EFFECT OF *Heterotrigona itama* BEE BREAD ON METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE PARAMETERS IN OBESE MALE RATS

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2022

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by

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Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

September 2022

ACKNOWLEDGEMENT

Above all, I thank Allah the Almighty for the breadth of knowledge He has bestowed upon me and other numerous reasons that made all these possible.

My deepest gratitude is to my supervisor, Assoc. Prof. Dr. Mahaneem Mohamed for her dedicated supervision, guidance and constructive criticism. I have been amazingly fortunate to be granted a supervisor who gave me freedom to explore on my own and at the same time the guidance to recover my steps faltered. I also would like to give my special appreciation to my co-supervisors, Dr. Nur Asyilla Che Jalil, Dr. Wan Syaheedah Wan Ghazali and Dr. Khairul Mohd Fadzli Mustaffa, for their valuable guidance and advice throughout this study.

My special thanks are to all the staff in the laboratories of Physiology and Centre Research Laboratory of School of Medical Sciences, Universiti Sains Malaysia for the assistance in providing guidance and technical support for doing my research. I also would like to acknowledge the financial supports from GIPS-PhD Grant (311/PPSP/4404813) and Research University Grant (1001/PPSP/8012378).

My special appreciation to all my colleagues especially Dr. Zaidatul Akmal Othman, Dr. Joseph Bagi Suleiman, and Dr. Victor Udo Nna for their hands and motivations. To my mother and late father, and siblings, thank you for your prayers and always being there for me and helping me in achieving this lifelong dream of mine.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
Acox-1	Acyl-CoA oxidase 1
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
Apo	Apolipoprotein
AST	Aspartate aminotransferase
ATGL	Adipose triacylglycerol lipase
ATGL	Adipose triglyceride lipase
Bax	Bcl-2 associated X protein
Bcl-2	β-cell lymphoma-2
BMI	Body mass index
CAT	Catalase
CETP	Cholesterol ester transfer protein
ChREBP	Carbohydrate response element binding protein
CPT-1	Carnitine palmitoyltransferase 1
CRP	C-reactive protein
CYP7α1	Cholesterol 7α-hydroxylase
FAS	Fatty acid synthase
FATP	Fatty acid transport protein
FFA	Free fatty acid
GGT	γ-glutamyl transferase
GPx	Glutathione peroxidase

GR	Glutathione reductase
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HFD	High-fat diet
HPLC	High performance liquid chromatography
HSL	Hormone-sensitive lipase
IDL	Intermediate-density lipoprotein
IL-6	Interleukin-6
IR	Insulin resistance
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LXR	Liver X receptors
MAFLD	Metabolic dysfunction-associated fatty liver disease
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κβ	Nuclear factor-kappa β
Nrf2	Nuclear factor erythroid 2-related factor 2
PPARγ	Proliferator-activated receptor γ
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species

SCD-1	Stearoyl-CoA desaturase-1
Sirt1	Sirtuin 1
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element binding protein-1c
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-α
VLDL	Very low-density lipoprotein

KESAN ROTI LEBAH *Heterotrigona itama* TERHADAP PARAMETER KETIDAKFUNGSIAN METABOLIK BERKAIT PENYAKIT HATI BERLEMAK DALAM KALANGAN TIKUS JANTAN OBES

ABSTRAK

Hampir 13% penduduk dunia mengalami obesiti. Ketidakfungsian metabolik berkait penyakit hati (MAFLD) ialah pengumpulan patologi lemak hati yang berpunca dari obesiti dan berkait rapat dengan banyak gangguan metabolik, tekanan oksidatif, inflamasi dan apoptosis. Roti lebah telah dilaporkan mengandungi sebatian fenolik dan mempamerkan sifat antioksida, anti-inflamasi dan anti-apoptosis. Di samping itu, ia mengurangkan dengan ketara tahap gen lipogenik hati dalam model tikus MAFLD. Walau bagaimanapun, sehingga kini, ianya masih belum diketahui sama ada roti lebah juga boleh memperbaiki lain-lain parameter dalam MAFLD. Oleh itu, kajian ini bertujuan untuk menentukan komposisi fenolik roti lebah *Heterotrigona itama* dan untuk menentukan kesan roti lebah ini terhadap parameter MAFLD dalam kalangan tikus jantan obes. Empat puluh ekor tikus *Sprague Dawley* jantan dewasa dengan berat antara 200-230 g telah dibahagikan secara rawak kepada empat kumpulan (n=10/kumpulan): kawalan normal (NC), diet tinggi lemak (HFD), roti lebah (HFD+Bb, HFD+0.5 g/kg/hari roti lebah) dan orlistat (HFD+Or, HFD+10 mg/kg/hari orlistat). Pada akhir minggu ke-12, tikus telah dimatikan untuk mendapatkan serum, tisu adipos dan tisu hati. Sembilan sebatian fenolik telah ditemui di dalam roti lebah yang mana asid trans 3-hidroksisinamik ialah sebatian yang paling tinggi telah ditemui dalam roti lebah. Roti lebah telah memperbaiki fungsi hati dan perubahan histopatologi hati dengan ketara dalam tikus MAFLD. Ia juga menurunkan pengawalaturan gen-gen berkaitan dengan pengambilan asid lemak

