SHORT TERM GRANT FINAL REPORT

Characterisation of Low Density Lipoprotein Subfraction Profile and APOE Genotype Among Diabetic Patients

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BAHAGIAN PENYELIDIKAN & PEMBANGUNAN

CANSELORI

UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: **Prof. Madya Faridah Abdul Rashid**

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2) Pusat Pengajian /Pusat/Unit: **Sains Perubatan**

3) Tajuk Projek: **Characterization of Low Density Lipoprotein Subfraction Profile and Apo E Genotype Among Diabetic Patients**

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).

Abstrak Bahasa Malaysia

Diabetes mellitus ialah penyakit metabolik yang bercirikan hiperglisemia dan dislipidemia, yang memberi pesakit diabetes risiko tinggi untuk mendapat penyakit jantung. Adalah dipercayai bahawa LDL kolesterol (LDLC), trigliserida (TG) dan genotip apolipoprotein E (apo E) berkait rapat dalam menentukan risiko tinggi ini. Kajian ini bertujuan untuk mencirikan dislipidemia diabetes dengan penentuan saiz LDL dan genotip apo E. Seramai 30 subjek Normal dan 35 Diabetes mellitus jenis 2 dikaji. Analisis biokimia lipid ditentukan secara automatik menggunakan kit komersial. Saiz LDL ditentukan menerusi mikroskop elektron transmisi. LDL1 dan LDL2 adalah bersaiz besar manakala LDL3, LDL4, dan LDL5 adalah bersaiz kecil dan dianggap memudaratkan kesihatan. Genotip apo E pula ditentukan menerusi tindakbalas rantai polimerase dan polimorfisme kepanjangan fragmen restriksi.

Penemuan

- Pesakit diabetes mellitus jenis 2 yang berlebihan berat badan mempunyai lebih banyak LDL4 berbanding seseorang normal
- Kandungan LDL4 berkadar terus dengan aras TG plasma. Ini menunjukkan hipertrigliseridemia menyumbang kepada pembentukan LDL4 dalam kes diabetes yang dikaji
- Kandungan LDL1 berkadar songsang dengan LDL3. Ini menunjukkan LDL1 bertukar menjadi LDL lebih kecil, pertukaran ini berterusan dan mengakibatkan banyak LDL3 terbentuk
- Genotip ε4/2 bercirikan LDLC *paling rendah* dan HDLC *paling tinggi*. Genotip ini adalah baik bagi pesakit diabetes yang berlebihan berat badan kerana mempunyai risiko *paling rendah* untuk mendapat penyakit jantung iskemia
- Genotip ε4/3 bercirikan TG *paling tinggi*, LDLC *paling tinggi*, TC *paling tinggi*, dan HDLC *paling rendah*. Genotip ini tidak baik bagi pesakit diabetes yang berlebihan berat badan kerana mempunyai risiko *paling tinggi* untuk mendapat penyakit jantung iskemia
- Genotip ε3/3 (normal) mempunyai risiko *perantara* untuk mendapat penyakit jantung iskemia

Kesimpulan

Saiz LDL dan genotip apo E perlu ditentukan untuk pesakit diabetes mellitus jenis 2 yang berlebihan berat badan kerana mereka berpotensi tinggi untuk mendapat penyakit jantung iskemia. Semasa mengurus perawatan pesakit berkenaan, kedua-dua LDLC dan TG perlu dipantau rapi sehingga 4 jam postprandial jika boleh.

Abstrak Bahasa Inggeris

Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia and dyslipidaemia, which places diabetics at increased risk of cardiovascular disease. We strongly believed that LDL cholesterol (LDLC), triglycerides (TG) and apolipoprotein E (apo E) genotype are closely related in determining this high risk. This study aims to study the effect of LDL subfraction and apo E genotype on diabetic dyslipidaemia. Normal (n=30) and diabetes mellitus type 2 (n=35) subjects who were not on any drug treatment were studied. Lipid biochemical analysis was performed by automated methods using commercial kits. LDL size was determined by transmission electron microscopy. LDL1 and LDL2 are large particles while LDL3, LDL4, and LDL5 are smaller particles and are considered detrimental to health. Apo E genotype was determined by polymerase chain reaction and restriction fragment length polymorphism.

Findings

- Overweight diabetes mellitus type 2 subjects have significantly more LDL4 compared to normal
- LDL4 content varies directly with plasma TG. This indicates that hypertriglyceridaemia contributes to the formation of LDL4 in the diabetics who were studied
- LDL1 content varies indirectly with LDL3. This indicates that LDL1 is converted to smaller LDL, this conversion continues and results in abundance of LDL3
- ε4/2 genotype is characterised by *lowest* LDLC and *highest* HDLC. This genotype is considered good for overweight diabetics as it confers the lowest risk for ischaemic heart disease
- ε4/3 genotype is characterised by *highest* TG, *highest* LDLC, *highest* TC, and *lowest* HDLC. This genotype is considerd not good for overweight diabetics as it confers the highest risk for ischaemic heart disease
- Normal ε3/3 genotype confers intermediate risk for ischaemic heart disease

Conclusion

LDL size and apo E genotip should be obtained for overweight diabetes mellitus type 2 patients because they are at increased risk for getting ischaemic heart disease. When managing the treatment of these these patients, both LDLC dan TG should be closely monitored up to 4 hours postprandially if possible.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
Diabetes mellitus jenis 2	Diabetes mellitus type 2
hiperglisemia	hyperglycaemia
dislipidemia	dyslipidaemia
saiz LDL	LDL size
genotip apo E	apo E genotype

5) *Output* Dan Faedah Projek

(a) Penerbitan *(termasuk laporan/kertas seminar)*
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbitkan/dibentangkan).

