacting with other inactive forms of GLP-1. The measurement is based on the capture of active GLP-1 from serum samples by a monoclonal antibody, immobilized in the wells of a microwell plate. After incubation and washing of unbound materials, anti GLP-1-alkaline phosphatase detection conjugate was added to the immobilized GLP-1. After appropriate washing to remove unbound or free conjugate, quantification of bound anti GLP-1-alkaline phosphatase conjugate was achieved by adding methyl umbelliferyl phosphate (MUP), which forms the fluorescent product umbelliferone in the presence of alkaline phosphatase. The amount of fluorescence generated was directly proportional to the concentration of active GLP-1 in the serum sample. The reaction was stopped by the addition of stop solution. The fluorescence of the reaction mixture an excitation/emission wavelength at 355 nm/460 nm was then read using a fluorescence unit (RFU) read-out of plate reader by (Hydroflex Elisa, Chemopharm, Austria). The minimum detectable of Active GLP-1 levels assay that can be detected by this assay is 2 pM.

University of Malaya

## 3.9 GENETIC ANALYSIS

### 3.9.1 DNA Extraction

Genomic DNA was isolated from peripheral blood leukocytes by using a commercially Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) as follows:

To each sterile 1.5ml labeled micro-centrifuge tube, 900µl of cell lysis solution was added and 300µl EDETA blood was added to each tube after gently rock the blood until thoroughly mixed. Then, the mixture was mixed by inverting the tubes 5–6 times and incubated for 10 minutes at room temperature (RT) with inversion (2–3 times) to lyse the red blood cells (RBC), followed by centrifugation at 16,000×g for 30 seconds at RT. The supernatant was removed and discard as much as possible without disturbing the visible white pellet. The tubes were vortexed vigorously until the white blood cells (WBC) are re-suspended (10–15 seconds). To each tube containing the re-suspended cells, 300µl nuclei lysis solution was added to the tube, the solution was pipetted 5–6 times to lyse the WBC until became very viscous, then 1.5µl of RNase Solution was added, mixed by inverting the tube 2–5 times and incubated at 37°C for 15 minutes, and then cooled to RT. After that, 100µl of protein precipitation solution was added to the nuclear lysate, vortexed vigorously for 10–20 seconds and centrifuged at 16,000×g for 3 minutes at RT. To another labeled 1.5ml tubes containing 300µl of RT isopropanol, the supernatant containing DNA was transferred, gently mixed by inversion until the white thread-like strands of DNA form a visible mass and centrifuged at 16,000×g for 1 minute at RT. The supernatant was decanted from each tube, to which 300µl of RT 70% ethanol was added and gently inverted several times to wash the DNA pellet and the sides of the tube. After centrifugation as in previous step for 1 minute, the ethanol was carefully aspirated using a drawn Pasteur pipette, and the pellet was air-dried for 10–15 minutes. To each tube, 100µl DNA rehydration was added, incubating at 65°C for 1 hour with periodically

University of Malaya

mixing by gently tapping the tube. Each tube with the rehydrated DNA was separated to four Eppendorf tube and kept at 80°C until analysis. The concentration and purification of extracted DNA were screened by 1% agarose gel electrophoresis. (APPENDIX C).

### 3.9.2 Genotyping of Single Nucleotide polymorphisms (SNPs)

#### 3.9.2.1 Selection of Polymorphisms Markers

The candidate DPP4 SNPs: rs1558957, rs3788979, rs4664443, rs1861978, rs12617656, rs17574, rs7608798 based on previous reports with T2DM-Associated Diseases (Aghili *et al.,* 2012; Bailey *et al.,* 2014; Bouchard *et al.,* 2009), and one SNP rs7633162 was associated with DPP-IV inhibition (Woodard-Grice *et al.*, 2010). In addition, we selected two SNPs; rs2160927, rs1014444 with a minor allele frequency of > 0.10, for a more coverage of the *DPP4* gene.

#### 3.9.2.2 TaqMan SNP Genotyping Assay

TaqMan SNP genotyping assay is also called "TaqMan Allelic Discrimination Technique". It is one of the robust and high throughput SNP scoring method used for genotyping experiments. This method based on the principle of the polymerase chain reaction (PCR) technique. In PCR, DNA amplification is achieved by using two oligonucleotides, called primers, which anneal to and are complementary to the DNA sequences flaking the desired region.

The determination of allele at a particular SNP is achieved via the use of hydrolysis probes (ie: TaqMan probes). These probes are sequence specific; they work by hybridizing to the target sequence between the two PCR primers during the annealing and extension phases of the reaction (Ong & Irvine, 2002). This intact probe is a dual labelled oligonucleotide with a quencher dye (TAMRA) attached to the 3' end and a reporter dye

(VIC or FAM) attached to the 5′ end. When the probe is intact, the quencher dye absorbs the fluorescence emitted by the reporter dye (Gibson et al., 1996). Florescence is produced during primer extension when the DNA polymerase cleaves the 5` reporter dye via its 5` exonuclease activity, separating it from the quencher and allowing increased fluorescence of the reporter dye (Gibson et al., 1996). This process is repeated in every cycle without interfering with the accumulation of PCR products. Therefore, fluorescence is directly proportional to the fluorophore released and the amount of DNA template present.

The TaqMan SNP genotyping assay selected for use in this experiment requires three elements: (i) DNA sample (ii) TaqMan universal Master Mix, and (iii) a SNP Genotyping assay, which contains sequence-specific forward and reveres primers to amplify the polymorphic sequences of interest, and two TaqMan Minor Grove Binder.

The two TaqMan MGB probes were labelled with the VIC dye and the other with the FAM dye at the 5′-end. A VIC dye is linked to the Allele 1 probe and a FAM dye linked to the Allele 2 probe. The 3′ end of the probe contained the non-fluorescent quencher (NFQ) which quenched the fluorescence dye at the 5′ end. The minor groove binder (MGB) serves to increase the melting temperature (MT) and allows the design of shorter probes (Afonina et al., 1997; Kutyavin et al., 2000). Finally, the probes combined with matched DNA templates and were decomposed by Taq DNA Polymerase followed by release of the fluorescent signal by the fluorescent dye (Holland et al., 1991) (Figure 3.1).

Analysis of the different signal strengths of the two types of fluorescence in each well determines the pattern of SNP alleles: if a sample shows enhanced fluorescence of either FAM or VIC, the SNP was classified as a homozygote; if a sample shows enhanced fluorescence of both FAM and VIC, the SNP was classified as heterozygote.

University of Malaya

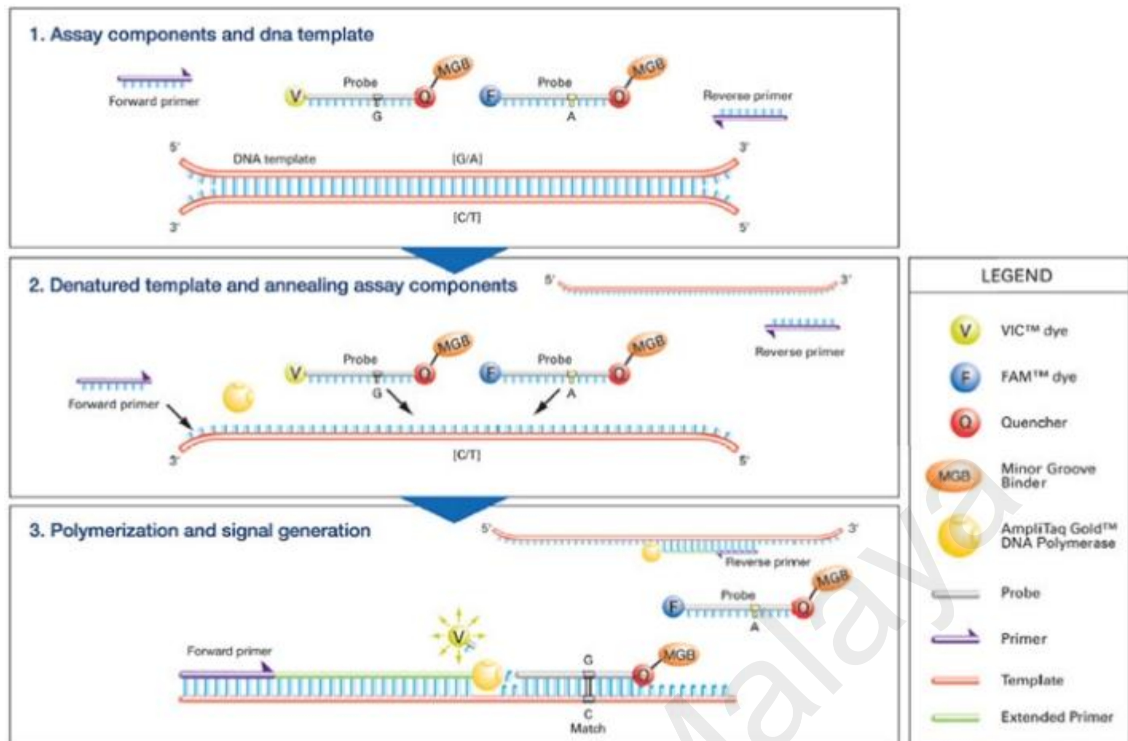


**Figure 3.1:** TaqMan SNP genotyping assay protocol.

Adapted from Applied Biosystems, 2006.

University of Malaya

The forward and reveres primers and probes used in this study were designed by Applied Biosystems. The primers and probes were designed to target the following context sequence (Table 3.1).

**Table 3.1: Context sequence for primers and probe design**

| SNP and Assay IDs | Necieotide sequence and (SNP) | Daye Allele |
|---|---|---|
| rs12617656<br>C___2789674_10 | TTATCATGCTGGTTTCACTTTAGAA[C/T]TACTCTGCC ACACATTTACTGAACA | VIC-C<br>FAM-T |
| rs2160927<br>C___2789703_10 | ATTATTACGGTACTAGTGAAGAATT[C/T]CTGGTTCC CTTCTAAACCCCATCTC | VIC-C<br>FAM-T |
| rs6733162<br>C__29330757_10 | AACTAAATCAACCAGTCCTGTGACC[C/G]TCACCCAT AAAATGACTCAGTGCAT | VIC-C<br>FAM-G |
| rs4664443<br>C___2789697_20 | CAGTGAATCACCCGAGGAAGCGGGC[A/G]TACTGCC AGTGCTGGCCTCAGGCTC | VIC-A<br>FAM-G |
| rs1014444<br>C___2789687_1_ | AAATGATGATGACAACAATAAATGC[A/G]GCTTTGG ATATCTTTCCATCTCAGC | VIC-A<br>FAM-G |
| rs3788979<br>C___2789710_10 | ATGCAGTAGGGAATGGTTTGCTTGG[T/C]CTGAGTAC AGCAGTGTTGGTGTGTG | VIC-C<br>FAM-T |
| s7608798<br>C__27055353_10 | TTGACAAAAAAAAAAAAGTAACATC[A/G]TTGCACA CATTTTAAAACTGCACAA | VIC-A<br>FAM-G |
| rs1558957<br>C___8944622_20 | TTCACAGCTCCTGCCTAGGAAAAAA[C/T]AATCACA GAATTGGTATTGACAAAA | VIC-C<br>FAM-T |
| rs1861978<br>C___2789733-10 | ACAAGACGTGAAAGCCGCAAGAGTT[G/T]CAGCCTA AACACTGAAGACCCCGCG | VIC-G<br>FAM-T |
| rs17574<br>C__25621701_10 | GCGCAGCAGCACCCAGCAGTCCCAG[A/G]AGAACCT TCCACGGTGTCTGCAAGC | VIC-A<br>FAM-G |

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The TaqMan SNP assay reaction mix was prepared as directed by applied Biosystem TaqMan Biosystemes Taqman SNP Genotyping Protocol (2006). The TaqMan Genotyping Mix for 10 µl reaction was prepared by adding the following components in the order below (Table 3.2).

**Table 3.2: TaqMan genotyping mix per 10 µl reaction**

| **Component** | **1x Reaction** |
|---|---|
| TaqMan Universal Master Mix | 5 µl |
| TaqMan SNP Genotyping Assay Mix (40X) | 0.20 µl |
| UltraPureTM DNase-free Distilled water | 3.8 µl |
| DNA | 1 µl (10 ng) |
| Total Volume | 10 µl |

10 µl of reaction mix was added into each well of the DNA reaction plate. The plate was sealed with the MicroAmp® Optical Adhesive Film and centrifuged briefly to spin down the contents and eliminate any air bubbles. To performed the PCR, the thermal cycling condition were as following (Table 3.3).

**Table 3.3: PCR thermal cycler conditions**

<table>
<tr><td colspan="4">Standard Protocol</td></tr>
<tr><td>Lid Temperature</td><td colspan="3">95 °C</td></tr>
<tr><td>Procedure</td><td>Time</td><td colspan="2">Temperature</td></tr>
<tr><td>Hold</td><td>10 min</td><td colspan="2">95 °C</td></tr>
<tr><td>Denature</td><td>15 sec</td><td>92 °C</td><td rowspan="2">40 Cycles</td></tr>
<tr><td>Anneal/Extend</td><td>1 min</td><td>60 °C</td></tr>
<tr><td>Hold</td><td colspan="3">10 °C</td></tr>
</table>

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### 3.9.3 Data processing

All of the assays were conducted in 96-well PCR plates. The samples were read on a StepOnePlus Real-Time PCR system and were analyzed using StepOne software v2.1 (Applied Biosystems). For each plate read, the following sample information was provided by the program: plate well number, marker number, allele X reaction, allele Y reaction, allele call and quality value. A plotted fluorescence graph was used to determine which allele were present in each sample (Figure 3.2).

Quality of clustering was determined by the closeness of fluorescence distribution for each allele group and if there was clear separation between each cluster. The genotyping success rates for SNPs rs1558957, rs3788979, rs17574, rs7608798, rs1014444, rs2160927, rs12617656, rs4664443, rs6733162, and rs1861978 were 99.6%, 99.7%, 99.5%, 99.1%, 99.5%, 99.6%, 99.6%, 98.8%, 99.6%, and 98.7% respectively. The sample and their respective genotypes were organized using Microsoft Excel.

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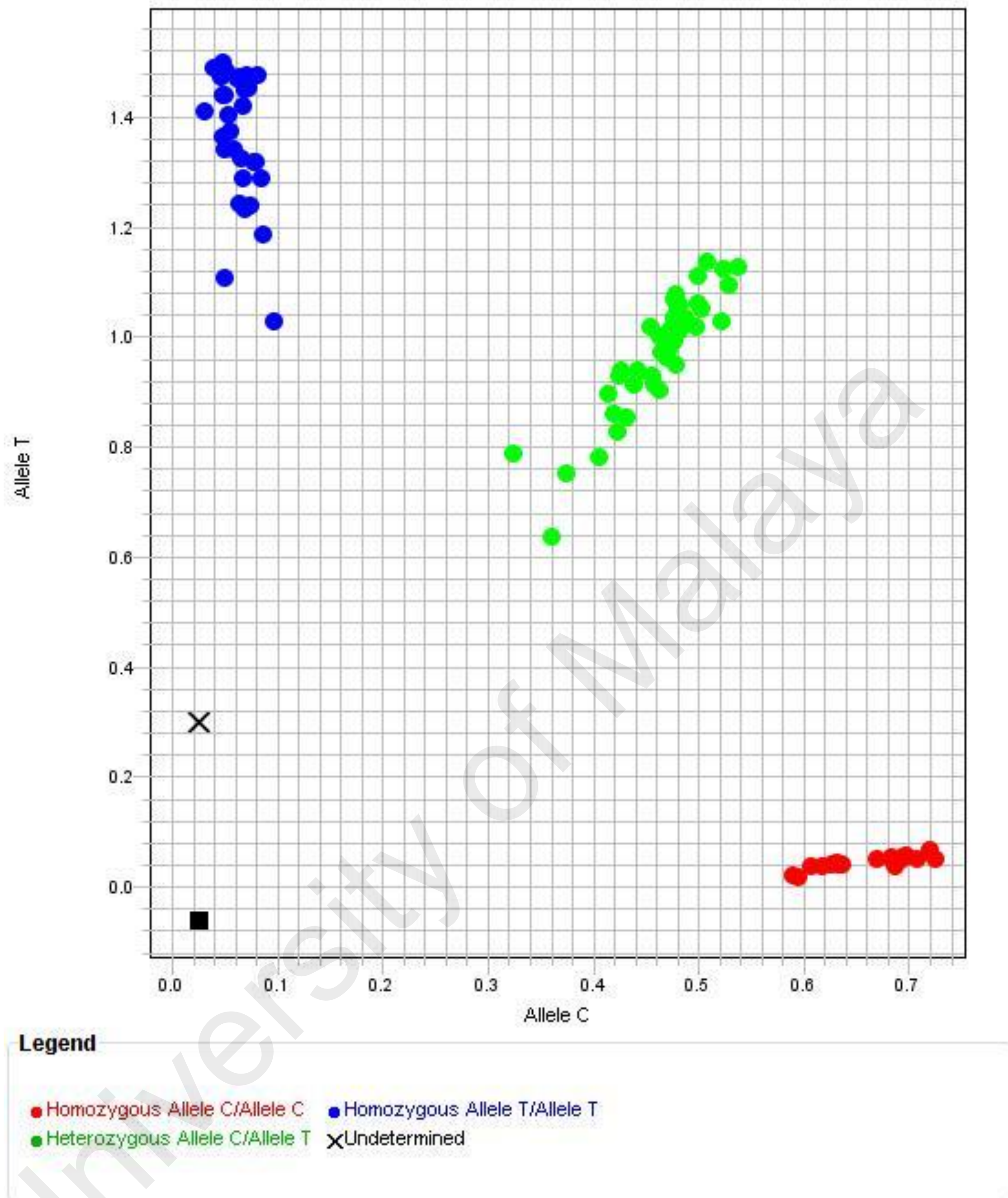


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**Figure 3.2: TaqMan allelic discrimination output signals.**
The output signals are plotted in a scatterplot with one fluorescent colour signal intensity on the y-axis (FAM) and the other on the x-axis (VIC). *DPP4* rs12617656, three clusters represent the three genotypes (C-C, C-T and T-T) could be determined. Signals that fall outside of these clusters are “undetermined”

## 3.10 QUALITY CONTROL

Pathological and normal controls were included with each run of biochemical analysis (FPG, Insulin, AIC, TG, TC, HDL-c, and LDL-c). Quality Controls with different concentrations were also included with each run of active GLP-1 assay. For SNPs genotyping; the genotyping success rate was more than 98% and the concordance rate was 100% that genotyped in duplicate samples. No template controls (NTCs) were detected together with samples in each run.