dan *de novo* lipogenesis, menaikkan pengawalaturan gen-gen berkaitan lipolisis, β-oksidasi asid lemak dan sintesis asid hempedu di dalam hati dengan ketara, justeru itu, memperbaiki metabolisme lemak. Tambahan pula, roti lebah juga mengurangkan penanda tekanan oksidatif (bahan reaktif asid tiobarbiturik, nitrik oksida, protein karbonil), meningkatkan aktiviti enzim antioksida (superoksida dismutase, katalase, glutation peroksidase, glutation S-transferase, glutation reduktase) dan translokasi faktor nuklear eritroid 2-berkait faktor 2 ke dalam nukleus, di samping mengurangkan penanda pro-inflamasi (tumor nekrosis faktor-α, nuklear faktor-kappa β, interleukin-1β, monosit pengkemotarik protin-1) dan penanda pro-apoptosis (Bax, caspase-3) dengan ketara, yang mana mungkin boleh dikaitkan dengan kehadiran sebatian-sebatian fenolik dalam roti lebah. Walau bagaimanapun, kajian lanjut diperlukan bagi menentukan mekanisme molekular yang lain dalam roti lebah sebelum ianya digunakan sebagai terapi alternatif ataupun komplementari dalam kalangan pesakit obesiti dengan MAFLD.

EFFECT OF *Heterotrigona itama* BEE BREAD ON METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE PARAMETERS IN OBESE MALE RATS

ABSTRACT

Almost 13 % of the world population suffers from obesity. Metabolic dysfunction-associated fatty liver disease (MAFLD) is a pathological accumulation of hepatic lipid resulted from obesity and closely linked with many metabolic disorders, oxidative stress, inflammation and apoptosis. Bee bread has been reported to contain phenolic compounds and exhibits antioxidant, anti-inflammatory, and anti-apoptotic properties. In addition, it significantly reduced the hepatic levels of lipogenic genes in MAFLD rat model. However, to date, it is not known whether bee bread may also improve other parameters in MAFLD. Therefore, the present study aimed to determine the phenolic composition of *Heterotrigona itama* bee bread and the effect of this bee bread on MAFLD parameters in obese male rats. Forty adult male *Sprague Dawley* rats weighing between 200-230 g were randomly divided into four groups (*n*=10/group): normal control (NC), high-fat diet (HFD), bee bread (HFD+Bb, HFD+0.5 g/kg/day bee bread) and orlistat (HFD+Or, HFD+10 mg/kg/day orlistat) groups. At the end of 12th week, rats were sacrificed to obtain serum, adipose and liver tissues. Nine phenolic compounds were discovered with trans 3-hydroxycinnamic acid as the highest amount of compound to be found in the bee bread. Bee bread significantly improved liver function and histopathological changes in MAFLD rats. It also significantly down-regulated genes related to fatty acid uptake and *de novo* lipogenesis, up-regulated genes related to lipolysis, fatty acid β-oxidation and bile acid synthesis in the liver, hence improved hepatic lipid