Jenis	Tajuk	Pengarang	Tahun Terbitan	Tempat Diterbit/ Dibentangkan
Full paper; poster presentation LAMPIRAN 1	Preliminary studies on apolipoprotein E genotypes and allele frequency among healthy subjects	Shahrul BSH, Faridah AR	29-30 April 2000	Simposium Sains Kesihatan Kebangsaan Ke-3, FSKB, UKM
Abstract; poster presentation LAMPIRAN 2	Relationship between genotype and allele of apolipoprotein E with the lipid status among Malays	Shahrul BSH, Mohd Rafi, Wan MWB, Faridah AR	18-21 May 2001	1st ASEAN Conference on Medical Sciences, Renaissance Kota Bharu Hotel. Book of Abstracts, Abstract No. P-22, page 69
Abstract; oral presentation LAMPIRAN 3	A study of postprandial patterns of lipaemia and glycaemia in patients with ischaemic heart disease and those with glucose intolerance	Faridah Abdul Rashid, Shahrul Bariyah Sahul Hamid, Mafauzy Mohamed, Wan Mohamad Wan Bebakar, Nazmi Mohd Noori, and Ruhani Halim	30 June – 1 July 2001	IRPA Top Down Research Workshop 8. Concorde Inn, KLIA 43900 Sepang, Selangor
Abstract; poster presentation LAMPIRAN 4	Palm olein load causes alteration of lipid kinetics during postprandial state in a paired study group	Shahrul BSH, Wan MWB, Mafauzy M, and Faridah AR	18-20 April 2002	1st PENSMA National Congress, Renaissance Melaka Hotel. *Submitted*
Abstract; to request for oral presentation LAMPIRAN 5	Characterization of low density lipoprotein subfraction profile and apo E genotype among diabetic patients	Shahrul Bariyah Sahul Hamid, Faridah Abdul Rashid and Wan Mohamad Wan Bebakar	17-18 May 2002	7th National Conference on Medical Sciences, Universiti Sains Malaysia Health Campus, Kubang Kerian, Kelantan. *To be submitted*

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten
(Jika ada dan jika perlu, sila gunakan kertas berasingan)

(c) Latihan Gunatenaga Manusia

i) Pelajar Siswazah: 4 orang pelajar di bawah penyeliaan:

- Shahrul Bariyah Sahul Hamid – PhD
- Mohd. Rafi Mustapha – MSc / Pegawai Sains
- Eid Mohammad s/o Akhtar Mohammad – MSc
- Julia Omar – MMed (posting Makmal Lipid)

ii) Pelajar Prasiswazah: 6 orang pelajar DTMP Tahun 3 yang menjalani projek penyelidikan akhir kursus yang diselia:

- Ang Wooi Lee
- Kong Siaw Huong
- Joel Jeebaseelan a/l William
- Marsitah Omar
- Yong Yau Lee
- Zamani Mohd. Zain

iii) Lain-lain: 2 orang teknologis makmal perubatan dari Jabatan yang turut bersama atas jemputan:

- Zakaria Abu Samah
- Lau Yoke chie

6. Peralatan Yang Telah Dibeli: *(Vot 35000 sahaja)*
(For Asset Management System Tracking)

Jenis	Keterangan	Pembekal	Serial Number	Harga	Tarikh beli	PO/DO/Invoice
Peralatan elektroforesis LAMPIRAN 6	Dual cooled vertical electrophoresis system, Model SE 600 Cat No: 80-6171-96	Amersham Pharmacia Biotech, Hoefer	20058937	RM3,600	2 Oct 2000	PO: A18103 DO: 21887 Invoice: 17872
Peralatan elektroforesis LAMPIRAN 7	Gradient maker, Model SG 50 Cat No: 80-6197-99	Amersham Pharmacia Biotech, Hoefer	None	RM999	2 Oct 2000	PO: A18103 DO: 21887 Invoice: 17872
			JUMLAH	RM4,599		

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

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TANDATANGAN PENGERUSI
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PUSAT PENGAJIAN SAINS PERUBATAN

Charaterization of Low Density Lipoprotein Subfraction Profile and APOE Genotype among Diabetic Patients