## 3.11 STATISTICAL ANALYSIS

HWE analyses were applied to compare the observed and expected genotype frequencies among diabetes-free controls by a goodness-of-fit chi-square using the DeFinetti software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) of the Institute of Human Genetics. The Golden Helix SNP & Variation Suite (SVS v8.x software; Bozeman, MT, USA) was used to estimate the LD between SNPs and construct haplotypes of related SNPs. The other statistical analyses were performed using SPSS v. 20 (IBM SPSS Inc., Chicago, IL, USA). Missing data were deleted list wise when any of the variables were missing and the entire observation was omitted from the analysis.

Clinical data includes demographic data (Age, Weight, Height, BMI, Waist Circumference, Hip, WHR, SBP and DBP), biochemical parameters (FPG, AIC, Insulin, HOMA-β, HOMA-IR, TC, HDLc, LDLc and TG), and addition to the sCD26/DPP-IV and active GLP-1, were log-transformed before statistical analyses, because they were not normally distributed and then re-transformed and expressed as geometric means. The significances of all demographics and biochemical parameters among the groups were assessed by ANOVA.

Odds ratio (ORs), 95% (CI) and corresponding P- values were calculated by multiple logistic regression analysis to assess the associations of each *DPP4* SNPs, recessive

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model, dominant model and additive model in total T2DM and T2DM with or without MetS after adjusting for age, gender, race and BMI, and also with T2DM in each ethnic group after adjusting for age, gender, and BMI. P-values were corrected for multiple comparisons with the Benjamini-Hochberg false discovery rate (FDR).

General Linear Model (GLM) after adjusting for age, race, gender and BMI was applied to determine associations of genotype groups in *DPP4* (SNPs) with sCD26/DPP-IV levels (dependent variables) in diabetic patients, and with MetS parameters, and HOMA-IR in obese non-diabetic subjects and normal subjects.

Assessment of sCD26/DPP-IV levels and active GLP-1 levels in T2DM and non-T2DM patients, both with and without MetS as well as in obesity group were evaluated by GLM analysis after adjusting for age, race, and gender as covariates. Spearman partial correlation coefficient ($r^s$) analysis was used to determine possible correlations between sCD26/DPP-IV levels and active GLP-1 levels.

A multiple linear regression analysis was used to examine the associations between sCD26/DPP-IV levels (as the dependent variable) and metformin therapy, AIC levels, and metabolic parameters in diabetic patients, as well as between sCD26/DPP-IV levels and insulin resistance in obese non-diabetic subjects. Hierarchical linear regression was applied to evaluate the associations between metabolic and diabetic parameters (as the dependent variable) and serum levels of sCD26/DPP-IV and active GLP-1 in normal and obese subjects respectively. *P*-values $< 0.05$ were considered to be significant.

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# CHAPTER 4: RESULTS

A total of 549 subjects were enrolled in the current study. Of these, 314 subjects with T2DM (257 with MetS and 57 without MetS) and 235 were non-diabetic control. The MetS criteria (Alberti *et al.*, 2009) of non-diabetic (control) subjects were confirmed by attending physicians and endocrinologists at UMMC. There were 164 control subjects without both MetS and diabetes and 71 non-diabetic participants with MetS.

## 4.1 DEMOGRAPHIC AND BIOCHEMICAL ANALYSIS

Demographic and biochemical parameters of all groups are shown in (Table 4.1 and Table 2). BMI, waist circumference, WHR, SBP, insulin and insulin resistance were significantly higher in the three target groups, non-diabetic MetS and T2DM with and without MetS subjects, compared to control. In contrast, HDL-c was significantly lower in the three target groups as compared to control subjects. Weight, hip circumference and TG were significantly higher in non-diabetic MetS subjects and T2DM with MetS compared with the control group with no significant differences between T2DM without MetS and control groups.

On the other hand, TC, FBG and AIC were significantly higher in T2DM with and without MetS subjects as compared to control group, with no significant differences observed between non-diabetic MetS and control groups. In contrast, HOMA-β in both T2DM with and without MetS was significantly lower than that in the control subjects. Furthermore, the LDL-c in both T2DM with and without MetS was significantly lower as compared to control subjects.

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**Table 4.1:** Demographic parameters among normal control, non-diabetes metabolic syndrome, type 2 diabetes mellitus with and without metabolic syndrome.

| Parameters | | Non-diabetes n=235 | | Type 2 diabetes n=314 | |
|---|---|---|---|---|---|
| | | Control n=164(29.9%) | MetS n=71(12.9%) | without MetS n=57(10.4%) | with MetS n=257(46.8%) |
| **Gender%** | **Male/Female** | 39.0/61.0 | 59.2/40.8 | 56.1/43.9 | 44.4/55.6 |
| **Races %** | **Malay** | 52.4 | 43.6 | 36.8 | 51.4 |
| | **Chinese** | 31.7 | 28.2 | 26.3 | 14.8 |
| | **Indian** | 15.9 | 28.2 | 36.9 | 33.8 |
| **Age (years)** | | 48.3(46.2-50.4) | 50.9(48.6-53.4) | 50.0(47.5-52.7) | 50.7(49.7-52.7) |
| | **P-Value** | | [a] 0.443 | [a, b] 0.999 | [a] 0.123, [b ,c] 0.999 |
| **Weight (kg)** | | 60.5(58.6-62.6) | 74.0(70.9-77.3) | 62.6(59.1-66.4) | 72.1(70.3-74.0) |
| | **P-Value** | | [a]***$1.3\times10^{-10}$*** | [a] 0.999, [b]***$4.3\times10^{-5}$*** | [a]***$1.3\times10^{-15}$***, [b]0.999, [c]***$2.5\times10^{-5}$*** |
| **Height (m)** | | 1.61(1.60-1.63) | 1.64(1.62-1.66) | 1.60(1.58-1.63) | 1.58(1.57-1.59) |
| | **P-Value** | | [a]*0.426* | [a] 0.999, [b] *0.273* | [a]***0.005***, [b]***$7.6\times10^{-5}$***, [c]0.660 |
| **Body Mass Index (kg/m$^2$)** | | 23.3(22.7-24.0) | 27.6(26.6-28.7) | 24.3(23.1-25.6) | 28.9(28.3-29.5) |
| | **P-Value** | | [a]***$3.2\times10^{-10}$*** | [a]*0.765*, [b]***$3.8\times10^{-4}$*** | [a]***$3.8\times10^{-29}$***, [b]*0.344*, [c]***$4.5\times10^{-10}$*** |
| **Waist Circumference (cm)** | | 81.5(79.6-83.4) | 94.9(92.4-97.4) | 86.0(82.7-89.4) | 96.6(95.2-98.0) |
| | **P-Value** | | [a]***$1.01\times10^{-14}$*** | [a]*0.047*, [b]***$1.5x10^{-4}$*** | [a]***$2.3\times10^{-33}$***, [b]0.999, [c]***$1.1\times10^{-8}$*** |
| **Hip (cm)** | | 98.4(97.2-99.7) | 105.5(103.3-107.8) | 97.0(94.8-99.2) | 104.1(102.8-105.3) |
| | **P-Value** | | [a]***$1.02\times10^{-6}$*** | [a]*0.999*, [b]***$2.1\times10^{-6}$*** | [a]***$1.1\times10^{-80}$***, [b]*0.999*, [c]***$1.02\times10^{-6}$*** |
| **Waist-to-Hip ratio** | | 0.83(0.82-0.84) | 0.90(0.88-0.92) | 0.89(0.87-0.91) | 0.93(0.92-0.94) |
| | **P-Value** | | [a]***$4.2\times10^{-9}$*** | [a]***$2.13\times10^{-5}$***, [b]*0.999* | [a]***$3.\ 3\times10^{-30}$***, [b]0.086, [c]***0.002*** |
| **Systolic Blood Pressure (mmHg)** | | 130(127-133) | 143(139-146) | 120(116-125) | 138(135-140) |
| | **P-Value** | | [a]***$1.3\times10^{-5}$*** | [a]***0.001***, [b]***$4.02\times10^{-11}$*** | [a]***$1.7\times10^{-4}$***, [b]*0.315*, [c]***$2.1\times10^{-10}$*** |
| **Diastolic Blood Pressure (mmHg)** | | 81(79-82) | 86(84-89) | 76(74-78) | 83(82-84) |
| | **P-Value** | | [a]***$3.5\times10^{-4}$*** | [a]***0.012***,[b]***$3.4\times10^{-08}$*** | [a]0.052,[b]*0.118*, [c] ***$3.4\times10^{-6}$*** |

The results presented represent geometric means (95% confidence interval of mean), [a]*vs* normal control group: [b]*vs* non-diabetes MetS group: [c]*vs* type 2 diabetes mellitus without metabolic syndrom which evaluated by ANOVA. Bold values are significant. MetS: metabolic syndrome.

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**Table 4.2:** Biochemical parameters among normal control, non-diabetes metabolic syndrome, type 2 diabetes mellitus with and without metabolic syndrome.

| Parameters | | Non-diabetes n=235 | | Type 2 diabetes n=314 | |
|---|---|---|---|---|---|
| | | **Control n=164(29.9%)** | **MetS n=71(12.9%)** | **without MetS n=57(10.4%)** | **with MetS n=257(46.8%)** |
| **Fasting Plasma Glucose (mmol/l)** | | 5.01(4.94-5.07) | 5.55(5.39-5.71) | 8.09(7.23-9.04) | 7.79(7.44-8.15) |
| | **P-Value** | | *$^{a}0.083$* | ***$^{a}1.8\times10^{-23}$, $^{b}8.8\times10^{-12}$*** | ***$^{a}1.4\times10^{-42}$, $^{b}3.5\times10^{-16}$***, *$^{c}0.999$* |
| **Glycosylated AIC (%)** | | 5.58(5.50-5.66) | 6.00(5.87-6.13) | 7.91(7.33-8.54) | 8.18(8.96-8.41) |
| | **P-Value** | | *$^{a}0.171$* | ***$^{a}2.7\times10^{-23}$, $^{b}4.2\times10^{-12}$*** | ***$^{a}1.9\times10^{-48}$, $^{b}1.2\times10^{-22}$***, *$^{c}0.999$* |
| **Insulin (pmol/l)** | | 48.8 (44.8-53.1) | 83.1(73.6-93.9) | 60.8(49.5-74.6) | 114(105.2-123.6) |
| | **P-Value** | | ***$^{a}1.8\times10^{-08}$*** | *$^{a}0.133$*, **$^{b}0.029$** | ***$^{a}5.4\times10^{-36}$, $^{b}0.001$, $^{c}7.7\times10^{-11}$*** |
| **HOMA-β (%)** | | 98(93-104) | 116(105-127) | 48(38-62) | 81(73-89) |
| | **P-Value** | | *0.518* | ***$^{a}1.99\times10^{-10}$, $^{b}1.01\times10^{-11}$*** | ***$^{a}0.023$, $^{b}4.7\times10^{-4}$, $^{c}2.4\times10^{-6}$*** |
| **HOMA-IR** | | 1.04(0.96-1.14) | 1.81(1.61-2.05) | 1.60(1.29-1.99) | 2.88(2.65-3.13) |
| | **P-Value** | | ***$^{a}1.09\times10^{-8}$*** | ***$^{a}9.4\times10^{-5}$***, $^{b}0.999$ | ***$^{a}1.5\times10^{-46}$, $^{b}5.6\times10^{-07}$, $^{c}3.3\times10^{-09}$*** |
| **Total-Cholesterol (mmol/l)** | | 5.16(5.01-5.32) | 5.04(4.81-5.28) | 4.61(4.33-4.90) | 4.75(4.62-4.89) |
| | **P-Value** | | $^{a}0.999$ | ***$^{a}0.004$***, *$^{b}0.111$* | ***$^{a,}$ 0.001***, $^{b}0.250$, $^{c}0.999$ |
| **High-Density Lipoprotein Cholesterol (mmol/l)** | | 1.50(1.45-1.55) | 1.18(1.12-1.23) | 1.36(1.29-1.42) | 1.11(1.08-1.14) |
| | **P-Value** | | ***$^{a}1.2\times10^{-12}$*** | ***$^{a}0.025$, $^{b}0.003$*** | ***$^{a}3.4\times10^{-34}$***, *$^{b}0.335$*, ***$^{c}1.5\times10^{-08}$*** |
| **Low-Density Lipoprotein Cholesterol (mmol/l)** | | 4.19(3.47-5.06) | 3.37(2.77-4.11) | 2.73(2.47-3.02) | 2.75(2.64-2.87) |
| | **P-Value** | | $^{a}0.300$ | ***$^{a}0.002$***, *$^{b}0.777$* | ***$^{a}3.4\times10^{-07}$***, $^{b}0.324$, $^{c}0.999$ |
| **Triglycerides (mmol/l)** | | 1.04(0.97-1.12) | 1.72(1.57-1.90) | 0.96(0.87-1.07) | 1.75(1.64-1.88) |
| | **P-Value** | | ***$^{a}7.8\times10^{-12}$*** | *$^{a}0.999$*, ***$^{b}2.5\times10^{-10}$*** | ***$^{a}2.1\times10^{-23}$***, *$^{b}0.999$*, ***$^{c}1.9\times10^{-15}$*** |

The results presented represent geometric means (95% confidence interval of mean), $^{a}$*vs* normal control group: $^{b}$*vs* non-diabetes MetS group: $^{c}$vs type 2 diabetes mellitus without Mets which evaluated by ANOVA. Bold values are significant. MetS: metabolic syndrome

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## 4.2 SERUM LEVELS OF SOLUBLE DEPEPTIDYLE PEPTIDASE 4 IN TYPE 2 DIABETES AND ASSOCIATION WITH METABOLIC SYNDROME AND ANTIDIABETIC AGENTS AMONG MALAYSIAN SUBJECTS

### 4.2.1 Prevalence of Metabolic Syndrome among Subjects with Type 2 Diabetes.

The prevalence of MetS among T2DM subjects was (81.8%) (Table 4. 3)**.** Among all subjects, Malays with T2DM have the highest prevalence of MetS (86.3%) followed by Indians (80.6%) and Chinese subjects (62.8%), (Table 4.3). Meanwhile, diabetic females have a higher prevalence of MetS than diabetic male subjects (85.1% versus 78.1% respectively) (Table 4.4).

**Table 4.3:** Prevalence of metabolic syndrome among subjects with type 2 diabetes mellitus.

| Race | | Type 2 diabetes | | Total |
|---|---|---|---|---|
| | | **Without MetS** | **With MetS** | |
| **Malay** | Count | 21 | 132 | 153 |
| | % within Race | 13.7 | 86.3 | 100.0 |
| **Chinese** | Count | 15 | 38 | 53 |
| | % within Race | 28.3 | 62.8 | 100.0 |
| **Indian** | Count | 21 | 87 | 108 |
| | % within Race | 19.4 | 80.6 | 100.0 |
| **Total** | Count | 57 | 257 | 314 |
| | % within Race | 18.2 | 81.8 | 100.0 |

Prevalence is shown as Race and metabolic syndrome Cross tabulation.

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**Table 4.4:** Prevalence of metabolic syndrome among Malaysian males and females**.**

| Gender | | Type 2 diabetes | | Total |
|---|---|---|---|---|
| | | **Without MetS** | **With MetS** | |
| **Male** | Count | 32 | 114 | 146 |
| | % within Gender | 21.9 | 78.1 | 100.0 |
| **Female** | Count | 25 | 143 | 168 |
| | % within Gender | 14.9 | 85.1 | 100.0 |
| **Total** | Count | 57 | 257 | 314 |
| | % within Gender | 18.2 | 81.8 | 100.0 |

Prevalence is shown as gender and metabolic syndrome cross tabulation.