metabolism. Furthermore, bee bread significantly reduced liver oxidative stress markers (thiobarbituric acid reactive substance, nitric oxide, protein carbonyl), elevated antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, glutathione reductase) and translocation of nuclear factor erythroid 2-related factor 2 to the nucleus, as well as mitigated pro-inflammatory (tumour necrosis factor-α, nuclear factor-kappa β, interleukin-1β, monocyte chemoattractant protein-1) and pro-apoptosis (Bax, caspase-3) markers, which may be attributed to the presence of phenolic compounds in the bee bread. However, further studies are needed to elucidate other molecular mechanisms of bee bread before it is used as an alternative or complementary therapy among obese patients with MAFLD.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Obesity has become a remarkable life-threatening disease worldwide. The excessive deposition of adipose tissue due to extreme disequilibrium between energy uptake and expenditure results into numerous chronic illnesses such as coronary heart disease, type 2 diabetes mellitus (T2DM), sleep apnoea, cancer, and non-alcoholic fatty liver disease (NAFLD) (Kyle et al., 2016). Malaysia has been reported to have the highest obesity rate among adults in Southeast Asian Countries (Ng et al., 2014) and the current prevalence of overweight and obesity among Malaysian adults has increased from 29.1% and 14.5% in 2016 to 30.4% and 19.7% in 2019, respectively (NHMS, 2020). Although NAFLD has been reported to be a common cause of chronic liver disease in Western countries (Muhidin et al., 2012), the prevalence of NAFLD in Asia has been demonstrated to be from 12% to 37% among the general population (Amarapurkar et al., 2007). Furthermore, based on a latest study carried out among 628 adult subjects in Klang Valley, Malaysia, 235 (37.4%) of the participants were diagnosed with NAFLD (Khammas et al., 2019), hence indicating the increasing prevalence of NAFLD among Malaysian.

The liver has an important role in lipid metabolism, importing serum free fatty acid (FFA), as well as manufacturing, storing, and distributing lipids and lipoproteins (Mirza, 2011). However, with the development of obesity, increased hepatic lipogenesis and serum FFA levels lead to overload accumulation of liver lipid which can further develop into liver damage, including impaired liver function, and eventually liver failure (Donnelly et al., 2005), and occasionally, hepatocellular

carcinoma (HCC) (Marchesini et al., 2003). NAFLD is one of the liver diseases that normally affect overweight and obese individuals (Chan et al., 2013; Goh et al., 2013). It is recognised as a variety of liver disorders ranging from hepatic steatosis (presence of macro-vesicular steatosis only) to non-alcoholic steatohepatitis (NASH) (presence of macro-vesicular steatosis with hepatocyte ballooning, lobular and/or portal inflammation and with/without fibrosis), progressing to advanced fibrosis, cirrhosis, and rarely, may develop into HCC (Perumpail et al., 2017). NAFLD is commonly connected with the main features of metabolic syndromes such as obesity (Golabi et al., 2020; Younossi et al., 2016), hyperglycaemia (Younossi et al., 2019), insulin resistance (IR) (Tanase et al., 2020; Coccia et al., 2020), hyperlipidaemia (Chen et al., 2020) and hypertension (Zhao et al., 2020). Consequently, a new disease named metabolic dysfunction-associated fatty liver disease (MAFLD) has been recommended to replace the old term NAFLD (Eslam et al., 2020a; 2020b), hence the new term MAFLD is used throughout this study.

The imbalance in hepatic lipid metabolism, such as the difference between lipid acquisition (i.e., high fatty acid uptake and *de novo* lipogenesis) and removal (i.e., low mitochondrial fatty acid oxidation and export of lipids), results in hepatic fat accumulation. Previous studies reported that significantly increased level of liver triglyceride (TG) (Ikeuchi et al., 2007) is associated with elevated levels of fatty acid synthase (FAS) (Chen et al., 2018) and serum FFA (Alkhouri et al., 2010), down-regulated protein and mRNA expression of cholesterol 7α-hydroxylase (CYP7α1) (Rahman et al., 2017; Chen et al., 2012a), up-regulated protein expressions of sterol regulatory element binding protein-1c (SREBP-1c) and stearoyl-CoA desaturase-1 (SCD-1) (Cintra et al., 2012; Kim et al., 2010), increased mRNA expression of acetyl-CoA carboxylase (ACC) (Ryu and Cha, 2003), down-regulated protein and