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The present study aimed to examine the association between diabetes mellitus type 2 with low density lipoprotein particle size distribution and the influence of apolipoprotein E genotype in altering lipid profile. A number of 35 subjects with diabetes mellitus type 2 without having any drug treatment were enrolled in this study. Results obtained were compared with 30 normal control subjects. Blood samples were taken after an overnight fast of 10-12 hours. Biochemical analysis was done using enzymatic automated method (Hitachi 912) for the determination of total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, and glucose concentration. LDL subfraction area under curve percentage (% AUC) was determined by using non-denaturing 2%-16 % polyarcylamide gel electrophoresis and APOE gene analysis was by restriction fragment length polymorphism (RFLP) method. Lipid profile test results showed the male diabetic subject had higher triglyceride and LDL cholesterol value. Whereas, female diabetic subjects had higher total cholesterol, triglyceride, HDL cholesterol, VLDL cholesterol and glucose concentration. Correlation studies indicate a positive and significant between triglyceride concentration and AUC LDL 4. Triglyceride also correlated positively and significantly with glucose concentration. This indicates that hypertriglyceridaemia could lead to the formation of small LDL particles. Looking into the correlation between the LDL subfraction, the AUC of LDL 1 correlated negatively with the AUC of LDL 3. Diabetic subject generally were found to have higher AUC value for the smaller LDL particles which comprises LDL 3, LDL 4, and LDL 5. The study on the APOE gene showed the ε3 and ε4 subjects had high total cholesterol and glucose concentration. Diabetic individuals with the ε2 allele did not have any significant difference in concentration with the ε3 and ε4 allele carriers when the triglyceride concentration was compared between the three allele carriers. Allele frequency seen in this groups of diabetic subjects was ε2 (0.143), ε3 (0.714) and ε4 (0.143). Frequency distribution obtained was similar to the findings from

study on diabetic mellitus type 2 subjects by Boemi *et. al* (1995). Frequency comparison with the non-diabetic subjects show the ε2 allele frequency was higher in diabetic subjects.

INTRODUCTION

An increase in the number of individuals with type 2 diabetes has been reported worldwide, with the most dramatic increase occurring in developing countries. It was reported by Mafauzy et. al (1999) that the prevalence of diabetes mellitus increased when subjects are beyond 40 years of age (10.1%). Almost 72% of the diabetic subjects had hypercholesterolemia and 31.9% had mixed hyperlipidaemia. The prevalence of type 2 diabetes varies with population age, genetic, and environmental factors. Besides it, other confounding factors are dietary intake, ethnic group, body mass index (BMI), and lack of exercise.

Diabetes mellitus is a vascular disease as it is related to macro vascular and micro vascular diseases. Nearly 75% of diabetic patients die of an atherosclerotic event, and the incidence of coronary artery disease is increased 2- to 4-fold in the diabetic compared to non-diabetic subjects (Hsueh & Law, 1998). The clustering of type 2 diabetes, a well documented risk factor for cardiovascular disease (CVD) with other established risk factors such as dyslipidaemia, hypertension, and abdominal obesity is well recognized. Each risk factor conveys its significant CVD risk and in combination they place the person with type 2 diabetes at substantial CVD risk. This clustering has been labeled the metabolic syndrome, dysmetabolic syndrome or insulin resistance syndrome. Recognition of these features in people with type 2 diabetes has special importance in treating these subjects with appropriate treatment regimens.

Lifestyle changes interact with underlying genetic factors and this accelerates the manifestation of metabolic syndrome. The recent National Cholesterol Education Program Adult Treatment Panel (NCEP ATP) III guidelines recognize the importance of multiple metabolic syndrome disorders, as seen in the metabolic syndrome, and

recommended an aggressive lifestyle modification approach followed by drug therapy in those who fail to respond to non pharmacological measures. However, the absolute percent of coronary artery disease (CAD) reduction is only 1% to 2%. The greater treatment effect is hindered by a number of possible explanations, such as influence of other traditional CAD risk factors (hypertension, low HDL-cholesterol, high-fat diet, obesity, lack of exercise, lack of compliance with drug regimen, and not reaching recommended LDL goal by NCEP ATP III. However, a critical factor that often remains after treatment is the presence of small, dense LDL particle in large quantity which is not detected by current LDL determination. It is calculated based on Friedewald calculation (NCEP ATP III report, 2001). This indicates the importance of early identification of individuals at high risk of CVD who have increased atherogenic small LDL particles.

Apolipoprotein E (apoE) is the key protein in transportation of lipids, and its polymorphism is recognized as one of the most important genetic determinants for CAD (Mahley, 1988). On a population basis it has the strongest impact on plasma lipid levels presently known for a single gene polymorphism. Genetic polymorphism is an important cause of aberrant lipoprotein metabolism and increases the CAD risk (Kataoka et. al, 1997; Kalix, et. al, 2001). The gene for apoE is polymorphic. It has three common alleles, which consist of epsilon 2, epsilon 3, and epsilon 4, and each code for three major isoforms, resulting in six common phenotypes. These isoforms vary in their receptor-binding affinity, with apoE 4 having the greatest receptor binding and apoE2 having less than two percent binding affinity. Individuals with apoE2 are reported to have higher levels of triglycerides and in homozygote even great increase in concentrations of remnants were observed, which results in type III dyslipidaemia (Zhao et. al, 1994). Individuals with epsilon 4, conversely, tend to have striking increase in LDL cholesterol (Kesäniemi et al, 1987). We designed this study in order to characterize the LDL subfraction pattern and to study the APOE gene polymorphism among type 2 diabetics compared to non-diabetic subjects.