### 4.2.2 Comparison of Fasting Serum Levels of sCD26/DPP-IV and Active GLP-1 between Groups of Study.

The fasting serum levels of sCD26/DPP-IV and active GLP-1 were assessed by general linear model (univariate), after adjusting for age, gender, and race as covariates. Serum levels of sCD26/DPP-IV were found to be significantly higher in T2DM with MetS [1199(1163-1236) ng/mL] than in normal subjects [1089(1048-1131) ng/mL] ($p$ = $1.2\times10^{-4}$). Similarly, serum sCD26/DPP-IV levels were also considerably higher in T2DM without MetS [1195(1120-1275) ng/mL] than in normal subjects [1089(1048-1131) ng/mL] ($p$ = 0.015). On the other hand, serum levels of sCD26/DPP-IV were notably higher in T2DM with MetS [1199(1163-1236) ng/mL] than in non-diabetic MetS subjects [1120(1057-1186) ng/mL] ($p$ = 0.041). Additionally, no significant difference in serum sCD26/DPP-IV levels was noted between T2DM patients with or without MetS, or between control and non-diabetic MetS subjects (Table 4.5).

In contrast to the observed increase in sCD26/DPP-IV levels, the fasting serum levels of active GLP-1 were significantly lower in T2DM with MetS [3.74 (2.29) pmol/L] than in normal subjects [4.26 (3.89) pmol/L] ($p$ = 0.020). Likewise, serum active GLP-1 levels were prominently lower in T2DM without MetS [3.53 (2.24) pmol/L] than in normal subjects [4.26 (3.89) pmol/L] ($p$ = 0.028) (Table 4.5).

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**Table 4.5**: Comparison of fasting serum levels of sCD26/DPP-IV and active GLP-1 between normal, non-diabetic metabolic syndrome, type 2 diabetes mellitus subjects with and without metabolic syndrome and total type 2 diabetes mellitus.

| Parameters | Normal (n=164) | Non-diabetic MetS (n=71) | Type 2 diabetes mellitus | | Total Type 2 diabetes mellitus (n=314) |
|---|---|---|---|---|---|
| | | | without MetS (n=57) | with MetS (n=257) | |
| **sCD26/DPP-IV (ng/mL)** | 1089(1048-1131) | 1120(1057-1186) | 1195(1120-1275) | 1199(1163-1236) | 1198(1166-1232) |
| **p-value** | | [a]***0.427,*** | [a]**0.015**, [b]*0.139* | [a]***$1.2\times10^{-4}$, *** [b]**0.041,**[c]*0.933* | [a]***$5.7\times10^{-5}$***, [b]**0.038** |
| **Active GLP-1 (pmol/L)** | 4.26(3.92-4.71) | 3.94(3.44-4.56) | 3.53(3.05-4.09) | 3.74 (3.50-4.02) | 3.71(3.48-3.94) |
| **p-value** | | [a]*0.340* | [a]**0.028**, [b]*0.270* | [a]***0.020,*** [b]*0.487,*[c]*0.479* | [a]***0.010***, [b]*0.397* |

The results presented represent geometric means (95% confidence interval of mean), adjusted for age, gender and race, [a]vs control group: [b]vs non-diabetic metabolic syndrome group: [c]vs type 2 diabetes mellitus without metabolic syndrome which evaluated by univariate (General Linear Model). Bold values are significant. MetS: metabolic syndrome.

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### 4.2.3 Correlation between sCD26/DPP-IV Levels and Active GLP-1 Levels among Normal, Non-Diabetic Metabolic Syndrome, Type 2 Diabetes Mellitus Subjects with and without Metabolic Syndrome

The Spearman partial correlation showed that sCD26/DPP-IV levels were negatively correlated with active GLP-1 levels in both T2DM patients with MetS (rs = -0.324; $p < 0.001$), and T2DM patients without MetS (rs = −0.299; $p < 0.001$), after adjustment for the confounders (age, race, and gender), and the results are depicted in Table 4.6.

**Table 4.6:** Correlation between of fasting serum levels of sCD26/DPP-IV and active GLP-1 among normal, non-diabetic metabolic syndrome, type 2 diabetes mellitus subjects with and without metabolic syndrome.

| Group | | $r^s$ | (P-value) |
|---|---|---|---|
| Active GLP-1 (pmol/L) | | | |
| Normal Control | 164 | -0.198 | *0.019* |
| Non-diabetes | 71 | -0.139 | *0.303* |
| Type 2 diabetes without MetS | 57 | -0.299 | *< 0.001* |
| Type 2 diabetes with | 257 | -0.324 | *< 0.001* |

The results are presented as rs and (P-value) assessed by Spearman partial correlation adjusted for, age, race and gender. Bold values are significant. MetS: metabolic syndrome.

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### 4.2.4 Associations between sCD26/DPP-IV Levels with Metabolic Syndrome, and AIC among Type 2 Diabetes Mellitus Patients.

The multiple linear regression with sCD26/DPP-IV as dependent variable in diabetic patients has shown that sCD26/DPP-IV was significantly associated with increased AIC levels (B = 19.96, $p = 0.009$), but not associated with insulin resistance (B = 2.44, $p = 0.525$), triglyceride and HDL-cholesterol (B = 0.492, $p = 0.964$), (B = 27.6, $p = 0.610$), respectively, after adjustment for the possible confounders (age, race, gender, and disease duration of T2DM) and the results are depicted in Table 4.7. Serum sCD26/DPP-IV levels were not associated with any parameters of diabetes, blood pressure, or dyslipidemia.

**Table 4.7:** Association of fasting serum levels of sCD26/DPP-IV with metabolic syndrome, and AIC among type 2 diabetes mellitus patients.

| Parameters | B | (P-value) |
|---|---|---|
| **Body Mass Index (kg/m$^2$)** | 4.64 | 0.999 |
| **HOMA-β (%)** | -0.068 | 0.693 |
| **Insulin resistance** | 2.44 | 0.525 |
| **Glycosylated AIC (%)** | 19.96 | ***0.009*** |
| **Triglyceride (mmol/L)** | 0.492 | 0.964 |
| **HDL-cholesterol(mmol/L)** | 27.60 | 0.610 |

The results are presented as unstandardized coefficients; B and (P-value) assessed using multiple linear regression adjusted for, age, race, gender, and duration of diabetes. Bold values are significant. B: coefficient for the relationship between the dependent variable “sCD26/DPP-IV level” and the independent variable “diabetic and metabolic biomarker”. The positive sign of the coefficient implies a direct relationship, and the negative sign implies an inverse relationship.

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### 4.2.5 Associations between sCD26/DPP-IV Levels and Antidiabetic Agents

Diabetic subjects were divided into 4 subgroups: those receiving monotherapy with metformin (n = 34); those receiving combination therapy without metformin (instead receiving SU and TZD with or without insulin; n = 28); those receiving combination therapy with metformin (metformin, SU, and TZD with or without insulin) (n = 241); and non-treated diabetes (n = 8) who were newly diagnosed by physicians/endocrinologists.

The general linear model indicated that patients treated with monotherapy (metformin) had significantly lower serum levels of sCD26/DPP-IV [1048(972-1130) ng/mL] than patients receiving combination therapy without metformin [1355(1228-1451) ng/mL] ($p = 2.9\times10\text{-}5$). Likewise, patients treated with combination therapy that included metformin had significantly lower serum levels of sCD26/DPP-IV [1205(1172-1240) ng/mL] than patients receiving combination therapy without metformin [1355(1228-1451) ng/mL] ($p = 0.023$) (Table 4. 8)

This association was confirmed by multiple linear regression analysis, and the results remained significant only for patients treated with monotherapy that included metformin (B = -201.6, $p = 0.041$), after adjustment for the possible confounders (age, gender, race, BMI, AIC, and duration of T2DM) (Table 4. 9).

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**Table 4.8:** Comparison of fasting serum levels of sCD26/DPP-IV between combination therapy without metformin, combination therapy with metformin, monotherapy with metformin and non- treated among subjects with type 2 diabetes.

| Parameters | Sulfonylurea + Thiazolidinedione with/without insulin (n=28) | Metformin + Sulfonylurea + Thiazolidinedione with/without insulin (n=241) | Monotherapy with metformin (n=34) | Non-treated (n=8) |
|---|---|---|---|---|
| **sCD26/DPP-IV (ng/mL)** | 1355(1228-1451) | 1205(1172-1240) | 1048(972-1130) | 1123(960-1314) |
| **p-value** | | [a]***0.023*** | [a]***$2.9\times10^{-5}$*** , [b]***0.001*** | [a]*0.056*, [b]*0.385*, [c]*0.432* |

The results are presented represent geometric means (SD), adjusted for age, gender, and race, [a]vs combination therapy without metformin (Sulfonylurea + Thiazolidinedione with or without insulin): [b]vs combination therapy with metformin (metformin + Sulfonylurea + Thiazolidinedione with or without insulin): [c]vs monotherapy with Metformin which evaluated by univariate (General Linear Model). Bold values are significant.

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**Table 4.9:** Association of fasting serum levels of sCD26/DPP-IV with antidiabetes medications groups among type 2 diabetes patients.

| Parameters | B | (P-value) |
|---|---|---|
| **Sulfonylurea + Thiazolidinedione with/without insulin** | 24.0 | *0.760* |
| **Metformin + Sulfonylurea + Thiazolidinedione with/without insulin** | -131.6 | *0.120* |
| **Monotherapy with Metformin** | -201.6 | ***0.041*** |
| **Non-treated** | -97.4 | *0.085* |

The results are presented as unstandardized coefficients; B and (P-value) assessed using multiple linear regression adjusted for, age, race, gender, BMI, AIC, and duration of diabetes. Bold values are significant. B: coefficient for the relationship between the dependent variable "sCD26/DPP-IV levels" and the independent variable "diabetic drugs". The positive sign of the coefficient implies a direct relationship, and the negative sign implies an inverse relationship. *(Sulfonylurea (SU), Thiazolidinedione (TZD) with or without insulin.

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### 4.2.6 Association of sCD26/DPP-IV Levels with Metabolic Syndrome and Type 2 Diabetes Mellitus Parameters among Normal Malaysian Subjects

The associations between serum levels of sCD26/DPP-IV, MetS and T2DM parameters in subjects without either MetS or diabetes mellitus (control group) were assessed by hierarchical linear regression after adjustment for (age, race, and gender). The analysis showed that serum levels of sCD26/DPP-IV were associated with increased in BMI (B = 0.003, $p = 0.023$), cholesterol (B = 0.001, $p = 0.001$), and LDL-c (B = 0. 001, $p = 0.001$) and the results are depicted in (Table 4. 10).

**Table 4.10:** Association of fasting serum levels of sCD26/DPP-IV with diabetic and metabolic parameters among normal subjects

| Parameters | B | $r^2$ | (P-value) |
|---|---|---|---|
| **Body Mass Index (kg/m2)** | 0.003 | 0.105 | **0.023** |
| **Waist circumference(cm)** | 0.006 | 0.284 | 0.055 |
| **Triglyceride (mmol/L)** | 0.0003 | 0.116 | 0.134 |
| **Cholesterol (mmol/L)** | 0.001 | 0.107 | **0. 001** |
| **LDL-cholesterol(mmol/L)** | 0.001 | 0.094 | **0.001** |
| **HDL-cholesterol(mmol/L)** | -2.461 | 0.333 | 0.796 |
| **Fasting Blood Sugar (mmol/L)** | 0.0001 | 0.077 | 0.420 |
| **Glycosylated AIC (%)** | 0.0001 | 0.026 | 0.531 |
| **HOMA-β (%)** | 0.017 | 0.053 | 0.125 |
| **Insulin resistance** | 0.0003 | 0.070 | 0.073 |

The results are presented as unstandardized coefficients; B, $r^2$ and (P-value) assessed using hierarchical linear regression adjusted for, age, race, and gender. Bold values are significant. B: coefficient for the relationship between the dependent variable "metabolic syndrome and T2DM parameters" and the independent variable "sCD26/DPP-IV level." The positive sign of the coefficient implies a direct relationship, and the negative sign implies an inverse relationship.

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### 4.2.7 Active GLP-1 Levels and sCD26/DPP-IV in Obese Non-Diabetic Subjects and their Association with Insulin Resistance

In the current study, active GLP-1 levels and sCD26/DPP-IV were also assessed in the non-Diabetic Subjects. They were divided based on BMI measurements of the obesity and overweight diagnostic criteria in the Asian and Malaysian population (Ismail *et al.*, 2004; WHO, 2004); 66 subjects were defined as normal weight (BMI between 18.5 and 22.9 kg/m$^2$), 97 subjects were overweight (BMI between 23 and 27.4 kg/m$^2$) and 59 subjects were obese (BMI ≥ 27.5 kg/m$^2$). 13 subjects were underweight (BMI < 18.5 kg/m2) and were excluded from subsequent analysis.

The general linear model after adjusting for possible confounders (gender, age and race) has shown that serum sCD26/DPP-IV levels were significantly higher in obese subjects [1229(1156-1302) ng/mL] ($p = 0.034$) compared to normal weight subjects [1083(1013-1153) ng/mL]. In line with the prononced increase in sCD26/DPP-IV levels, active GLP-1 levels were significantly lower in obese [3.88(3.33-4.53) pmol/L] ($p = 0.021$) and borderline in overweight [4.16 (3.69-4.69) pmol/L] ($p = 0.057$) in comparison to normal weight subjects [5.37(4.63-6.22) pmol/L] (Table 4. 11).

Association of sCD26/DPP-IV levels with insulin resistance and other metabolic parameters were evaluated after adjusting for age, gender, and race using linear regression. sCD26/DPP-IV levels were associated with insulin resistance (B = 82.28, $p$ = 0.023), in obese subjects. Remarkably, sCD26/DPP-IV levels were not associated with any parameters of dyslipidemia, diabetes, or blood pressure (Table 4. 12).

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**Table 4.11:** Comparison of fasting serum levels of sCD26/DPP-IV and active GLP-1 between normal weight, overweight, and obese subjects

| Parameters | Normal weight subjects =66 | Overweight subjects =97 | P-Value | Obese subjects = 59 | P-Value |
|---|---|---|---|---|---|
| **sCD26/DPP-IV (ng/mL)** | 1083(1013-1153) | 1112(1056-1169) | *[a]0.372* | 1229(1156-1302) | ***[a]0.034,*** *[b]0.082* |
| **Active GLP-1 (pmol/L)** | 5.37(4.63-6.22) | 4.16 (3.69-4.69) | *[a]0.057* | 3.88(3.33-4.53) | ***[a]0.021***, *[b]0.999* |

The results represent geometric means and 95% confidence interval of mean, adjusted for age, race and gender. [a]vs normal weight group: [b]vs over weight group which were assessed by the General Linear Mode. Bold values are significant.

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**Table 4.12:** Association of sCD26/DPP-IV levels with insulin resistance and other metabolic parameters among obese subjects

| Parameters | B | P-Value | 95.0% Confidence Interval for B | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| **Waist Circumference (cm)** | 3.36 | 0.414 | -4.86 | 11.9 |
| **Systolic Blood Pressure (mmHg)** | 0.643 | 0.828 | -5.31 | 6.59 |
| **Diastolic Blood Pressure (mmHg)** | -2.79 | 0.511 | -11.31 | 5.72 |
| **Plasma Glucose (mmol/L)** | -94.4 | 0.090 | -214.3 | 15.5 |
| **Total-Cholesterol (mmol/L)** | -55.2 | 0.703 | -345.5 | 235.2 |
| **HDL-cholesterol (mmol/L)** | -80.28 | 0.677 | -305.9 | 466.4 |
| **LDL-cholesterol (mmol/L)** | 113.8 | 0.401 | -157.3 | 385.1 |
| **Triglyceride (mmol/L)** | 75.4 | 0.376 | -94.6 | 245.3 |
| **Insulin resistance** | 82.28 | **0.023** | 11.88 | 152.7 |

Linear regression analysis was performed between serum sCD26/DPP-IV levels and insulin resistance as well as other metabolic parameters adjusted for age, age, race and gender.

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## 4.3 GENETIC ANALYSIS

In this study, ten *DPP4* SNPs were successfully genotyped in 314 patients with T2DM and 235 non-diabetic. The potential effects of *DPP4* loci on T2DM susceptibility in Malaysian case-control samples were explored.

### 4.3.1 Hardy-Weinberg Equilibrium Test for the *DPP4* SNPs Genotyped in Malaysian Subjects.

*DPP4* SNPs; rs1558957, rs17574, rs7608798, rs1014444, rs2160927, rs12617656, rs4664443, rs6733162, rs1861978 did not deviated from Hardy-Weinberg Equilibrium (P-value = 0.180, 0.77, 0.34, 0.35, 0.12, 0.11, 0.730, 0.06, 0.39 in the control group respectively) (Table 4.13). However, SNP, rs3788979 showed deviation from Hardy Weinberg equilibrium (*P*-value = 0.031); consequently, it was excluded from the subsequent analysis (Table 4.13).