mRNA expression of adipose triacylglycerol lipase (ATG) (Turpin et al., 2011) and down-regulated mRNA expression of carnitine palmitoyltransferase 1 (CPT-1) (Cintra et al., 2012) in the liver of obese rats showing that obesity can cause disturbance in hepatic lipid metabolism in obese rats. The exact mechanism of the progression of MAFLD in obesity is not known. However, it has been suggested that there is increased oxidative stress as the production of reactive oxygen species (ROS) is increased selectively in adipose tissue of obese mice, accompanied by augmented expression of nicotinamide adenine dinucleotide phosphate oxidase expression and decreased expression of antioxidant enzymes. This oxidative stress, in turn, causes dysregulated production of adipocytokines, including adiponectin, plasminogen activator inhibitor-1, interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) (Furukawa et al., 2004). Oxidative stress due to an imbalance between oxidants and antioxidants can cause DNA hydroxylation, lipid peroxidation, protein denaturation, apoptosis and inflammation (Zhu et al., 2012). Similarly, significantly higher levels of lipid peroxidation such as malondialdehyde (MDA), and reduced activity of antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are found in the liver obese rats (Halima et al., 2018; Moreno-Fernández et al., 2018; Rahman et al., 2017). Excessive oxidative stress contributes to the advancement and pathological findings of liver disease, and therefore, an antioxidant may be beneficial in treating treat liver disease (Chen et al., 2018; Mousavi et al., 2018; Zhu et al., 2012). It is also has been suggested that there is apoptosis in hypertrophied adipocytes as a result of local hypoxia in an expanding adipose tissue, and leads to infiltration of adipose tissue by macrophages which produce some pro-inflammatory cytokines such as tumour necrosis-α (TNF-α) and IL-6 (Alkhouri et al., 2010).

To date, effective and proven pharmacological regimens for treating MAFLD are still being investigated. Although various pharmacological agents have been proposed to treat MAFLD, the current recommended pharmacotherapies for NAFLD are pioglitazone and vitamin E, showing limited efficacy (Sumida and Yoneda, 2018). Besides, scientists have shown an interest in exploring the potential of natural products from plants/herbs and dietary sources, including bee bread that may be able to treat MAFLD. Bee bread is a bee product used traditionally to maintain and improve general health and liver function. It is a fermented mixture of bee pollen, honey and bee saliva that the worker bees use as a food source for larvae, and also for young bees to produce royal jelly. It is later packed into the cells of the honeycomb or pots, in which it goes through a chemical change to finally develop a product known as bee bread (Markiewicz-Żukowska et al., 2013). Bee bread has been demonstrated to exhibit excellent digestibility and abundant chemical compositions (Habryka et al., 2016). It consists of proteins, vitamins, lipids, microelements, phenolic and flavonoid compounds that are identified as the added values to its nutritional and therapeutic properties. Bee bread possesses antibacterial (Abouda et al., 2011), antioxidant (Baltrušaityte et al., 2007) and anticancer (Sobral et al., 2017) properties. A previous study has reported that bee bread-honey mixture (1:2) administered 15 g twice a day after meal for five weeks exerted its hepatoprotective properties as shown by improved levels of serum C-reactive protein (CRP), liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and disappearances of yellowing eye sclera and skin pigmentation as well as reduced liver size in alcoholic fatty liver disease patients (Čeksterytė et al., 2012). It is also reported that bee bread was able to reduce liver damage in aluminium-induced hepatotoxicity rats by alleviating the levels of CRP, ALT, and AST, and improving

the levels of monocytes and lymphocyte counts, demonstrating its protective effects against the hepatotoxicity rat model, which could be accredited to its high radical scavenging activity. In addition, bee bread has also shown its medicinal effects in other varieties of illnesses, such as cardiovascular diseases, as shown by improved aortic antioxidant enzymes, FAS activity, size of adipocytes and area of a necrotic patch in the myocardium (Othman et al., 2020), male fertility as demonstrated by improved testicular oxidative stress, inflammation and apoptosis, as well as the testis lactate transport (Suleiman et al., 2020a), and renal dysfunction as exhibited by improved serum renal function parameters, oxidative stress, inflammatory and Bcl-2 associated X protein (Bax) in the kidney (Eleazu et al., 2020) in rats and glioblastoma cell line (Markiewicz-Żukowska et al., 2013). Therefore, this study was conducted to enrich more knowledge in the field of treatment in combating MAFLD.