METHODS

Subjects

New diagnosed subjects attending the lipid clinic at Hospital Univerisiti Sains Malaysia were recruited after screening the folders at the record office. Type 2 diabetic patients were confirmed by fasting venous blood glucose levels according to the WHO year 2000. After informed consent was given, fasting blood samples were taken for measurement of lipids and lipoproteins.

Biochemical analyses

Total cholesterol, triglycerides, and glucose were determined by colorimetric enzymatic assay with Hitachi chemistry analyzer using BioMëriux reagents. LDL cholesterol was calculated based on the Friedewald formula and HDL cholesterol was measured after precipitation. Lipid and other lipoprotein were precipitated using the phosphotungstic acid and magnesium ion precipitation method. Anthropometric measurements included weight and height. Individuals were categorized into normal weight, overweight, and obese based on BMI < 25 kgm^{-2}, ≥ 25 kgm^{-2} and ≤ 29 kgm^{-2}, and >29 kgm^{-2} respectively (Boemi et. al, 1995). Persons were classified as diabetic when fasting venous plasma glucose was ≥ 7.0 mmol/L. In the absence of symptoms, diagnosis was confirmed by an additional high glucose level result on another day. Absence of diabetes among the control subjects was confirmed by measurement of fasting glycaemia.

LDL subfractionation

The contents and conditions of electrophoresis were the two main parts involved in optimizing of a non-denaturing linear gradient PAGE. Preparation of materials was based on Li et al. (1997). Gel contents, and electrophoresis conditions were optimization before and gel calibration step.

The polyacrylamide gel was standardized with lipid control, lipoprotein deficient serum (LPDS), purified HDL, polystyrene latex beads, and purified albumin. Electron micrographs were obtained using the Philips CM 12 transmission electron microscope (TEM) at 80 kV. After the electron micrographs have been obtained, further size

determination of the standard particles was done using the Leica Q Win size determination software.

The migration of the gel standards was measured from the point of application (origin) to the middle of each band. Migration distance of each calibrator and their mean particle size (diameter) were measured based on the micrographs captured with transmission electron microscope. A standard curve was plotted based on mean particle size (diameter) measured for each of the calibrators and its migration distance from origin. This plot was later used to determine the size of each LDL subclass. Scanning results of sample migration were later referred to the standard curve to identify the LDL subclasses present in each sample and its area under curve percentage (AUC %).

APOE genotyping

The amount of blood drawn was 5 ml. The EDTA and plain were used to collect the blood samples and 2.5 ml was placed in each tube before centrifuging at 3,500 rpm for 5 minutes.

For apo E genotyping, genomic DNA was isolated from leukocytes by standard method and amplification was done using the modified method of Hixon & Vernier (1990). The 5' part of exon 4 of the human apo E gene at chromosome 19q13.13-19q13.32, coding for amino acids 61-174 was amplified by the polymerase chain reaction (PCR). The human apoE gene exhibits three common variants (E2, E3, and E4) which differ from each other at residue 112 and 158 in the mature protein. E4 has arginine (DNA sequence for the site GCGC) and E2 has cysteine (GTGC) at both sites, whereas E3 has cysteine (GTGC) at site 112 and arginie (GCGC) at site 158 (Wang et. al, 1995). A section of apoE DNA that contains the genotype differentiating sites was amplified by PCR. The primers sequences are as stated following:

F6 upstream primer 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3'

F4 downstream primer 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3'

The PCR cycles consist of sample preheating at 95°C for 5 minutes in 50 µl PCR reaction mixture including dNTP and *Taq* polymerase. The preheating was followed by denaturation at 95°C for 1 minute and subjected to annealing of the primers to the single-stranded DNA at 60°C for 1 minute, and extension of the primers in both directions along the template at 70°C for 2 minutes. Newly synthesized fragments in turn serve as templates for a chain reaction which results in the exponential amplification of the target sequence. The second cycle begins with 95°C for 1 minute until 30th cycle is completed after 3 hours.

Partial amplification of exon 4 of the apoE gene generates a DNA fragment of 244 base pairs (bp) was then subjected to *Hha*I from *Haemophilus haemolyticus* digestion for overnight at 37°C. HhaI cleaves at GCG*C. The fragment contains four constant and two polymorphic *Hha*I sites (Fig.1). Restriction enzyme digestion of the fragment and electrophoresis allow the products of the six different apoE genotype as shown in table 1. Fragments were visualized by ethidium bromide staining after electrophoresis in 17.8% polyacrylamide gel. Electrophoresis was conducted at the voltage of 140 for 110 minutes with the tris-acetate-EDTA (TAE 5X) buffer.