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**Table 4.13:** Association Hardy-Weinberg Equilibrium test for the *DPP4* SNPs genotyped in Malaysian subjects.

| ***DPP4* SNPs** | **Alleles** | **SNP Position on Chromosome 2*** | **HWE P-Value** | | **Call Rate %** |
|---|---|---|---|---|---|
| | | | **Control** | **Case** | |
| rs1558957 | C > T | 162,033,665 | 0.18 | 0.002 | 99.6 |
| rs3788979 | A < G | 162,044,379 | **0.031** | 0.57 | 99.7 |
| rs17574 | T < C | 162,073,469 | 0.77 | 1.000 | 99.5 |
| rs7608798 | A < G | 162,033,707 | 0.34 | 0.04 | 99.1 |
| rs1014444 | G < A | 162,012,348 | 0.35 | 0.08 | 99.5 |
| rs2160927 | T < C | 162,034,565 | 0.12 | 0.03 | 99.6 |
| rs12617656 | C < T | 161,994,637 | 0.11 | 0.09 | 99.6 |
| rs4664443 | G > A | 162,029,379 | 0.730 | 0.30 | 98.8 |
| rs6733162 | G < C | 162,056,988 | 0.06 | 0.04 | 99.6 |
| rs1861978 | G < T | 162,073,199 | 0.39 | 0.53 | 98.7 |

HWE: Hardy-Weinberg Equilibrium.* Location on chromosome 2 based on dbSNP Hap-Map (forward strand at NCBI build 38).

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### 4.3.2 Association of *DPP4* Polymorphism with Type 2 Diabetes Mellitus among Malaysian Subjects.

The dominant, additive, and recessive genetic models were employed to test the association of *DPP4* polymorphisms with T2DM. The logistic regression models were adjusted for age, race, gender and BMI. Frequency of risk allele of rs12617656 (T), in normal subjects were 0.38, versus 0.62, 0.62, 0.63 in total T2DM patients, T2DM with MetS and T2DM without MetS respectively.

The logistic regression models showed that SNP rs12617656 in Malaysian subjects was associated with Total T2DM, under the recessive genetic model, (OR (95%CI) = 1.98(1.22-3.20); $p$ = 0.006), and with T2DM subjects with MetS OR (95%CI) = 2.20(1.30-3.72); $p$ = 0.003). The dominant model showed association between SNP rs12617656 with Total T2DM OR (95%CI) =1.95(1.19-3.19); $p$ = 0.008), and T2DM subjects without MetS OR (95%CI) = 4.48(1.73-11.56); $p$ = 0.002). The additive model showed an association of rs12617656 with Total T2DM, OR (95%CI) =1.63(1.22-2.19); $p$ = 0.001), and with T2DM subjects with MetS OR (95%CI) = 1.59 (1.16-2.18); $p$ = 0.004) and T2DM without MetS OR (95%CI) = 1.86 (1.18-2.92); $p$ = 0.007). On the other hand, no association was noted between rs12617656 and non-diabetic subjects with MetS. (Table 4.14).

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**Table 4.14:** Association of *DPP4* rs12617656 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs12617656 Polymorphisms C < T | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | Control (n=164) | MetS* (n=71)) | without MetS* (n=57) | With MetS* (n=257) | |
| **Risk Allele frequency (T) n(frequency)** | 75(0.46) | 37(0.52) | 36(0.63) | 160(0.62) | 196(0.62) |
| **Homozygous (C-C) n(frequency)** | 54(0.33) | 16(0.22) | 6(0.10) | 46(0.18) | 52(0.17) |
| **Heterozygous (C-T) n(frequency)** | 71(0.43) | 36(0.51) | 30(0.53) | 103(0.40) | 133(0.42) |
| **Homozygous (T-T) n(frequency)** | 39(0.24) | 19(0.27) | 21(0.37) | 108(0.42) | 129(0.41) |
| **Recessive Model** | Odds ratio | 1.14 | 1.59 | 2.20 | 1.98 |
| | 95% CI | 0.55-2.37 | 0.80-3.16 | 1.30-3.72 | 1.22-3.20 |
| | P-Value | 0.73 | 0.19 | **0.003** | **0.006#** |
| **Dominant Model** | Odds ratio | 1.48 | 4.48 | 1.64 | 1.95 |
| | 95% CI | 0.71-3.06 | 1.73-11.56 | 0.96-2.80 | 1.19-3.19 |
| | P-Value | 0.30 | ***0.002*** | 0.07 | **0.008#** |
| **Additive Model** | Odds ratio | 1.21 | 1.86 | 1.59 | 1.63 |
| | 95% CI | 0.78-1.87 | 1.18-2.92 | 1.16-2.18 | 1.22-2.19 |
| | P-Value | 0.40 | ***0.007*** | **0.004** | **0.001#** |

Recessive model; odds ratio (T-T *vs.* (C-T + C-C)). Dominant model; odds ratio ((T-T + C-T) *vs.* C-C). The additive model; common odds ratio (T-T *vs.* C-T *vs.* C-C), additive model was re-coded for the count of the risk allele T, which is 0 within genotype C-C, 1 within genotype C-T, and 2 within genotype T-T.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded. # Adjusted for multiple comparisons using the false discovery rate at the 5% level. Bold values are significant.

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Frequency of risk allele of rs4664443 (A), in normal subjects were 0.13, versus 0.21, 0.21, and 0.25 in total T2DM patients, T2DM with MetS and without MetS respectively.

The logistic regression models (adjusted for age, race, gender and BMI) showed that SNP rs4664443 was associated with Total T2DM, under the additive genetic model, OR (95%CI) =1.53 (1.02-2.28); $p = 0.039$), and with T2DM subjects with MetS OR (95%CI) = 1.60 (1.01-2.52); $p = 0.046$). The dominant model showed a borderline association with Total T2DM OR (95%CI) = 1.60 (0.98-2.62); $p = 0.061$), and no association with T2DM with or without MetS, while, the additive model of rs4664443 showed no association with T2DM. On the other hand, no association was noticed between rs12617656 and non-diabetic subjects with MetS. Despite this, association for SNP rs4664443 with T2DM were no longer significant after multiple comparisons testing, with a false discovery rate of 5%, was applied but it may still have clinical significance (Table 4.15).

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**Table 4.15:** Association of *DPP4* rs4664443 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs4664443 Polymorphisms G > A | Non-diabetes Control (n=164) | Non-diabetes MetS* (n=71)) | Type 2 diabetes mellitus without MetS* (n=57) | Type 2 diabetes mellitus With MetS* (n=257) | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| **Risk Allele frequency (A) n(frequency)** | 22(0.13) | 13(0.18) | 14(0.25) | 53(0.21) | 67(0.21) |
| **Homozygous (A-A) n(frequency)** | 5(0.03) | 5(0.07) | 5(0.09) | 12 (0.05) | 19(0. 06) |
| **Heterozygous (A-G) n(frequency)** | 34(0.20) | 16(0.23) | 17(0.30) | 81(0.31) | 96(0.31) |
| **Homozygous (G-G) n(frequency)** | 125(0.76) | 50(0.70) | 35(0.61) | 164(0.64) | 199(0.63) |
| **Recessive Model** | Odds ratio | 2.95 | 3.50 | 2.96 | 2.21 |
| | 95% CI | 0.57-15.17 | 0.77-15.97 | 0.72-12.10 | 0.75-6.54 |
| | P-Value | 0.20 | 0.11 | 0.13 | 0.153 |
| **Dominant Model** | Odds ratio | 1.02 | 1.50 | 1.60 | 1.60 |
| | 95% CI | 0.48-2.18 | 0.75-3.02 | 0.94-2.73 | 0.98-2.62 |
| | P-Value | 0.96 | *0.25* | 0.085 | 0.061 |
| **Additive Model** | Odds ratio | 1.19 | 1.58 | 1.60 | 1.53 |
| | 95% CI | 0.64-2.20 | 0.90-2.80 | 1.01-2.52 | 1.06-2.28 |
| | P-Value | 0.58 | *0.11* | **0.046[#]** | **0.039[#]** |

Recessive model; odds ratio (A-A *vs.* (A-G + G-G)). Dominant model; odds ratio ((A-A + A-G) *vs.* G-G). The additive model; common odds ratio (A-A *vs.* A-G *vs.* G-G), additive model was re-coded for the count of the risk allele A, which is 0 within genotype G-G, 1 within genotype A-G, and 2 within genotype G-G.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded. [#] Adjusted for multiple comparisons using the false discovery rate at the 5% level. Bold values are significant.

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Frequency of risk allele of rs7633162 (C), in normal subjects were 0.54, versus 0.61, 0.61, 0.67 in total T2DM patients, T2DM with MetS and without MetS, respectively. Logistic regression models (adjusted for age, race, gender and BMI) showed that rs7633162 was associated with Total T2DM, under the additive genetic model, OR (95%CI) =1.42 (1.06-1.90); $p$ = 0.020), and showed a borderline association in T2DM subjects with MetS OR (95%CI) = 1.37 (0.99-1.89); $p$ = 0.057), as well as. T2DM subjects without MetS OR (95%CI) = 1.53 (0.99-2.38); $p$ = 0.058).

The dominant model of rs7633162 showed a borderline association with Total T2DM OR (95%CI) = 1.67 (1.00-2.82); $p$ = 0.052), and no association with T2DM subjects with or without MetS. Likewise, the recessive model of rs7633162 showed a borderline association with Total T2DM OR (95%CI) = 1.56 (1.00-2.46); $p$ = 0.053) and no association with T2DM subjects with or without MetS. On the other hand, no association was noticed between rs12617656 and non-diabetic subjects with MetS.

Despite this, association for SNP rs7633162 with T2DM did not remain significant after multiple comparisons testing was applied, with a false discovery rate of 5%, but may have clinical significance (Table 4.16).

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**Table 4.16:** Association of *DPP4* rs7633162 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs7633162 Polymorphisms G < C | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | **Control (n=164)** | **MetS* (n=71))** | **without MetS* (n=57)** | **With MetS* (n=257)** | |
| **Risk Allele frequency (C) n(frequency)** | 88(0.54) | 37(0.52) | 38(0.67) | 156(0.61) | 194(0.61) |
| **Homozygous (C-C) n(frequency)** | 53(0.32) | 24(0.34) | 26(0.46) | 101(0.39) | 127(0.40) |
| **Heterozygous (C-G) n(frequency)** | 69(0.42) | 25(0.35) | 23(0.40) | 109(0.42) | 132(0.42) |
| **Homozygous (G-G) n(frequency)** | 42(0.26) | 22(0.31) | 8(0.14) | 47(0.18) | 55(0.18) |
| **Recessive Model** | Odds ratio | 0.93 | 1.69 | 1.42 | 1.56 |
| | 95% CI | 0.46-1.90 | 0.89-3.23 | 0.87-2.34 | 1.00-2.46 |
| | P-Value | 0.85 | 0.11 | 0.16 | **0.053**[#] |
| **Dominant Model** | Odds ratio | 0.75 | 1.98 | 1.69 | 1.67 |
| | 95% CI | 0.37-1.53 | 0.84-4.69 | 0.95-3.02 | 1.00-2.82 |
| | P-Value | 0.43 | *0.12* | 0.08 | **0.052**[#] |
| **Additive Model** | Odds ratio | 0.88 | 1.53 | 1.37 | 1.42 |
| | 95% CI | 0.57-1.36 | 0.99-2.38 | 0.99-1.89 | 1.06-1.90 |
| | P-Value | 0.56 | *0.058* | 0.057 | **0.020**[#] |

Recessive model; odds ratio (C-C *vs.* (C-G + G-G)). Dominant model; odds ratio (C-C + C-G) *vs.* G-G). The additive model; common odds ratio (C-C *vs.* C-G *vs.* G-G), additive model was re-coded for the count of the risk allele C, which is 0 within genotype G-G, 1 within genotype C-G, and 2 within genotype C-C.

*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded. * Adjusted for multiple comparisons using the false discovery rate at the 5% level. Bold values are significant.

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Risk allele frequencies of rs1861978 (T), rs2160927(C), rs1558957 (T), rs17574 (C), rs7608798 (G), and rs1014444 (A), in normal subjects were 0.89, 0.52, 0. 10, 0.84, 0.54, and 0.49 versus 0.90, 0.63, 0.11, 0.86, 0.61, and 0.59 in diabetic patients respectively.

The logistic regression models (adjusted for age, race, gender and BMI) showed that SNPs rs1861978, rs2160927, rs1558957, rs17574, rs7608798, rs1014444 were not associated with total T2DM and/or T2DM with or without MetS (Table 4. 17, Table 4. 18, Table 4. 19, Table 4. 20, Table 4. 21 and Table 4. 22).

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**Table 4.17:** Association of *DPP4* rs1861978 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs1861978 Polymorphisms G < T | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | Control (n=164) | MetS* (n=71)) | Without MetS* (n=57) | With MetS* (n=257) | |
| **Risk Allele frequency (T) n(frequency)** | 146(0.89) | 61(0.86) | 50(0.88) | 233(0.91) | 283(0.90) |
| **Homozygous (G-G) n(frequency)** | 3(0.02) | 2(0.03) | 0(0.00) | 4(0.02) | 4(0.01) |
| **Heterozygous (G-T) n(frequency)** | 30(0.18) | 16(0.22) | 15(0.26) | 40(0.15) | 55(0.18) |
| **Homozygous (T-T) n(frequency)** | 131(0.80) | 53(0.75) | 42(0.74) | 213(0.83) | 255(0.81) |
| **Recessive Model** | Odds ratio | 1.19 | 0.91 | 1.81 | 1.56 |
| | 95% CI | 0.54-2.61 | 0.43-1.94 | 0.96-3.41 | 0.89-2.74 |
| | P-Value | 0.67 | 0.81 | 0.06 | 0.12 |
| **Dominant Model** | Odds ratio | 0.53 | 8.3E | 1.16 | 1.61 |
| | 95% CI | 0.07-4.19 | 0.00-0.00 | 0.19-6.96 | 2.95-8.82 |
| | P-Value | 0.55 | *0.99* | 0.87 | 0.58 |
| **Additive Model** | Odds ratio | 1.07 | 1.04 | 1.59 | 1.48 |
| | 95% CI | 0.54-2.11 | 0.52-2.10 | 0.92-2.76 | 0.90-2.43 |
| | P-Value | 0.85 | *0.91* | 0.09 | 0.12 |

Recessive model; odds ratio (T-T *vs.* (G-T + G-G)). Dominant model; odds ratio ((T-T + G-T) *vs.* G-G). The additive model; common odds ratio (T-T *vs.* G-T *vs.* G-G), additive model was re-coded for the count of the risk allele T, which is 0 within genotype G-G, 1 within genotype G-T, and 2 within genotype T-T.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.18:** Association of *DPP4* rs2160927 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs2160927 Polymorphisms T< C | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | Control (n=164) | MetS* (n=71)) | Without MetS* (n=57) | WithMetS* (n=257) | |
| **Risk Allele frequency (C) n(frequency)** | 85(0.52) | 37(0.52) | 40(0.70) | 157(0.61) | 197(0.63) |
| **Homozygous (C-C) n(frequency)** | 49(0.30) | 18(0.25) | 21(0.37) | 105(0.41) | 126(0.40) |
| **Heterozygous (C-T) n(frequency)** | 72(0.44) | 37(0.52) | 28(0.49) | 103(0.40) | 131(0.42) |
| **Homozygous (T-T) n(frequency)** | 43(0.26) | 16(0.23) | 8(0.14) | 49(0.19) | 57(0.18) |
| **Recessive Model** | Odds ratio | 0.61 | 0.98 | 1.36 | 1.29 |
| | 95% CI | 0.29-1.31 | 0.49-1.98 | 0.82-2.23 | 0.82-2.05 |
| | P-Value | 0.21 | 0.96 | 0.23 | 0.28 |
| **Dominant Model** | Odds ratio | 0.89 | 1.78 | 0.99 | 1.21 |
| | 95% CI | 0.42-1.88 | 0.76-4.17 | 0.56-1.73 | 0.73-2.01 |
| | P-Value | 0.76 | *0.19* | 0.96 | 0.46 |
| **Additive Model** | Odds ratio | 0.80 | 1.18 | 1.13 | 1.18 |
| | 95% CI | 0.51-1.26 | 0.76-1.85 | 0.82-1.54 | 0.88-1.58 |
| | P-Value | 0.33 | *0.46* | 0.46 | 0.27 |

Recessive model; odds ratio (C-C *vs.* (C-T + T-T)). Dominant model; odds ratio ((C-C + C-T) *vs.* T-T). The additive model; common odds ratio (C-C *vs.* C-T *vs.* T-T), additive model was re-coded for the count of the risk allele C, which is 0 within genotype T-T, 1 within genotype C-T, and 2 within genotype C-C.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.19:** Association of *DPP4* rs1558957 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs1558957 Polymorphisms C > T | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | Control (n=164) | MetS* (n=71)) | without MetS* (n=57) | With MetS* (n=257) | |
| Risk Allele frequency (T) n(frequency) | 16(0.10) | 8(0.11) | 6(0.11) | 27(0.11) | 33(0.11) |
| Homozygous (C-C) n(frequency) | 135(0.82) | 59(0.83) | 47(0. 82) | 211(0.82) | 258(0.82) |
| Heterozygous (C-T) n(frequency) | 26(0.16) | 9(0.13) | 9(0.16) | 38(0.15) | 47(0.15) |
| Homozygous (T-T) n(frequency) | 3(0.02) | 3(0.04) | 1(0.02) | 8(0.03) | 9(0.03) |
| Recessive Model | Odds ratio | 2.43 | 0.79 | 1.87 | 2.03 |
| | 95% CI | 0.39-15.22 | 0.07-8.36 | 0.42-8.26 | 0.50-8.33 |
| | P-Value | 0.34 | 0.84 | 0.41 | 0.33 |
| Dominant Model | Odds ratio | 0.56 | 0.68 | 0.79 | 0.85 |
| | 95% CI | 0.22-1.40 | 0.29-1.63 | 0.42-1.48 | 0.48-1.50 |
| | P-Value | 0.21 | *0.39* | 0.46 | 0.56 |
| Additive Model | Odds ratio | 0.78 | 0.73 | 0.92 | 0.98 |
| | 95% CI | 0.37-1.64 | 0.34-1.57 | 0.55-1.54 | 0.61-1.55 |
| | P-Value | 0.50 | *0.42* | 0.76 | 0.92 |