Table 1: Genotype diagnostic fragment size

		Fragment size (bp)			
ε2/2	**ε3/3**	**ε4/4**	**ε3/2**	**ε4/2**	**ε4/3**
91	91		91	91	91
83			83	83	
		72		72	72
	48	48	48	48	48
38	38	38	38 x 2	38 x 2	38 x 2
	35	35	35	35	35 x 2

Diagnostic fragments are only 91 bp, 83 bp, 72 bp, and 48 bp. Difference between the six genotypes could be determined by these specific combinations. Symbol x indicates the presence of two fragment with the same size and it reflects double florescence under ultra-violet visualization.

Alelle frequency was calculated based on the gene-counting method (Garry et. al 1999).

$$\varepsilon 2=\frac{N_{2/2}+(0.5)N_{3/2}+(0.5)N_{4/2}}{*N_{total}}$$

$$\varepsilon 3=\frac{N_{3/3}+(0.5)N_{3/2}+(0.5)N_{4/3}}{N_{total}}$$

$$\varepsilon 4=\frac{N_{4/4}+(0.5)N_{4/2}+(0.5)N_{4/3}}{N_{total}}$$

- N is the number of subjects

Statistical analyses

Statistical analyses were performed using the SPSS version 10 for windows software. Students t-tests were used for continuous variables and chi-square (χ^2) tests were used for categorical variables to test for statistical significance. Statistical significance was defined as a two-tailed p-value <0.05. Results are presented as mean ± standard deviation for the parameters in each group.

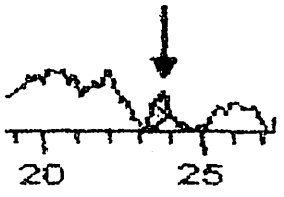
RESULTS

LDL subfraction

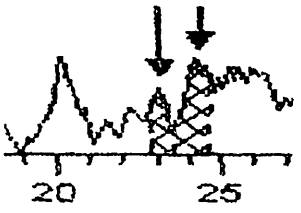
Up to five distinct peaks were seen within the range of 23 mm-25 mm from the origin (Fig.1). LDL subclass sizes were: LDL 1 (27.8 nm), LDL 2 (25.1 ± 1.35 nm), LDL 3 (22.4 ± 1.35 nm), LDL 4 (19.7 ± 1.35 nm), and LDL 5 (17 nm).

APOE genotyping

Six of the genotypes of APOE are depicted in figure 2, 3, and 4. DNA fragments size varies in each genotype based on the presence of base substitution at sites 112 and 158 within the amplified region of the gene. The determination of each genotype was done by the diagnostic fragment sizes.

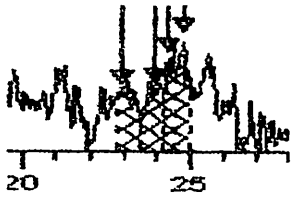


(A) LDL 1 subfraction

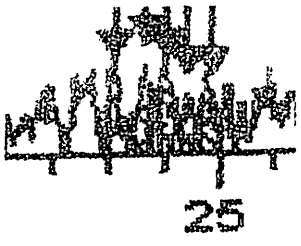


(B) LDL 2 subfraction

(C) LDL 3 subfraction



(D) LDL 4 subfraction



(E) LDL 5 subfraction

Figure 1: Densitometric scans of LDL subfractions

These are the scanning images of the 2% - 16% gradient gel after staining with Oil Red O. Electrophoresis was performed for 5 hours with citrate-borate-EDTA buffer, pH 8.3 at 20 °C.

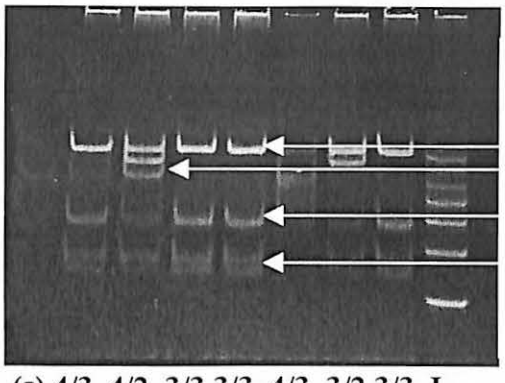


Figure 2: Four genotypes comprising of ε4/3, ε4/2, ε3/3, and ε3/2.

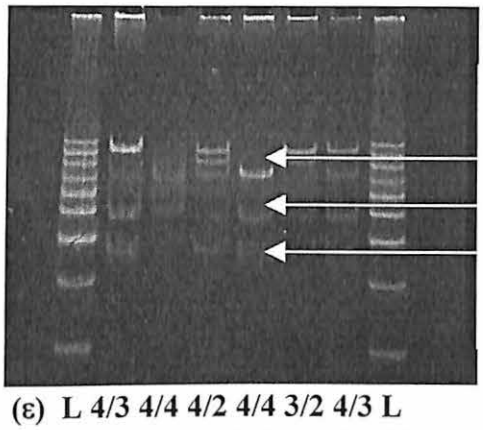


Figure 3: Fragment sizes of ε4/4 genotype.

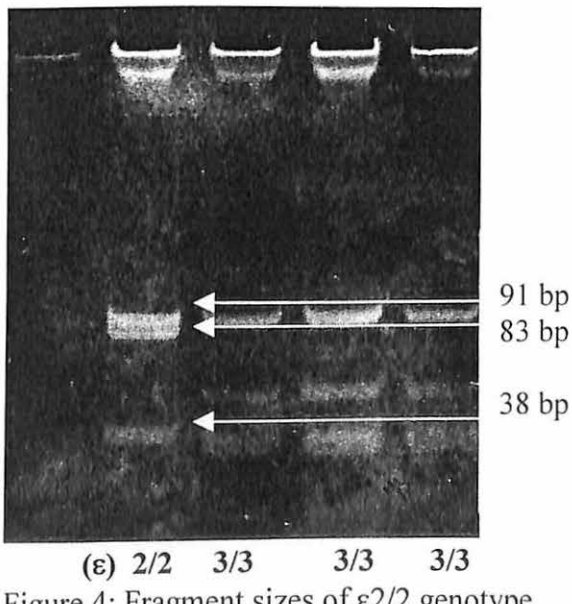


Figure 4: Fragment sizes of ε2/2 genotype

Gender effect on lipid profile

Herein, triglycerides was higher among diabetic females compared to non-diabetic females. Presence of estrogen in females decreases the apoE retention time on VLDL particle (Kushwaha et. al, 1991). However, in males, testosterone increases lipoprotein lipase activity. As a result, triglycerides are increased among females and reduced in males. Female subjects in either diabetic or non-diabetic group had higher total cholesterol and LDL cholesterol. However, there was no significant differences in diabetic group for both of these parameters when gender effect was compared. Hiperinsulinemia results in increased cholesterol and there is no significant gender effect. Triglycerides were only raised in diabetic female subjects. HDL was higher among females and in contrary male subjects had lowered HDL cholesterol level. Inhibition of LpL activity among females by estrogen contributes to the increase in triglyceride but indirectly it causes an increase in HDL cholesterol.

Total cholesterol and LDL cholesterol

The diabetic ε4 allele carriers were found to develop high LDL cholesterol compared to the ε2 allele carriers. Similar results was reported by (Kataoka et. al, 1996). However, total cholesterol was increased among the ε3 and ε4 allele carriers with diabetes. Generally, ε4 allele was found to be correlated with increased LDL cholesterol in both groups. Negative feedback down-regulation happens when the amount of cholesterol is increased beyond its bound. Further intake of cholesterol is suppressed by reducing the number of LDL receptors at hepatocytes (Davignon, et. al, 1988).

Triglycerides

In the presence of the ε4 allele distinct increase of triglyceride to above normal cut off value was seen in this study. Unexpectedly, subjects with the ε3 allele were found to be having increased triglycerides. We had presumed that subjects the with ε3 allele would show lowest triglyceride level as have been reported previously. The increase in triglyceride level among the ε4 allele carriers having diabetes was due to the inhibition of lipoprotein lipase activity (Dallongeville et. al, 1992).

HDL cholesterol

Generally, ε2 allele carriers either diabetic or non-diabetic had the highest HDL cholesterol. However, ε3 allele carrier of both group showed the lowest level. Female subjects, those with the ε3/2 allele were among the group with the highest HDL cholesterol. It was found that apoE2 and apoE3 form stable complexes with HDL and subjects with this particular isoforms show high HDL level (Weisgraber, 1990). In vivo and in vitro studies have shown that apoE plays an important role in the regulation of cholesteryl ester transfer protein (CETP) activity. This may explain the relationship between apoE polymorphism with HDL cholesterol. ApoE has also been suggested to modulate lipoprotein lipase (LpL) activity, and individuals with higher LpL activity are expected to have high HDL cholesterol.

DISCUSSION

Formation of small LDL is found to be influenced by triglycerides concentration in this study. However, reduction in AUC% of large LDL particles inversely correlated with changes of smaller LDL particles especially LDL 3 AUC%. Similar findings have been reported, where LDL 4 correlated positively with VLDL and IDL (Shen et. al, 1981; Krauss & Burke, 1982). Coresh et. al (1993) found triglycerides to be a determinant of the LDL subfraction characteristic and to be correlated with development of coronary artery. However, they reported age and gender will not influence lipid profile. We found triglycerides concentration to be marginally significant in its positive correlation with glucose concentration. Thus, we hypothesize that hyperglycemia could contribute to the formation of small, dense LDL particle especially among diabetic subjects. Nishina et. al (1992) claimed that LDL B subclass which is a atherogenic phenotype has a connection with the LDL receptor and insulin gene at the short arm of the 19th chromosome.