Recessive model; odds ratio (T-T *vs.* (C-T + C-C). Dominant model; odds ratio ((T-T + C-T) *vs.* C-C). The additive model; common odds ratio (T-T *vs.* C-T *vs.* C-C), additive model was re-coded for the count of the risk allele T, which is 0 within genotype C-C, 1 within genotype C-T, and 2 within genotype T-T.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.20:** Association of *DPP4* rs17574 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs17574 Polymorphisms T < C | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | Control (n=164) | MetS* (n=71)) | Without MetS* (n=57) | With MetS* (n=257) | |
| **Risk Allele frequency (C) n(frequency)** | 137(0.84) | 59(0.83) | 47(0.82) | 224(0.87) | 271(0.86) |
| **Homozygous (C-C) n(frequency)** | 115(0.70) | 50(0.71) | 38(0.67) | 195(0.76) | 233 (0.74) |
| **Heterozygous (C-T) n(frequency)** | 44(0.27) | 18(0.25) | 18(0.31) | 57(0.22) | 75(0.24) |
| **Homozygous (T-T) n(frequency)** | 5(0.03) | 3(0.04) | 1(0.02) | 5(0.02) | 6(0.02) |
| **Recessive Model** | Odds ratio<br>95% CI<br>P-Value | 1.12<br>0.55-2.31<br>0.75 | 1.00<br>0.51-1.97<br>0.99 | 1.52<br>0.89-2.61<br>0.13 | 1.34<br>0.83-2.16<br>0.24 |
| **Dominant Model** | Odds ratio<br>95% CI<br>P-Value | 0.45<br>0.09-2.37<br>0.35 | 1.66<br>0.18-15.54<br>*0.66* | 1.27<br>0.27-5.94<br>0.77 | 1.36<br>0.34-5.34<br>0.66 |
| **Additive Model** | Odds ratio<br>95% CI<br>P-Value | 0.99<br>0.54-1.81<br>0.96 | 1.04<br>0.57-1.91<br>*0.89* | 1.42<br>0.88-2.28<br>0.15 | 1.29<br>0.84-1.96<br>0.24 |

Recessive model; odds ratio (C-C *vs.* (C-T + T-T)). Dominant model; odds ratio ((C-C + C-T) *vs.* T-T). The additive model; common odds ratio (C-C *vs.* C-T *vs.* T-T), additive model was re-coded for the count of the risk allele C, which is 0 within genotype T-T, 1 within genotype C-T, and 2 within genotype C-C.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.21:** Association of *DPP4* rs7608798 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| rs7608798 Polymorphisms A < G | Non-diabetes | | Type 2 diabetes mellitus | | Total type 2 diabetes* (n=314) |
|---|---|---|---|---|---|
| | **Control (n=164)** | **MetS* (n=71))** | **Without MetS* (n=57)** | **With MetS* (n=257)** | |
| **Risk Allele frequency (G) n(frequency)** | 88(0.54) | 38(0.54) | 36(0.63) | 154(0.60) | 190(0.61) |
| **Homozygous (A-A) n(frequency)** | 39(0.24) | 15(0.21) | 8(0.14) | 50(0.20) | 58(0.19) |
| **Heterozygous (A-G) n(frequency)** | 75(0.46) | 36(0.51) | 27(0.47) | 106(0.41) | 133(0.42) |
| **Homozygous (G-G) n(frequency)** | 50(0.30) | 20(0.28) | 22(0.39) | 101(0.39) | 123(0.39) |
| **Recessive Model** | Odds ratio<br>95% CI<br>P-Value | 0.88<br>0.43-1.79<br>0.72 | 1.21<br>0.62-2.36<br>0.58 | 1.35<br>0.82-2.23<br>0.24 | 1.32<br>0.84-2.09<br>0.24 |
| **Dominant Model** | Odds ratio<br>95% CI<br>P-Value | 1.25<br>0.57-2.73<br>0.58 | 1.57<br>0.66-3.70<br>*0.31* | 0.81<br>0.46-1.43<br>0.47 | 1.01<br>0.60-1.70<br>0.96 |
| **Additive Model** | Odds ratio<br>95% CI<br>P-Value | 0.88<br>0.55-1.39<br>0.58 | 1.24<br>0.79-1.95<br>*0.35* | 1.06<br>0. 77-1.46<br>0.73 | 1.13<br>0.84-1.51<br>0.43 |

Recessive model; odds ratio (G-G *vs.* (A-G + A-A)). Dominant model; odds ratio ((G-G + A-G) *vs.* A-A). The additive model; common odds ratio (G-G *vs.* A-G *vs.* A-A), additive model was re-coded for the count of the risk allele G, which is 0 within genotype A-A, 1 within genotype A-G, and 2 within genotype G-G.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.22:** Association of *DPP4* rs1014444 polymorphism with diabetes with and without metabolic syndrome and total type 2 diabetes mellitus.

| **rs1014444 Polymorphisms G < A** | **Non-diabetes** | | **Type 2 diabetes mellitus** | | **Total type 2 diabetes* (n=314)** |
|---|---|---|---|---|---|
| | **Control (n=164)** | **MetS* (n=71))** | **without MetS* (n=57)** | **With MetS* (n=257)** | |
| **Risk Allele frequency (A) n(frequency)** | 80(0.49) | 34(0.48) | 34(0.60) | 150(0.58) | 184(0.59) |
| **Homozygous (A-A) n(frequency)** | 42(0.26) | 14(0.20) | 17(0.30) | 98(0.38) | 115(0.37) |
| **Heterozygous (A-G) n(frequency)** | 76(0.46) | 39(0.55) | 33(0.58) | 104(0.41) | 137(0.43) |
| **Homozygous G-G) n(frequency)** | 46(0.28) | 18(0.25) | 7(0.12) | 55(0.21) | 62(0.20) |
| **Recessive Model** | Odds ratio | 0.56 | 0.88 | 1.48 | 1.40 |
| | 95% CI | 0.25-1.25 | 0.42-1.84 | 0.88-2.48 | 0.87-2.26 |
| | P-Value | 0.16 | 0.73 | 0.14 | 0.17 |
| **Dominant Model** | Odds ratio | 0.99 | 2.38 | 1.00 | 1.23 |
| | 95% CI | 0.48-2.05 | 0.98-5.77 | 0.58-1.71 | 0.75-2.02 |
| | P-Value | 0.98 | *0.056* | 0.99 | 0.41 |
| **Additive Model** | Odds ratio | 0. 82 | 1.25 | 1.16 | 1.22 |
| | 95% CI | 0.52-1.29 | 0.79-1.97 | 0.84-1.59 | 0.91-1.64 |
| | P-Value | 0.38 | *0.35* | 0.36 | 0.18 |

Recessive model; odds ratio (A-A *vs.* (A-G + G-G)). Dominant model; odds ratio ((A-A + A-G) *vs.* G-G). The additive model; common odds ratio (A-A *vs.* A-G *vs.* G-G), additive model was re-coded for the count of the risk allele A, which is 0 within genotype G-G, 1 within genotype A-G, and 2 within genotype A-A.
*Controlled for age, race, gender and BMI. MetS: metabolic syndrome. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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### 4.3.3 Association of *DPP4* Polymorphism with Type 2 Diabetes Mellitus among Malaysian Ethnic Groups

Analysis of the association of *DPP4* SNPs with T2DM subjects among the three main Malaysian races (Malay, Chinese and Indian), was performed by using logistic regression model after adjustment for possible covariates (age, gender and BMI). Frequency of risk allele of rs12617656 (T), in normal Malay, Chinese, and Indian (0.49, 0.38, and 0.50) versus T2DM (0.56, 0.49, and 0.79 respectively).

The additive genetic model revealed that SNP rs12617656 was associated with T2DM among Malaysians of Indian ethnicity OR (95%CI) =2.29 (1.24-4.24) *p* =0.0009).The recessive genetic model revealed that rs12617656 with T2DM among Malaysians of Indian ethnicity OR (95%CI) =3.21 (1.21-8.62); *p* =0.019). Likewise, the dominant genetic model revealed that OR (95%CI) =3.72 (1.23-10.43); *p* =0.003) On the other hand, SNP rs12617656 slowed no association with T2DM among subjects of Malay and Chinese ethnicity (Table 4.23).

The additive genetic model showed that SNP rs7633162 was significantly associated with T2DM among of the Malay ethnicity (additive, OR (95%CI) =1.53 (1.01-2.30); *p* =0.044) with T2DM (Table 4.24). However, this association did not remain significant after multiple testing with a false discovery rate of 5% was applied. The dominant genetic model showed a borderline association between SNP rs7633162 with T2DM in the Malay subjects (Dominant OR = 1.97, *p* = 0.062) (Table 4.24).

SNP rs4664443 showed no association between T2DM and ethnic groups (Table 4.25). Similarly, the logistic regression models (adjusted for age, gender and BMI) showed that SNPs rs1861978, rs2160927, rs1558957, rs17574, rs7608798, rs1014444 were not significantly associated with T2DM in any ethnic groups (Table 4. 26, Table 4. 27, Table 4. 28, Table 4. 29, Table 4. 30 and Table 4. 31).

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**Table 4.23:** Association of *DPP4* rs12617656 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups

| rs12617656 Polymorphisms C < T | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | **Malay n=86** | **Chinese n=52** | **Indian n=26** | **Malay n=153** | **Chinese n= 53** | **Indian n=108** |
| **Risk Allele frequency (T) n(frequency)** | 42(0.49) | 20(0.38) | 13(0.50) | 85(0.56) | 26(0.49) | 85(0.79) |
| **Homozygous (C-C) n(frequency)** | 24(0.28) | 20(0.38) | 10(0.38) | 27(0.18) | 16(0.30) | 9(0.08) |
| **Heterozygous (C-T) n(frequency)** | 40(0.46) | 25(0.48) | 6(0.23) | 82(0.53) | 22(0.42) | 29(0.27) |
| **Homozygous (T-T) n(frequency)** | 22(0.26) | 7(0.14) | 10(0.39) | 44(0.29) | 15(0.28) | 70(0.65) |
| **Recessive Model** | | Odds ratio | | 1.50 | 2.16 | 3.21 |
| | | 95% CI | | 0.74-3.03 | 0.65-7.13 | 1.21-8.62 |
| | | P-Value | | 0.250 | *0.208* | **0.019**[#] |
| **Dominant Model** | | Odds ratio | | 1.99 | 1.05 | 3.72 |
| | | 95% CI | | 0.99-4.21 | 0.40-2.73 | 1.23-10.43 |
| | | P-Value | | 0.051 | 0.924 | **0.003**[#] |
| **Additive Model** | | Odds ratio | | 1.52 | 1.30 | 2.29 |
| | | 95% CI | | 0.98-2.36 | 0.67-2.50 | 1.24-4.24 |
| | | P-Value | | 0.130 | *0.439* | **0.0009**[#] |

Recessive model; odds ratio (T-T *vs.* (C-T + C-C)). Dominant model; odds ratio ((T-T + C-T) *vs.* C-C). The additive model; common odds ratio (T-T *vs.* C-T *vs.* C-C), additive model was re-coded for the count of the risk allele T, which is 0 within genotype C-C, 1 within genotype C-T, and 2 within genotype T-T.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded. [#]Adjusted for multiple comparisons using the false discovery rate at the 5% level. Bold values are significant.

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**Table 4.24:** Association of *DPP4* rs6733162 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs6733162 Polymorphisms G < C | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | Malay n=86 | Chinese n=52 | Indian n=26 | Malay n=153 | Chinese n= 53 | Indian n=108 |
| **Risk Allele frequency (C) n(frequency)** | 46(0.53) | 23(0.44) | 20(0.77) | 89(0.58) | 31(0.58) | 78(0.72) |
| **Homozygous (C-C) n(frequency)** | 29(0.34) | 10(0.19) | 14(0.54) | 55(0.36) | 15(0.28) | 57(0.53) |
| **Heterozygous (C-G) n(frequency)** | 33(0.38) | 25(0.48) | 11(0.42) | 67(0.44) | 23(0.43) | 42(0.39) |
| **Homozygous (G-G) n(frequency)** | 24(0.28) | 17(0.33) | 1(0.04) | 31(0.20) | 15(0.28) | 9(0.08) |
| **Recessive Model** | | Odds ratio | | 1.67 | 2.00 | 0.99 |
| | | 95% CI | | 0.87-3.22 | 0.71-5.61 | 0.39-2.50 |
| | | P-Value | | 0.12 | *0.19* | 0.98 |
| **Dominant Model** | | Odds ratio | | 1.97 | 1.78 | 0.37 |
| | | 95% CI | | 0.97-4.02 | 0.66-4.80 | 0.04-3.24 |
| | | P-Value | | 0.062 | 0.26 | 0.37 |
| **Additive Model** | | Odds ratio | | 1.53 | 1.60 | 0.86 |
| | | 95% CI | | 1.01-2.30 | 0.86-2.99 | 0.41-1.178 |
| | | P-Value | | **0.044**# | *0.14* | 0.68 |

Recessive model; odds ratio (C-C *vs.* (C-G + G-G)). Dominant model; odds ratio ((C-C + C-G) *vs.* G-G). The additive model; common odds ratio (C-C *vs.* C-G *vs.* G-G), additive model was re-coded for the count of the risk allele C, which is 0 within genotype G-G, 1 within genotype C-G, and 2 within genotype C-C.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded. #Adjusted for multiple comparisons using the false discovery rate at the 5% level. Bold values are significant.

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**Table 4.25:** Association of *DPP4* rs4664443 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs4664443 Polymorphisms G > A | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | **Malay n=86** | **Chinese n=52** | **Indian n=26** | **Malay n=153** | **Chinese n= 53** | **Indian n=108** |
| **Risk Allele frequency (A) n(frequency)** | 10(0.13) | 3(0.06) | 9(0.35) | 20(0.14) | 4(0.08) | 43(0.40) |
| **Homozygous (A-A) n(frequency)** | 1(0.01) | 1(0.02) | 3(0.12) | 6(0.03) | 1(0.02) | 12(0.11) |
| **Heterozygous (A-G) n (frequency)** | 19(0.22) | 3(0.06) | 12(0.46) | 30(0.21) | 5(0.09) | 61(0.56) |
| **Homozygous (G-G) n(frequency)** | 66(0.77) | 48(0.92) | 11(0.42) | 117(0.76) | 47(0.89) | 35(0.32) |
| **Recessive Model** | | Odds ratio<br>95% CI<br>P-Value | | 3.35<br>0.55-52.1<br>0.123 | 3.32<br>0.18-60.3<br>*0.418* | 1.41<br>0.32-6.09<br>0.65 |
| **Dominant Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.30<br>0.63-2.68<br>0.472 | 1.83<br>0.40-8.43<br>0.436 | 1.61<br>0.62-4.17<br>0.328 |
| **Additive Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.43<br>0.77-2.66<br>0.256 | 1.61<br>0.62-4.17<br>*0.328* | 1.41<br>0.69-2.91<br>0.35 |

Recessive model; odds ratio (A-A *vs.* (A-G + G-G)). Dominant model; odds ratio ((A-A + A-G) *vs.* G-G). The additive model; common odds ratio (A-A *vs.* A-G *vs.* G-G), additive model was re-coded for the count of the risk allele A, which is 0 within genotype G-G, 1 within genotype A-G, and 2 within genotype G-G.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded

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**Table 4.26:** Association of *DPP4* rs1861978 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs1861978 Polymorphisms G < T | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | Malay n=86 | Chinese n=52 | Indian n=26 | Malay n=153 | Chinese n= 53 | Indian n=108 |
| **Risk Allele frequency (T) n(frequency)** | 77(0.90) | 51(0.98) | 19(0.73) | 144(0.94) | 53(1.0) | 86(0.80) |
| **Homozygous (G-G) n(frequency)** | 1(0.01) | 0(0.00) | 2(0.08) | 1(0.006) | 0 (0.02) | 3(0.03) |
| **Heterozygous (G-T) n(frequency)** | 17(0.20) | 3(0.06) | 10(0.38) | 16(0.10) | 1(0.55) | 38(0.35) |
| **Homozygous (T-T) n(frequency)** | 68(0.79) | 49(0.94) | 14(0.54) | 136(0.89) | 52(0.26) | 67(0.62) |
| **Recessive Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.40<br>0.61-3.23<br>0.43 | 2.39<br>0.21-27.67<br>*0.49* | 2.14<br>0.79-5.81<br>0.14 |
| **Dominant Model** | | Odds ratio<br>95% CI<br>P-Value | | 0.46<br>0.97-1.99<br>0.64 | 0.00<br>0.00-0.00<br>0.00 | 2.94<br>0.44-14.88<br>0.27 |
| **Additive Model** | | Odds ratio<br>95% CI<br>P-Value | | 2.39<br>1.28-2.78<br>0.49 | 1.28<br>0.21-27.67<br>*0.54* | 1.96<br>0.89-4.67<br>0.09 |

Recessive model; odds ratio (T-T *vs.* (G-T + G-G)). Dominant model; odds ratio ((T-T + G-T) *vs.* G-G). The additive model; common odds ratio (T-T *vs.* G-T *vs.* G-G), additive model was re-coded for the count of the risk allele T, which is 0 within genotype G-G, 1 within genotype G-T, and 2 within genotype T-T.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.27:** Association of *DPP4* rs2160927 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs2160927 Polymorphisms T< C | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | **Malay n=86** | **Chinese n=52** | **Indian n=26** | **Malay n=153** | **Chinese n= 53** | **Indian n=108** |
| **Risk Allele frequency (C) n(frequency)** | 46(0.53) | 20(0.38) | 20(0.77) | 87(0.57) | 21(0.40) | 84(0.78) |
| **Homozygous (C-C) n(frequency)** | 24(0.28) | 8(0.16) | 17(0.65) | 52(0.34) | 11(0.21) | 63(0.58) |
| **Heterozygous (C-T) n(frequency)** | 43(0.50) | 23(0.44) | 6(0.23) | 70(0.46) | 19(0.36) | 42(0.39) |
| **Homozygous (T-T) n(frequency)** | 19(0.22) | 21(0.40) | 3(0.12) | 31(0.20) | 23(0.43) | 3(0.08) |
| **Recessive Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.53<br>0.79-2.99<br>0.20 | 1.00<br>0.30-3.29<br>*0.99* | 0.84<br>0.32-2.22<br>0.72 |
| **Dominant Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.15<br>0.56-2.39<br>0.70 | 0.78<br>0.31-1.93<br>0.58 | 4.57<br>0.68-30.63<br>0.11 |
| **Additive Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.25<br>0.82-1.89<br>0.30 | 0.86<br>0.47-1.66<br>*0.70* | 1.14<br>0.52-2.50<br>0.75 |

Recessive model; odds ratio (C-C *vs.* (C-T + T-T)). Dominant model; odds ratio ((C-C + C-T) *vs.* T-T). The additive model; common odds ratio (C-C *vs.* C-T *vs.* T-T), additive model was re-coded for the count of the risk allele C, which is 0 within genotype T-T, 1 within genotype C-T, and 2 within genotype C-C.