Insulin resistance is associated with increased non-esterifies fatty acid flux to the liver, increased hepatic output of large VLDL which is not suppressed postprandially, hiperlipidaemia and increased cholesterol ester transfer protein activity. All of these factors are the possible reason for the formation of small LDL particles observed in this study. The mechanism responsible for the relationship of hyperglycemia or

hyperinsulinemia with high triglyceride and high LDL cholesterol concentration is due to the factor of reduced enzymatic activity involved in lipid metabolism. Differences in insulin action or plasma insulin level may affect the activity of both these enzymes. The presence of glycosylated LDL and small LDL increases the LDL of cholesterol. This is explained by the reduced binding ability to the LDL receptors than the normal LDL. A decrease in the size of LDL confers additional atherosclerotic risk to this group of individual with diabetes mellitus type 2.

Frequency of each genotype among diabetic subjects in our study differed when we compared with the non-diabetic Asian population APOE genotype frequency. However, comparison with result obtained in the study conducted by Boemi et. al (1995) on diabetic subjects revealed almost similar frequency. Further analyses were performed on the impact of these polymorphisms on lipid and lipoprotein metabolism. Epsilon (ε) which comprises ε2, ε3, and ε4 effects on plasma lipid and lipoprotein were studied separately and we noticed triglycerides, VLDL cholesterol, and glucose to be increased in all three allele carriers with diabetes. However, total cholesterol and LDL cholesterol was raised only in ε3 and ε4 allele carriers with diabetes. Apparently, ε3 allele carriers had high LDL cholesterol in the presence of diabetes mellitus type 2. Only non-diabetic ε2 allele carrier had the highest HDL cholesterol. HDL cholesterol level among ε2 and ε3 allele carriers with diabetes was reduced compared to their control non-diabetic group. This indicates impaired synthesis of HDL particles during hyperinsulinemia. In contrast, ε4 allele carriers did not show any reduction in HDL cholesterol compared to the ε4 non-diabetic subjects.

Triglycerides were similar between all three allele carriers among the diabetic subjects. However, previous studies have reported that only subjects with the ε2 allele have the tendency to have increased triglyceride concentration. We assume that in the presence of insulin resistance, diabetic subjects are prone to have increased triglyceride especially among the ε4 allele carriers. Generally, presence of insulin resistance exacerbates the effect of APOE gene polymorphism on lipid and lipoprotein metabolism.

In summary, there was a higher percentage of diabetic subjects with ε2 and ε4 alleles. The ε4 allele carriers of diabetic group could be categorized as having higher risk of developing atherosclerosis due to increased LDL cholesterol, total cholesterol, and triglyceride but reduced HDL cholesterol. Surprisingly, the ε3 allele carriers of diabetic group also had increased triglyceride level. This indicates in the presence of insulin resistance, aberration to normal lipid and lipoprotein metabolism occurs even among individuals with the normal ε3 variant of APOE gene. A conclusive result on the influence of APOE genotype polymorphism on plasma lipid and lipoprotein levels in diabetics could come from the analysis of a larger sample of subjects.

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KAJIAN AWAL MENGENAI GENOTIP APOLIPOPROTEIN E DAN FREKUENSI ALEL DI KALANGAN SUBJEK SIHAT

PRELIMINARY STUDIES ON APOLIPOPROTEIN E GENOTYPES AND ALLELE FREQUENCY AMONG HEALTHY SUBJECTS

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ABSTRAK/ABSTRACT

Kajian ini menentukan genotip apolipoprotein E dan frekuensi allel dalam kalangan individu sihat. Penentuan genotip apo E dianalisa dengan kaedah polimorfisma rangkaian fragmen terpilih (RFLP). Kolesterol dan trigliserida ditentukan dengan kaedah berautomasi. Kolesterol lipoprotein berketumpatan rendah (LDL) diperoleh dari pengiraan Friedewald manakala kolesterol lipoprotein berketumpatan amat rendah (VLDL) ditentukan dengan membahagikan kepekatan trigliserida dengan pemalar 2.2. Kolesterol lipoprotein berketumpatan tinggi (HDL) dotentukan dengan kaedah pemendakan asid fosfotungstik dan ion Mg. Frekuensi alel epsilon 3 didapati lebih tinggi di kalangan wanita berbanding dengan lelaki sebanyak 5.2% manakala terdapat peningkatan sebanyak 8.3% dan 66.7% pada subjek lelaki dengan alel epsilon 4 serta 2. Kepekatan trigliserida dalam pembawa E3/3 adalah 1.79 mmol/L manakala dalam E4/2, E2/2, E3/2 dan E4/3 adalah lebih tinggi iaitu sebanyak 60.3%, 53.6%, 47.5% dan 10.6% dalam turutan. HDL adalah hampir sama bagi setiap genotip dan kepekatan LDL didapati meningkat sebanyak 46.2% pada subjek E2/2 dan sebaliknya pada subjek E4/2 sebanyak 13.8%. Kepekatan VLDL didapati lebih tinggi pada subjek E4/2, E2/2, E3/2 sebanyak 67.9%, 60.3% dan 53.8% dalam turutannya. Genotip E4/2 dicirikan oleh kepekatan trigliserida dan VLDL yang tinggi. Ciri ini juga diperhati pada subjek dengan E2/2 yang mempunyai kandungan kolesterol LDL yang tinggi berbanding dengan genotip lain.

This study ascertained apolipoprotein (apo) E genotypes and allele frequency among healthy subjects. The apo E genotype was analyzed by restriction fragment length polymorphism (RFLP). Cholesterol and triglycerides were measured by automated method. Low density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula and very low density lipoprotein (VLDL) cholesterol determined by dividing triglycerides by a constant of 2.2. High density lipoprotein (HDL) cholesterol was determined by the phosphotungstic acid and Mg ion sedimentation method. The allele frequency of epsilon 3 was found to be higher in females compared to males by 5.2% whereas there was an increase of 8.3% and 66.7% in the percentage of epsilon 4 and epsilon 2 among males. Triglycerides concentration in E3/3 carriers was 1.79 mmol/L while in E4/2, E2/2, E3/2 and E4/3, the concentrations were higher by 60.3%, 53.6%, 47.5% and 10.6% respectively, HDL cholesterol concentration was found to be almost similar for each of the genotypes and LDL cholesterol concentration was raised by 46.2% in the E2/2 carrier and reduced in the E4/2 carrier by 13.8%. VLDL cholesterol concentration was found to be higher in the E4/2, E2/2, E3/2 by 67.9%, 60.3% and 53.8% respectively. The E4/2 genotype carriers were characterized by the higher triglyceride and VLDL cholesterol concentration. This was also seen in the E2/2 carrier whose LDL cholesterol was seen to be higher compared to the other genotypes.

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Characterization of Low Density Lipoprotein Subfraction Profile and Apo E Genotype Among Diabetic Patients

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The present study aimed to examine the association between diabetes mellitus type 2 with low density lipoprotein particle size distribution and the influence of apolipoprotein E genotype in altering lipid profile. A total of 35 subjects (19 males, 16 females, mean age 50±11 years, mean BMI 27±4 kg/m^2) with diabetes mellitus type 2 who were overweight and without any drug treatment were enrolled in this study. Results obtained were compared with that of 30 normal control subjects (14 males, 16 females, mean age 27±5 years, mean BMI 23±3 kg/m^2). Plain blood samples were taken after an overnight fast of 10-12 hours. Serum biochemical analysis was done using automated enzymatic methods (Hitachi 912) for the determination of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and glucose concentration. HDL cholesterol was performed after chemical precipitation. LDL cholesterol was calculated if triglycerides was less than 4.5 mmol/L. Otherwise, direct LDL cholesterol estimation was done. LDL subfraction area under curve percentage (% AUC) was determined by using non-denaturing 2-16 % polyacrylamide gel electrophoresis. APOE gene analysis was by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The lipid profil test results showed that male diabetics had higher triglycerides and LDL cholesterol whereas female diabetics had higher total cholesterol, triglycerides, HDL cholesterol, VLDL cholesterol and glucose. There was a positive and significant correlation between triglycerides and AUC of LDL 4. Triglycerides also correlated positively and significantly with glucose. This indicates that hypertriglyceridaemia could possibly lead to the formation of small LDL particles. The possibility of step-wise conversion of bigger LDL into smaller LDL was studied by looking into the correlation between the LDL subfractions. The AUC of LDL 1 correlated negatively with the AUC of LDL 3. Diabetics generally were found to have higher AUC values for the smaller LDL particles which comprise of LDL 3, LDL 4, and LDL 5. The study on APOE gene showed that ε3 and ε4 diabetics had elevated total cholesterol and glucose. Diabetics with the ε2 allele did not have any significant difference with the ε3 and ε4 subjects when the triglycerides concentration was compared with that of the 3 allele carrier. Allele frequency obtained for diabetics was ε2 (0.143), ε3 (0.714) and ε4 (0.143). The frequency distribution obtained was similar to the findings from the study on diabetic mellitus type 2 subjects by Boemi *et. al* (1995). Frequency comparison with the normal control showed that the ε2 allele frequency was higher in diabetics. Overweight ε4/3 diabetics portrayed the worst atherogenic profile with the highest triglycerides, LDL cholesterol and total cholesterol, and the lowest HDL cholesterol. Insulin resistance is associated with increased non-esterified fatty acid flux to the liver, increased hepatic output of large VLDL which is not suppressed postprandially, hyperlipidaemia and increased cholesterol ester transfer protein activity. All these factors could act in concert and possibly give rise to the formation of small LDL particles observed in this study. The mechanism responsible for the relationship of hyperglycaemia or hyperinsulinaemia with elevated triglycerides and elevated LDL cholesterol is due to reduced enzymatic activity of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) involved in lipid metabolism. Differences in plasma insulin level or insulin action may affect the activity of both these enzymes. The presence of glycosylated LDL and small LDL increases the contribution of LDL to total cholesterol estimation. This is explained by the reduced binding ability of both these abnormal LDL particles to LDL receptors compared to normal LDL. We therefore conclude that a decrease in LDL size with high propensity for small LDL 4 therefore confers additional atherosclerotic risk to overweight individuals with diabetes mellitus type 2, especially those with ε4/3 genotype.

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