*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.28:** Association of *DPP4* rs1558957 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs1558957 Polymorphisms C > T | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | Malay n=86 | Chinese n=52 | Indian n=26 | Malay n=153 | Chinese n= 53 | Indian n=108 |
| Risk Allele frequency (T) n(frequency) | 5(0.06) | 4(0.08) | 8(0.31) | 11(0.07) | 2(0.04) | 21(0.19) |
| Homozygous (C-C) n(frequency) | 77(0.90) | 45(0.87) | 13(0.50) | 135(0.88) | 51(0.96) | 72(0.67) |
| Heterozygous (C-T) n(frequency) | 8(0.09) | 7(0.13) | 11(0.42) | 15(0.10) | 1(0.02) | 31(0.29) |
| Homozygous (T-T) n(frequency) | 1(0.01) | 0(0.00) | 2(0.08) | 3(0.02) | 1(0.02) | 5(0.04) |
| Recessive Model | | Odds ratio | | 2.09 | 6.0E | 0.76 |
| | | 95% CI | | 0.21-21.29 | 0.00-0.00 | 0.13-4.55 |
| | | P-Value | | 0.53 | 1.00 | 0.76 |
| Dominant Model | | Odds ratio | | 1.45 | 0.35 | 0.52 |
| | | 95% CI | | 0.56-3.77 | 0.06-2.15 | 0.20-1.34 |
| | | P-Value | | 0.44 | 0.25 | 0.17 |
| Additive Model | | Odds ratio | | 1.40 | 0.70 | 0.64 |
| | | 95% CI | | 0.64-3.06 | 0.16-3.06 | 0.31-1.33 |
| | | P-Value | | 0.40 | 0.63 | 0.23 |

Recessive model; odds ratio (T-T *vs.* (C-T + C-C)). Dominant model; odds ratio ((T-T + C-T) *vs.* C-C). The additive model; common odds ratio (T-T *vs.* C-T *vs.* C-C), additive model was re-coded for the count of the risk allele T, which is 0 within genotype C-C, 1 within genotype C-T, and 2 within genotype T-T.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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**Table 4.29:** Association of *DPP4* rs17574 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs17574 Polymorphisms<br>T < C | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | Malay<br>n=86 | Chinese<br>n=52 | Indian<br>n=26 | Malay<br>n=153 | Chinese<br>n= 53 | Indian<br>n=108 |
| **Risk Allele frequency (C) n(frequency)** | 73(0.85) | 46(0.88) | 19(0.73) | 138(0.90) | 51(0.96) | 83(0.77) |
| **Homozygous (C-C) n(frequency)** | 61(0.71) | 41(0.79) | 13(0.50) | 124(0.81) | 48(0.91) | 61(0.56) |
| **Heterozygous (C-T) n(frequency)** | 23(0.27) | 9(0.17) | 12(0.46) | 27(0.18) | 5(0.09) | 43(0.40) |
| **Homozygous (T- T) n(frequency)** | 2(0.02) | 2(0.04) | 1(0.04) | 2(0.01) | 0(0.00) | 4(0.04) |
| **Recessive Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.29<br>0.64-2.61<br>0.47 | 1.58<br>0.43-5.79<br>*0.48* | 1.56<br>0.61-4.00<br>0.35 |
| **Dominant Model** | | Odds ratio<br>95% CI<br>P-Value | | 074<br>0.09-6.39<br>0.79 | 5.70<br>0.00-0.00<br>0.99 | 1.31<br>0.12-13.89<br>0.82 |
| **Additive Model** | | Odds ratio<br>95% CI<br>P-Value | | 1.20<br>0.64-2.22<br>0.57 | 1.67<br>0.51-5.45<br>*0.39* | 1.44<br>0.64-3.22<br>0.39 |

Recessive model; odds ratio (C-C *vs.* (C-T + T-T)). Dominant model; odds ratio ((C-C + C-T) *vs.* T-T). The additive model; common odds ratio (C-C *vs.* C-T *vs.* T-T), additive model was re-coded for the count of the risk allele C, which is 0 within genotype T-T, 1 within genotype C-T, and 2 within genotype C-C.

*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

University of Malaya

**Table 4.30:** Association of *DPP4* rs7608798 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| **rs7608798 Polymorphisms A < G** | **Non type 2 diabetes mellitus(n=164)** | | | **Type 2 diabetes mellitus* (n=314)** | | |
|---|---|---|---|---|---|---|
| | **Malay n=86** | **Chinese n=52** | **Indian n=26** | **Malay n=153** | **Chinese n= 53** | **Indian n=108** |
| **Risk Allele frequency (G) n(frequency)** | 47(0.55) | 22(0.42) | 19(0.73) | 87(0.57) | 20(0.38) | 83(0.77) |
| **Homozygous (A-A) n(frequency)** | 18(0.21) | 19(0.37) | 2(0.08) | 30(0.19) | 24(0.45) | 4(0.04) |
| **Heterozygous (A-G) n(frequency)** | 42(0.49) | 23(0.44) | 10(0.38) | 73(0.48) | 18(0.34) | 42(0.39) |
| **Homozygous (G-G) n(frequency)** | 26(0.30) | 10(0.19) | 14(0.54) | 50(0.33) | 11(0.21) | 62(0.57) |
| **Recessive Model** | | Odds ratio | | 1.45 | 0.82 | 1.42 |
| | | 95% CI | | 0.75-2.83 | 0.26-2.60 | 0.54-3.69 |
| | | P-Value | | 0.27 | 0.73 | 0.47 |
| **Dominant Model** | | Odds ratio | | 1.09 | 0.64 | 1.49 |
| | | 95% CI | | 0.52-2.29 | 0.25-1.61 | 0.22-10.06 |
| | | P-Value | | 0.82 | 0.34 | 0.68 |
| **Additive Model** | | Odds ratio | | 1.20 | 0.77 | 1.33 |
| | | 95% CI | | 0.78-1.83 | 0.41-1.44 | 0.62-2.84 |
| | | P-Value | | 0.40 | 0.41 | 0.46 |

Recessive model; odds ratio (G-G *vs.* (A-G + A-A)). Dominant model; odds ratio ((G-G + A-G) *vs.* A-A). The additive model; common odds ratio (G-G *vs.* A-G *vs.* A-A), additive model was re-coded for the count of the risk allele G, which is 0 within genotype A-A, 1 within genotype A-G, and 2 within genotype G-G.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

University of Malaya

**Table 4.31:** Association of *DPP4* rs1014444 polymorphism with type 2 diabetes mellitus among the main Malaysian ethnic groups.

| rs1014444 Polymorphisms G < A | Non type 2 diabetes mellitus(n=164) | | | Type 2 diabetes mellitus* (n=314) | | |
|---|---|---|---|---|---|---|
| | Malay n=86 | Chinese n=52 | Indian n=26 | Malay n=153 | Chinese n= 53 | Indian n=108 |
| **Risk Allele frequency (A) n(frequency)** | 44(0.51) | 18(0.35) | 19(0.73) | 81(0.53) | 22(0.42) | 81(0.75) |
| **Homozygous (A-A) n(frequency)** | 21(0.24) | 6(0.12) | 15(0.58) | 45(0.29) | 12(0.23) | 58(0.54) |
| **Heterozygous (A-G) n(frequency)** | 46(0.54) | 23(0.44) | 7(0.27) | 72(0.47) | 20(0.38) | 45(0.42) |
| **Homozygous (G-G) n(frequency)** | 19(0.22) | 23(0.44) | 4(0.15) | 36(0.24) | 21(0.40) | 5(0.05) |
| **Recessive Model** | | Odds ratio | | 1.48 | 1.91 | 0.95 |
| | | 95% CI | | 0.74-2.95 | 0.54-6.75 | 0.97-2.50 |
| | | P-Value | | 0.27 | *0.31* | 0.91 |
| **Dominant Model** | | Odds ratio | | 0.93 | 1.20 | 3.93 |
| | | 95% CI | | 0.46-1.80 | 0.48-2.97 | 0.79-19.41 |
| | | P-Value | | 0.84 | *0.70* | 0.09 |
| **Additive Model** | | Odds ratio | | 1.13 | 1.29 | 1.27 |
| | | 95% CI | | 0.74-1.72 | 0.68-2.46 | 0.61-2.63 |
| | | P-Value | | 0.56 | *0.43* | 0.53 |

Recessive model; odds ratio (A-A *vs.* (A-G + G-G)). Dominant model; odds ratio ((A-A + A-G) *vs.* G-G). The additive model; common odds ratio (A-A *vs.* A-G *vs.* G-G), additive model was re-coded for the count of the risk allele A, which is 0 within genotype G-G, 1 within genotype A-G, and 2 within genotype A-A.
*Controlled for age, gender and BMI. The results presented as frequency and corresponding odds ratio, and P-value. The outliers (studentized residual is greater than 2.0 or less than −2.0) were excluded.

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### 4.3.4 Association of *DPP4* SNPs Common Haplotypes with Type 2 Diabetes Mellitus among Malaysian Subjects.

The SNP haplotype blocks were identified via linkage disequilibrium (LD) and constructed from the SNPs that were included in the study. LD between SNPs were performed with SNP & Variation Suite (SVS) software. The degrees of LD with $r^2$ among *DPP4* SNPs are shown in Figure 4.1 and Table 4.32. It is noteworthy that a linkage disequilibrium block (haplotype block) was not observed among the SNPs (Table 4.32).

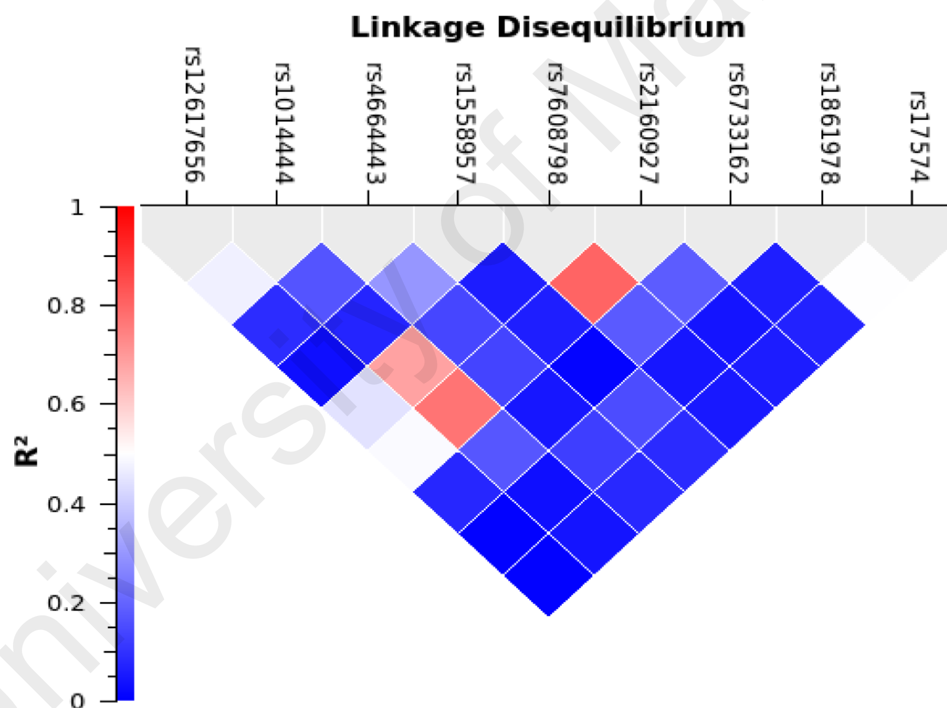


**Figure 4.1:** Pairwise linkage disequilibrium among *DPP4* SNPs in Malaysian subjects. Values in the upper axis represent *DPP4* SNPs, while values in the left axis represent the $R^2$ value.

University of Malaya

**Table 4.32:** The estimated values of linkage equilibrium analysis between the *DPP4* SNPs in Malaysian subjects.

| D' | rs12617656 (C < T) | rs1014444 (G < A ) | rs4664443 (G > A) | rs1558957 (C > T) | rs7608798 (A < G) | rs2160927 (T < C) | rs6733162 (G < C) | rs1861978 (G < T) | rs17574 (T < C) |
|---|---|---|---|---|---|---|---|---|---|
| rs12617656 (C < T) | - | 0.71 | 0.71 | 0.59 | 0.69 | 0.72 | 0.29 | 0.27 | 0.21 |
| rs1014444 (G < A) | 0.47 | - | 0.94 | 0.86 | 0.88 | 0.88 | 0.44 | 0.54 | 0.53 |
| rs4664443 (G > A) | 0.09 | 0.17 | - | 0.76 | 0.91 | 0.90 | 0.53 | 0.48 | 0.32 |
| rs1558957 (C > T) | 0.03 | 0.07 | 0.30 | - | 0.81 | 0.85 | 0.36 | 0.40 | 0.36 |
| rs7608798 (A < G) | 0.44 | 0.68 | 0.14 | 0.06 | - | 0.90 | 0.42 | 0.68 | 0.65 |
| rs2160927 (T < C) | 0.49 | 0.78 | 0.13 | 0.06 | 0.80 | - | 0.43 | 0.68 | 0.65 |
| rs6733162 (G < C) | 0.08 | 0.17 | 0.05 | 0.01 | 0.18 | 0.18 | - | 0.84 | 0.72 |
| rs1861978 (G < T) | 0.01 | 0.03 | 0.12 | 0.15 | 0.04 | 0.04 | 0.06 | - | 0.85 |
| rs17574 (T < C) | 0.01 | 0.04 | 0.08 | 0.08 | 0.06 | 0.06 | 0.07 | 0.50 | $r^2$ |

The correlation cofficients D' and $r^2$ between SNPs are shown in the above and below diagonal of this table, respectively.

University of Malaya

### 4.3.5 Association of Genotype Groups in *DPP4* (SNPs) with CD26/DPP-IV Levels

The association of genotype groups in *DPP4* (SNPs) with sCD26/DPP-IV levels were performed using the general linear model with Bonferroni correction after adjusting for age, race, gender and BMI. Univariate analysis showed that the SNP rs4664443 G>A polymorphism in subjects with T2DM was associated with increased sCD26/DPP-IV levels ($p$ = 0.042). Moreover, pairwise comparison showed that subjects with T2DM carrying the homozygous AA genotype of rs4664443 showed significantly higher serum sCD26/DPP-IV levels than subjects carrying the GG genotype (1346± 167 vs. GG 1215± 246 ng/mL, $p$ = 0.039) (Table 4.33).

**Table 4.33:** Association of rs4664443 polymorphism with sCD26/PP4 levels among the type 2 diabetes mellitus subjects

| **Parameter** | **Mean +SD** | | | **Univariate P-Value** | **Pairwise comparisons P-Value** | |
|---|---|---|---|---|---|---|
| | **A–A n(19)** | **A–G n(96)** | **G–G n(199) Ref** | | **A–A *vs* G–G** | **A–G *vs* G–G** |
| **sCD26/DPP-IV (ng/ml)** | 1346 ± 167 | 1220 ± 232 | 1215 ± 246 | **0.042** | **0.039** | **0.999** |

The results presented represent as means ± SD, adjusted for age, gender, race, and BMI as covariates which were evaluated using univariate (General Linear Model) with a Bonferroni adjustment applied for multiple comparison tests. Ref: reference, the protective genotype was selected to be a reference for the comparison. Bold values are significant.

University of Malaya

# CHAPTER 5: DISCUSSION

## 5.1 DEMOGRAPHIC AND BIOCHEMICAL ANALYSIS

The demographics and biochemical parameters were significantly different between the groups. The gender, race and age appeared to be different between groups of study. Therefore, these were controlled as covariates during analysis to ensure no effect modifiers or confounders.

## 5.2 SERUM LEVELS OF SOLUBLE CD26/DEPEPTIDYLE PEPTIDASE 4 IN TYPE 2 DIABETES AND ASSOCIATION WITH METABOLIC SYNDROME AND ANTIDIABETIC AGENTS AMONG MALYSIAN SUBJECTS

### 5.2.1 Prevalence of Metabolic Syndrome among Malaysian Subjects with Type 2 Diabetes.

The prevalence of metabolic syndrome (MetS) among Malaysian subjects with T2DM (81.8%) appears to be closely related with a previous study by Tan *et al.* (2013). However, our results are similar to those in other populations; 83% for Pakistanis (Kiani *et al.*, 2016), 89.5% for Spanish (Rodríguez *et al.*, 2010), 86% for Nigerian (Ogbera, 2010), 70% for White Americans, 65% for African Americans and 62% for Mexican Americans (Lin & Pi-Sunyer, 2007), and 63.2 % in the Iranians (Janghorbani & Amini, 2012).

MetS is estimated to be present in 75–80% of T2DM patients (Alexander *et al.*, 2003; Mogre *et al.*, 2014). Malaysia has a much higher prevalence of MetS compared with other Asian countries (Mohamud *et al.*, 2012). The differences may be due to the variable criteria used in defining MetS as well as ethnic differences (Mogre *et al.*, 2014). In Malaysian subjects, it was evident that diabetic females have a higher prevalence of MetS

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than diabetic males. In Malaysian subjects, it was evident that diabetic females have higher prevalence of MetS than their male counterpart. This is attributed primarily to their propensity for abnormally larger waist circumference compared to diabetic males. These results were in agreement with Mohamud *et al.* (2011) who showed that obesity was higher among Malaysian females compared to males. In addition, diabetic females were more likely than males to present with hypertension, low levels of HDL-c and high levels of TG in many populations (Legato *et al.*, 2006).

Several studies have shown that the prevalence of MetS varies among different ethnic populations living in the same country and is has been postulated to be associated with environmental and genetic factors (Poulsen *et al.*, 2001; Reilly & Rader, 2003). Similarly, our study showed that Malays with T2DM have the highest prevalence of MetS followed by Indians; the lowest prevalence of MetS was in Chinese subjects.

### 5.2.2 Association of sCD26/DPP-IV with Type 2 Diabetes Mellitus and Metabolic Syndrome

In recent years, studies on DPP-IV level and/or activity have mainly focused on the metabolic effects linked to GLP-1 degradation (Pala & Rotella, 2013). It is reasonable to assume that an increase in DPP-IV levels and/or activity might contribute to the impaired incretin effect observed in T2DM patients (Ryskjær *et al.*, 2006). However, sCD26/DPP-IV was suggested to be a new adipokine, and considered to be a source for circulating DPP-IV activity in serum/plasma, which is relevant for GLP1 degradation, suggesting that sCD26/DPP-IV could play a major role in metabolism (Zilleßen et al., 2016).

Numerous studies assessing DPP-IV activity in subjects with T2DM have shown conflicting results such as increased (Fadini *et al.,* 2012; Mannucci *et al.,* 2005; Vanderheyden *et al.,* 2009), or decreased activity (Korosi *et al.*, 2001; Meneilly *et al.*, 2000). However, these disparities might be partially explained by dissimilar patient

characteristics. Despite the body of research in this area, little is known about the associations between sCD26/DPP-IV levels, MetS, and T2DM.

In this study, the association of fasting serum sCD26/DPP-IV levels and active GLP-1 with T2DM and MetS were assessed; we showed that sCD26/DPP-IV levels was significantly higher in T2DM patients. In addition, we revealed significant elevation of sCD26/DPP-IV levels in T2DM patients either with or without MetS. This finding supports a previous study by Lee *et al.* (2013) which reported a significant elevation in sCD26/DPP-IV levels in subjects with T2DM. More recently, review article also suggested increased of sCD26/DPP-IV levels in T2DM subjects (Röhrborn *et al.*, 2015).

Considerable evidence demonstrated that sCD26/DPP-IV constitutes about 90–95% of the overall DPP-IV-like activity in serum/plasma (Busso *et al.*, 2005; Durinx *et al.*, 2000; Friedrich *et al.*, 2007; Kobayashi *et al.*, 2002; Lee *et al.*, 2013; Zilleßen *et al.*, 2016). There are several studies on increased DPP-IV activity in T2DM patients have been reported (Fadini *et al.*, 2012; Mannucci *et al.,* 2005) but the cause for this increase has not been clarified.

The mechanisms underlying increased DPP-IV activity and/or levels in T2DM remain unclear. Several assumptions are hypothetically based on the context of clinical features presented in T2DM. The first explanation stems from the impact of chronic hyperglycaemia. In experimental models, it was demonstrated that high glucose exposure in human glomerular endothelial cells lead to stimulation of DPP-IV biosynthesis (Pala *et al.,* 2003), and increased DPP-IV mRNA expression in human hepetic cells (Miyazaki *et al.*, 2012). In addition, another study demonstrated that DPP-IV activation was not induced in control subjects, impaired glucose tolerance, or T2DM patients, according to oral glucose loading (Pala *et al.,* 2010). Similarly, another study revealed a trend for increased DPP-IV activity in subjects with T2DM, but DPP-IV activity was not altered following meal ingestion and acute changes in plasma glucose (Ryskjær *et al.,* 2006). On



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the other hand, it was found that sCD26/DPP-IV levels in healthy subjects displayed an acute increase after oral glucose loading, and this abrupt increase may be linked with the existence of insulin resistance and/or non-alcoholic fatty liver (Aso *et al.,* 2013). Furthermore, chronic hyperglycaemia stimulates an increase in DPP-IV activity in T1DM and T2DM (Mannucci *et al.,* 2005). Recently, it has been reported that DPP-IV activity was positively correlated with the duration of diabetes (Osawa *et al.*, 2016). Taken together, these findings assumes that DPP-IV biosynthesis is associated with long-term exposure (chronic hyperglycemia) to elevated glucose levels.

The second hypothesis is derived from the involvement of advanced glycation end products (AGEs). It is reported that there are higher levels of AGEs in T2DM; serum sCD26/DPP-IV levels has been shown to be significantly correlated with serum AGE (Tahara *et al.*, 2013). Also, Tahara *et al.* (2013) proposed that AGEs may up-regulate cellular DPP-IV expression and subsequently increase serum levels of sCD26/DPP-IV in humans. Thus, chronic hyperglycemia itself and accumulated AGEs may result in higher sCD26/DPP-IV levels and/or activity T2DM (Tahara *et al.,* 2013).

DPP-IV also possesses non-catalytic functions that may be involved in T cell activation via interaction with a wide range of ligands (Gong *et al.*, 2015; Klemann *et al.*, 2016; Wagner *et al.*, 2016). sCD26/DPP-IV binds to insulin-like growth factor II/mannose-6-phosphate receptor (IGF-II/M6P-R) and increases T-cell proliferation (Ikushima *et al.*, 2002). Therefore, it has been suggested that sCD26/DPP-IV may be implicated in the development of inflammation (ZhongRao, *et al.,* 2013). Furthermore, a recent study, aimed to establish whether DPP-IV plays role in developing hyperglycemia through its pro-inflammatory actions have found that DPP-IV activity was positively correlated with the levels of inflammatory markers and IGF-II/M6P-R. Moreover, DPP-IV activity rises by itself and results in increased inflammation through its interaction with the IGF-II/M6P receptor, leading to a decrease in insulin secretion and an increase

in insulin resistance. This, in turn, eventually leads to the development of hyperglycemia. Zheng *et al.* (2015) showed that increase of DPP-IV activity leads to an increase in the risk of hyperglycemic events by 9.47%.

### 5.2.3 Association between Active GLP-1 Levels with Type 2 Diabetes Mellitus and Its Correlation with sCD26/DPP-IV Levels

The present study showed that fasting serum active GLP-1 was associated with T2DM with and without MetS in Malaysian subjects. However, in line with the observed increased in sCD26/DPP-IV levels, active GLP-1 levels were found to be significantly lower in T2DM subjects. Remarkably, serum levels of sCD26/DPP-IV were negatively correlated with active GLP-1 levels in T2DM patients both with and without MetS. A previous study conducted in Chinese subjects with T2DM showed that GLP-1 levels were lower compared to normal glucose tolerance subjects (Zhang *et al.,* 2012). A similar finding in Caucasian subjects discovered that GLP-1 levels decreased significantly in subjects with T2DM than in the normal subjects (Legakis *et al.*, 2003). Even in patients with T1DM, GLP-1 levels were lower than the levels recorded in healthy subjects (Blaslov *et al.,* 2015).

Impairment of GLP-1 action or release has a negative impact in diabetes (Wang *et al.*, 2015). Several studies examining GLP-1 levels in diabetic patients have revealed impaired GLP-1 levels in T2DM patients in comparison to healthy subjects (Bagger *et al.*, 2011; Calanna *et al.*, 2013; Knop *et al.*, 2012; Knop *et al.*, 2007; Madsbad, 2014; Muscelli *et al.*, 2008; Nauck *et al.*, 2011; Vilsbøll *et al.*, 2001). However, some studies revealed unaltered GLP (Knop *et al.*, 2012; Ryskjær *et al.*, 2006; Smushkin *et al.*, 2011; Vollmer *et al.*, 2008).

Impairment of the incretin effect in T2DM subjects appears to be primarily attributed to decreased GLP-1 levels, and may be secondarily caused by either raised degradation

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of DPP-IV or its reduced secretion by the gut (Freeman, 2009). Therefore, it is suggested that decrease in GLP-1 levels in the early stages of the disease is the most dominant basis of impairment due to deficiencies in its secretion. However, increased sCD26/DPP-IV activity plays a predominant role after prolonged duration of disease (Pala *et al.*, 2010). There has been some disagreement regarding the pathophysiology of this impairment. Studies have shown that the lengthy duration and increased severity of T2DM is associated with reduction in GLP-1 levels (Meier & Nauck, 2008; Nauck *et al.*, 2011). Another study revealed a moderate decrease in postprandial GLP-1 levels in T2DM patients compared to normal subjects. In addition, there was also minor decrease in GLP-1 levels in IGT subjects (Toft-Nielsen *et al.*, 2001). Taken together, these studies have provided better support to the notion that the decrease in GLP-1 activity in T2DM patients progresses secondary to chronic hyperglycaemia and goes along with an overall impairment of β-cell function in these subjects (Meier & Nauck, 2006; Nauck *et al.*, 2004; Singh, 2015). Additionally, impaired release or action of GLP-1 might be attributed to restored expression of GLP-1 receptors, which may be reduced in T2DM (Xu *et al.,* 2007), and glucotoxicity may plays a pivotal role in the reduction GLP-1R expression (Cho *et al.*, 2014). Furthermore, ethnic differences in GLP-1 levels and GLP-1 responsiveness has been suggested among East-Asians, particularly in Japanese and Koreans (Singh, 2015).

University of Malaya

### 5.2.4 Associations between sCD26/DPP-IV Levels with Metabolic Syndrome, and AIC levels among Type 2 Diabetes Mellitus Patients

The present study also established that elevated serum levels of sCD26/DPP-IV in T2DM patients were significantly associated with increased A1C. This finding is in agreement with a previous study (Lee *et al.*, 2013). On the other hand, it has been reported that treatment with DPP-IV inhibitors may improve A1C levels in T2DM patients (Kusunoki *et al.*, 2015). Furthermore, previous studies have reported that DPP-IV activity in diabetic patients showed significant correlations with serum A1C levels (Mannucci *et al.,* 2005; Ryskjær *et al.*, 2006). However, this finding is inconsistent with a previous research in which DPP-IV activity showed no correlation with A1C (Fadini *et al.*, 2012).

The findings of the present study did not show any significant results of sCD26/DPP-IV levels with insulin resistance in diabetic patients. This finding is in contrast with a previous report (Lee *et al.*, 2013), which may be attributed to differences in treatment profiles, and thus may influence insulin resistance state. Our study further showed that sCD26/DPP-IV levels seem to not be associated with any factors related to MetS, such as triglyceride and HDL-cholesterol. This finding is in agreement with a recently published study which demonstrated that serum sCD26/DPP-IV levels were not associated with blood pressure, triglyceride and HDL-cholesterol in men with T2DM (Tanaka *et al.*, 2016). However, most diabetic patients in this study received medications for dyslipidemia and diabetes; hence, additional basic studies are needed to clarify the association between serum levels of sCD26/DPP-IV and MetS.

### 5.2.5 Associations between sCD26/DPP-IV Levels and Antidiabetic Agents

Our findings revealed that levels of sCD26/DPP-IV were lower in subjects with T2DM receiving metformin monotherapy, which is in agreement with a study that reported that treatment with metformin reduced serum sCD26/DPP-IV levels (Lee *et al.,* 2013).

University of Malaya

Similarly, DPP-IV activity were significantly lower in subjects with T2DM on monotherapy (e.g., metformin) (Fadini *et al.*, 2012; Green *et al.*, 2006; Lindsay *et al.*, 2005). In effect, the pivotal role for metformin in reducing DPP-IV activity could be partially attributed to its ability to suppress the release of soluble isoforms of DPP-IV from cells into serum (Green *et al.*, 2006; Lenhard *et al.*, 2004). Other studies postulated that metformin lowers DPP-IV activity indirectly via its metabolic effects by increasing levels of GLP-1 in circulation and up-regulating GLP-1 receptor expression in pancreatic β-cells (Liu & Hong, 2014). It has been postulated that metformin modestly represses DPP-IV activity and augments intact GLP-1 without any effect on basal levels of GLP-1 in subjects with T2DM (Wu *et al.*, 2014).

### 5.2.6 Association of sCD26/DPP-IV Levels with Metabolic Syndrome and Type 2 Diabetes Mellitus Parameters among Normal Subjects.

Associations between sCD26/DPP-IV levels and MetS parameters were assessed in the control group. MetS is well-known to be associated with the risk of developing T2DM and CVD (O'Neill & O'Driscoll, 2015). In fact, it has been shown that adipose tissue enlargement and inflammation of adipocytes augments the release of sCD26/DPP-IV levels from fat cells into the circulation. In addition, serum DPP-IV levels are elevated in subjects with MetS (Lamers *et al.*, 2011). Previous studies have reported that circulating DPP-IV correlated with components of MetS, such as waist circumference, BMI, and plasma TG (Fadini *et al.*, 2012; Lamers *et al.*, 2011). It has recently been reported that increased DPP-IV activities were associated with chronic inflammation and predicted new-onset hyperglycemia (Zheng *et al.*, 2015).

In another study conducted in healthy Chinese subjects suggested that increased DPP-IV activity is a strong risk predictor of insulin resistance, MetS (Yang *et al.*, 2014) and increased risk of developing hypertension (Zheng *et al.*, 2014). In the current study, we

University of Malaya

showed that increased serum levels of sCD26/DPP-IV were associated with increased BMI, total cholesterol, and LDL-c. Our research provides evidence that sCD26/DPP-IV may be useful as a biomarker for increased risk of obesity or metabolic syndrome.

### 5.2.7 Active GLP-1 Levels and sCD26/DPP-IV Levels in Obese Non-Diabetic Subjects and their Association with Insulin Resistance.

In the current study, we examined serum levels of active GLP-1 and sCD26/DPP-IV in obese, overweight and normal weight subjects; we found that sCD26/DPP-IV levels were significantly higher in obese subjects compared to normal weight subjects. This finding is in concordance with results reported by Stengel *et al.* (2014) who similarly demonstrated that sCD26/DPP-IV levels in obese subjects were higher than normal subjects. We also observed that sCD26/DPP-IV levels in obese subjects were positively associated with insulin resistance. A study by Sell *et al.* (2013) highlighted that circulating DPP-IV was elevated in insulin resistant subjects. It has been reported that DPP-IV activities can promote inflammation, impairment of insulin sensitivity, and may mediate development of pathways implicated in obesity (Chielle *et al.*, 2016). In visceral adipose tissue, it was observed that sCD26/DPP-IV levels were positively associated with the degree of insulin resistance in obese subjects (ZhongRao, *et al.*, 2013).

However, the mechanism(s) underlying increased DPP-IV activity and/or levels remains unclear. Researchers have found that enlargement of visceral adipocytes in obesity may contribute to increased levels of circulating sCD26/DPP-IV in obese subjects. This may possibly be due to increased sCD26/DPP-IV expression and release from adipose tissue (Röhrborn *et al.*, 2015)

In recent years, studies on DPP-IV activity and/or level have mainly focused on its metabolic effects related to GLP-1 degradation (Pala & Rotella, 2013), it is reasonable to assume that an increase in sCD26/DPP-IV level and/or activity might contribute to the

University of Malaya

impaired GLP-1 levels. In the present study, we also found that the active GLP-1 levels were significantly lower in obese subjects as compared to normal weight subjects. Our results are in agreement with those that have reported reduced glucose-stimulated GLP-1 levels and GLP-1 secretion in obese subjects (Holst, 2007; Hussein *et al.*, 2014), but in contrast with the results obtained by Wadden *et al.* (2013). In a study by Carr *et al.* (2010) aimed to determine whether increase in plasma DPP-IV activity in obesity could affect the levels of active GLP-1 and GIP. They discovered increased DPP-IV activity and reduced GLP-1 secretion in obese subjects, concluding that augmented DPP-IV activity is not related with a generalized increased in GLP-1 metabolism.

Several evidence have indicated that the effects or actions of GLP-1 is impaired. However, it remains unclear whether this is due to reduction of either insulinotropic potency or GLP-1 secretion or both (Madsbad, 2014). However, Hussein *et al.* (2014) reported that an impaired secretory response of GLP-1 is related to the degree of insulin resistance state in obese subjects. Otherwise, the impact of GLP-1 in the process of developing obesity was proposed to be partially due to the physiological effects of the hormone on food intake and appetite (Verdich *et al.*, 2001). Hence, dysfunctions of GLP-1 has been implicated in obesity, postprandial reactive hypoglycemia, and T2DM (Holst, 2007). Furthermore, impairment of GLP-1 could be the pathophysiological link between T2DM and obesity (Madsbad, 2014).

This finding provides evidence that sCD26/DPP-IV and GLP-1 may play an important role in the pathogenesis of obesity, suggesting that sCD26/DPP-IV may be valuable as an early marker for the augmented risk of obesity and insulin resistance.

University of Malaya

## 5.3 GENETIC ANALYSIS

### 5.3.1 Genetic Analysis of *DPP4*

T2DM is a complex polygenic disease, rendering individual susceptible and protective genes more difficult to identify. Susceptibility to complex forms of T2DM is associated with SNPs polymorphisms. SNPs and the mechanisms by which these individual alleles contribute to an increased susceptibility to T2DM are largely unknown. However, they are thought to impact on susceptibility through changes in the regulation of those gene expressions (Gaulton *et al.*, 2015). In addition, the number of genes that contribute to T2DM remains unknown and whether any single susceptibility gene will be shared among all ethnic groups, or to some degree in a specific population exists. It is widely known that environmental and genetic factors contribute to the initiation and progression of T2DM through different mechanisms in gene-environment interactions (Ahlqvist & Groop, 2015; Groop & Pociot, 2014; Permutt *et al.*, 2005). This interaction could modulate gene expression via epigenetic mechanisms, including DNA methylation, histone modifications, and microRNA (Kwak & Park, 2016; Stančáková & Laakso, 2016; Zimmet *et al.*, 2014). Most risk variants for T2DM in healthy populations act through impairing insulin secretion (resulting in β-cell dysfunction) rather than insulin action (resulting in insulin resistance), which establishes that inherited abnormalities of β-cell function or mass (or both) are critical precursors of T2DM (Cerf, 2013; Florez, 2008; McCarthy, 2010; Petrie *et al.*, 2011; Voight *et al.*, 2010).

GWAS in humans have identified specific variants that are associated with T2DM in several genes; these have been extensively performed in various ethnic groups including Europeans, South Asians, East Asians and Mexican populations (Cho *et al.*, 2012; Dupuis *et al.*, 2010; Hara *et al.*, 2014; Imamura *et al.*, 2012; Mahajan *et al.*, 2014; Morris *et al.*, 2012; Perry *et al.*, 2012; Saxena *et al.*, 2012; Saxena *et al.*, 2007; Scott *et al.*, 2007; Shu *et al.*, 2010; Sladek *et al.*, 2007; Steinthorsdottir *et al.*, 2007; Unoki *et al.*, 2008; Voight

University of Malaya

*et al.*, 2010; Yamauchi *et al.*, 2010; Yasuda *et al.*, 2008; Zeggini, 2007; Zeggini *et al.*, 2008). GWAS on European populations have identified approximately 50 loci associated with T2DM. Additionally, GWAS on non-European populations and trans-ethnic GWAS meta-analysis have identified more than 30 susceptibility loci for T2DM, which were not detected in earlier European GWAS (Cho *et al.*, 2012; Hara *et al.*, 2014; Imamura *et al.*, 2012; Kooner *et al.*, 2011; Mahajan *et al.*, 2014; Unoki *et al.*, 2008; Williams *et al.*, 2014; Yamauchi *et al.*, 2010; Yasuda *et al.*, 2008). Further research is required to identify additional susceptibility loci associated with T2DM; the association between *DPP4* and T2DM has rarely been investigated and no such study has been carried out in Asian populations. The current study is the first to determine whether *DPP4* polymorphism is associated with T2DM in Malaysian subjects as well as in the three main Malaysian ethnic groups (Malay, Chinese and Indian), and to investigate *DPP4* allelic distributions and their association with sCD26/DPP-IV levels. Current available literature substantiates that some *DPP4* SNPs are significantly associated with elevated plasma lipid levels and cardiovascular risk. In effect, *DPP4* polymorphisms may have important clinical significance. Hence, further analysis was carried out on the association of *DPP4* with T2DM.

University of Malaya

### 5.3.2 Association of *DPP4* Polymorphisms with Type 2 Diabetes Mellitus among Malaysian Subjects

*DPP4* encodes the DPP-IV protein and it is expressed across a wide range of human cell types. It belongs to the S9b DPP family, which is relevant for GLP1 degradation, suggesting that DPP-IV could play a major role in regulating glucose homeostasis. Hence, it is thought to be a potential candidate gene influencing T2DM (Gong *et al.*, 2015; Röhrborn *et al.*, 2015; Zhong *et al.*, 2015).

The current study showed that *DPP4* polymorphisms are significantly associated with diabetes in Malaysian subjects. Associations were found for SNP rs12617656 under the recessive, additive and dominant genetic models. SNPs rs7633162 and rs4664443 were associated with T2DM under the additive model although this association did not remain significant after applied multiple testing with a false discovery rate of 5%, but it may still have statistical or clinical importance to studies future.

It is well-known that inflammation is as a major contributor to the development of insulin resistance and T2DM (Després & Lemieux, 2006; Horng & Hotamisligil, 2011; Hotamisligil, 2006), through activation of T-cells that mediate insulin resistance and adipose tissue inflammation (Zhong *et al.*, 2014), in which DPP-IV plays a major role (Klemann *et al.*, 2016; Ohnuma *et al.*, 2001; Ohnuma *et al.*, 2008; Wagner *et al.*, 2016). DPP-IV is up-regulated in pro-inflammatory states including T2DM and obesity (Zhong *et al.*, 2015). T-cells activation involves the binding of DPP-IV on the surface of T-cells to several matrix proteins including ADA and co-association with CD45 resulting in co-stimulatory signals (Pacheco *et al.*, 2005; Wagner *et al.*, 2016; Zhong *et al.*, 2015). In addition, the secretion of DPP-IV from skeletal muscle cells and adipocytes may exert paracrine effects on insulin signaling (Lamers *et al.*, 2011). A recent GWAS by Jiang *et al.* (2014) indicated that SNP rs12617656 at the DPP4 locus was associated with rheumatoid arthritis (RA) in the Han Chinese population. In line with this, our study found a strong association between rs12617656 at the *DPP4* locus and T2DM. Hence, it is noteworthy that chronic systemic inflammation has a key role in the pathophysiology of T2DM and RA (Lu *et al.*, 2014), which can be explained by the elevated levels of inflammatory mediators such as C-reactive protein (CRP) and IL-6 in patients with RA (Chung *et al.*, 2011), similar to T2DM (Wang *et al.*, 2013). We also observed that SNP rs4664443 was associated with T2DM and was linked to variations in sCD26/DPP-IV

levels. A recent study suggested the allele T of rs4664443, is related to higher serum lipid levels or increased BMI in Chinese T2DM individuals (Xing *et al.*, 2016).

Dyslipidemia is a common feature of T2DM, and quantifiable by the measurement of apolipoprotein B (ApoB) levels (Bailey *et al.,* 2014). A recent study identified novel SNPs in DPP4 gene associated with ApoB levels in the South Asian population, whom are prone to developing T2DM and MI at lower BMI and younger age. They reported that SNP rs4664443 of DPP4 was significantly associated with ApoB levels (Bailey *et al.*, 2014). Linking inflammation and inflammatory markers with DPP-IV and ApoB levels will help us understand how these genetic variations contribute to susceptibility to T2DM. ApoB, has been found to be a predictor of inflammation and considered to be more closely related to inflammatory markers than total, non-high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol (Sattar *et al.*, 2004). Research by Faraj *et al.* (2006) reported that higher levels of ApoB was associated with an increase in levels of diverse inflammatory markers, such as IL-6, tumor necrosis factor alpha (TNF-α) and CRP. Furthermore, Ley *et al.* (2010) demonstrated that elevated ApoB concentrations was associated with T2DM incident and was superior to HDL and LDL cholesterol in predicting the disease, and is an effective predictor for the development of MetS (Ryoo & Park, 2013). Another study reported that insulin resistance was associated with the serum ApoB levels (Sung & Hwang, 2005).

This study also demonstrated that SNP rs7633162 was significantly associated with T2DM. Woodard-Grice *et al.* (2010) analyzed the impact of DPP-IV inhibition on genotypic variations in *DPP4*, and showed that the haplotype of SNPs rs6733162-rs12469968-rs873826 was associated with a sitagliptin-induced decrease in DPP-IV activity. Finally, our results demonstrated that SNPs rs1558957, rs7608798, rs17574 were not associated with T2DM. These findings were consistent with a multi-stage analysis, searching for associations between *DPP4* genetic polymorphism and cardiovascular

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disease risk factors, such as lipids, diabetes-related phenotypes, and blood pressure in European ancestry. We also showed that rs17848915 merged into rs17574, rs1558957 and rs7608798 of the analyzed SNPs was associated with hyperglycaemia/diabetes, triacylglycerol and LDL-cholesterol in the first stage, but were not successfully replicated in stage 2 (Bouchard *et al.*, 2009).

### 5.3.3 Association of *DPP4* Polymorphisms with Type 2 Diabetes Mellitus among the Ethnic Groups

It is well known that the frequencies of genetic variations are different among ethnic groups, leading to differences in effect size and importance of the a particular susceptibility gene (Mahajan *et al.*, 2014; Saxena *et al.*, 2012; Waters *et al.*, 2010). The association of *DPP4* polymorphisms with T2DM among the three main Malaysian ethnic groups was evaluated. We found that the effects of *DPP4* SNPs on T2DM susceptibility were ethnically dependent; Malaysian Indian subjects were at higher risk for T2DM with regards to rs12617656 compared to the Malay and Chinese subjects. Although rs7633162 showed a significant association among the Malay subjects with T2DM, this association did not remain significant after multiple testing. Across ethnicities, allelic frequency distribution was also different. The Hap Map project showed that the allelic distribution of many *DPP4* SNPs in Chinese and Japanese was the opposite of that in Europeans (Buchanan *et al.*, 2012; Xing *et al.*, 2016).

The significant associations found with T2DM may be attributed to gene-environment interactions, while gene-gene interactions might have contributed to the differences in gene-disease associations among the different ethnic groups (Moonesinghe *et al.*, 2012). Genetic differences between ethnic groups and linkage disequilibrium patterns, compounded by the contribution of non-genetic factors (epigenetic factors) and lifestyle changes can modify the risk of T2DM (Saxena *et al.*, 2012) .

University of Malaya

### 5.3.4 Association of Haplotypes Block of *DPP4* SNPs with Type 2 Diabetes Mellitus among Malaysian Subjects

Haplotype block with LD was identified and constructed from the SNPs included in this study. It is noteworthy that a linkage disequilibrium block (haplotype block) was not observed among the SNPs analyzed. Notably, the SNPs of *DPP4* appear to have an independent effect on T2DM in Malaysian subjects.

## 5.4 SIGNIFICANCE OF FINDINGS

This study provides basic information of DPP4 genetic polymorphisms in Malaysian T2DM subjects and might explain of the variance in sCD26/DPP-IV level, suggest that identifying an individual's DPP4 SNPs might one day be a practical and useful clinical approach for prediction of T2DM incidence risk or for selecting patients for treatment with DPP4 targeting drugs.

The current findings add substantially to our understanding of how increased sCD26/DPP-IV levels enhance the degradation of GLP-1 and lipid accumulation, and that these factors may also promote the development of hyperglycemia. Conclusively, sCD26/DPP-IV is emerging as a new research focus, putting this molecule to the list of adipo-cytokines with pro-inflammatory functions. Combining the accumulated knowledge on *DPP4* gene polymorphisms and sCD26/DPP-IV levels will lead to an improved understanding of its impact on health and disease.

In general, genetic studies on T2DM has many potential clinical applications including; risk prediction of T2DM and its related complications, identification of novel therapeutic targets, genetic prediction of the efficacy of lifestyle interventions, and eventually optimization of patient care through personalized genomic medicine (Stančáková & Laakso, 2016). Hence, clinicians may be able to individualize T2DM

University of Malaya

therapy by genotypes (Pearson, 2009). So far, risk prediction of T2DM using genetic testing has had little value in clinical practice (Lyssenko & Laakso, 2013).

## 5.5 FUTURE WORK

The current study requires additional mechanistic and functional data to address the precise roles of the reported SNP associations in T2DM aetiology to increase the novelty of the study in future research. In addition, further investigation is required to determine the influence of *DPP4* gene polymorphism on the efficacy and safety of DPP-IV inhibitors. Although the list of susceptibility loci for T2DM is growing, the entire heritability of T2DM remains poorly understood and the mechanisms behind the plethora of association for this locus remains debatable and needs to be elucidated.

Additional association studies will be required to evaluate the genetic aspects of T2DM with a much larger trans-ethnic sample sizes to identify additional T2DM loci, increasing the power of detection. Future research should also focus on the interaction between various environmental, genetic and epigenetic factors. This will lead to the discovery of novel synergetic pathways that increases the risk of developing T2DM. Through these efforts, multi-layered genomics may provide a foundation for precision medicine and improve patient outcomes in the prevention and treatment of T2DM.

## 5.6 LIMITATIONS OF THE STUDY

One of the limitations of the current study is that because it is a hospital-based study, the sampling method was non-probability, thus findings of this study limits its generalization to the entire Malaysian population. The sub-grouping of T2DM subjects according to ethnic groups resulted in small sample size groups; provides insufficient power to support evidence for an association between *DPP4* SNPS and T2DM within the ethnic subgroups or to develop general conclusions. Finally, we unfortunately were not

University of Malaya

able to evaluate DPP-IV activity. Several studies have established that serum levels of sCD26/DPP-IV is strongly correlated with circulating DPP-IV activity in serum/plasma (Busso *et al.*, 2005; Durinx *et al.*, 2000; Friedrich *et al.*, 2007; Kobayashi *et al.*, 2002; Lee *et al.*, 2013; Zilleßen *et al.*, 2016), suggesting that serum sCD26/DPP-IV levels may reflect about 90–95% of the overall DPP-IV activity. The hormone GIP, cleaved by DPP-IV, was also not measured in this study.

University of Malaya

# CHAPTER 6: CONCLUSION

In our study, the prevalence of metabolic syndrome (MetS) among Malaysians with Type 2 Diabetes Mellitus (T2DM) was 81.8%. We found that the prevalence of MetS was higher in diabetic females than diabetic males. Malay subjects with T2DM have the highest prevalence of MetS (86.3%), followed by Indians (80.6%) and Chinese (62.8%) subjects. Soluble CD26/Dipeptidyl Peptidase-IV (sCD26/DPP-IV) levels were higher in T2DM subjects with and without MetS, whereas active glucagon-like peptide-1 (GLP-1) levels were lower. In addition, sCD26/DPP-IV levels were associated with AlC and negatively correlated with active GLP-1 levels. Metformin monotherapy was associated with reduced sCD26/DPP-IV levels. This study revealed that obese non-Diabetic Subjects had reduced serum GLP-1 levels and elevated sCD26/DPP-IV levels with positively associated with insulin resistance. In normal subjects, increased serum levels of sCD26/DPP-IV were associated with increased BMI, cholesterol, and LDL-c. In addition, active GLP-1 levels were lower in obese non-diabetic subjects.

*DPP4* SNPs rs12617656, rs7633162 and rs4664443 were significantly associated with T2DM in Malaysian subjects. SNP rs12617656 was associated with T2DM in Malaysian subjects, particularly pronounced among Malaysian Indian subjects. The association of SNPs rs7633162 with T2DM was more evident among the Malay ethnic group. In addition, SNP rs4664443 showed a significant association with sCD26/DPP-IV levels in T2DM patients.

University of Malaya

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

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University of Malaya