1.2 Justification and significant of study

To date, there is no particular therapy for MAFLD, although lifestyle interventions, for examples appropriate food consumption, regular exercise, and weight loss, have been shown to be effective in managing MAFLD (Adams et al., 2017). Nevertheless, these treatment options are intriguing and hard to follow due to adherence issues, mostly for long-term management. Numerous pharmacological therapies are available to treat MAFLD, including pioglitazone and vitamin E, nevertheless, these treatments have been reported to have limited efficacy in MAFLD (Sumida and Yoneda, 2018). Several anti-obesity drugs such as orlistat, sibutramine, phentermine, and diethylpropion also have been used to reduce obesity, which is an independent risk factor of MAFLD (Saunders et al., 2018). However, some cardiovascular effects have been reported in the patients after consuming sibutramine and phentermine, including tachycardia, palpitation, and an increased in

blood pressure (Arias et al., 2009; James et al., 2010). Thus, these drugs have been reported to be removed by Food and Drug Administration (FDA). Another drug such as diethylpropion, also has been prohibited for long-term usage due to its some neurological effects, including dry mouth, constipation, and insomnia (Arias et al., 2009), hence demonstrates some difficulties faced throughout the process of developing effective and safe anti-obesity treatments particularly for long-term usage. Orlistat, a potent gastric and pancreatic lipase inhibitor with a weight-reducing effect, has been recommended to treat obesity. The administration of orlistat has succeeded in decreasing the absorption of fat in the gastrointestinal tract and inhibiting the dietary TGs from entering the liver (Ye et al., 2019). Several studies have demonstrated that treatment with orlistat ameliorated metabolic variables (Sahebkar et al., 2018; Khan et al., 2017) and oxidative stress (Suleiman et al., 2020b; Othman et al., 2019a) in obese male rats. However, orlistat has also shown its side effects, for examples abdominal bloating, increased flatulence and diarrhoea (Lee and Dixon, 2017). Scientists have, therefore, become more captivated in researching antioxidant and/or anti-inflammatory and/or anti-apoptotic interventions as a useful approach to mitigate this disease progression. In a previous study, bee bread has been reported to contain carbohydrates, proteins and some lipids. It also has flavonoids and phenols as well as antioxidant activity (Othman et al., 2019b). Lee obesity index and levels of total cholesterol (TC), low-density lipoprotein (LDL), aortic oxidised-LDL and malondialdehyde (MDA) are significantly lower while activities of aortic antioxidant enzyme SOD and GPx are significantly higher in HFD-induced obese group supplemented with bee bread at 0.5 g/kg/day for six weeks compared to control obese group. Its FAS activity, size of adipocytes and area of the necrotic patch in the myocardium are also lower suggestive of some protective

effects of bee bread against the risks of cardiovascular disease in obese rats (Othman et al., 2020). In addition, a previous study reported by Zhen et al. (2021) demonstrated that the intake of bee bread (80, 400 and 800 mg/kg/day) for eight weeks down-regulated the expressions of hepatic lipogenic protein and genes such as FAS and ACC levels in HFD-induced fatty liver disease rats, hence inhibited hepatic lipid synthesis. However, to date, it is not known whether bee bread may also ameliorate other obesity-related liver changes such as the levels of lipid in the liver, the other hepatic lipid metabolism pathways including fatty acid uptake, lipolysis, and fatty acid oxidation, as well as the liver oxidative stress, inflammatory and apoptosis markers (Zhen et al., 2021). Therefore, the aim of this study is to determine the effect of *Heterotrigona itama* bee bread on MAFLD parameters in obese male rats.

1.3 Objective

1.3.1 General objective

To determine the effect of *Hetertrigona itama* bee bread on MAFLD parameters in obese male rats.

1.3.2 Specific objectives

1. To determine the *Heterotrigona itama* bee bread phenolic composition by high performance liquid chromatography (HPLC) analysis.
2. To determine body weight gain, Lee obesity index, body mass index (BMI) and food intake, in obese male rats supplemented with *Heterotrigona itama* bee bread.
3. To determine serum biochemical profiles (glucose, insulin, oral glucose tolerance test, lipid profile, liver function test, leptin, adiponectin and free fatty acid), and

liver and faecal lipid contents in obese male rats supplemented with *Heterotrigona itama* bee bread.

4. To determine liver lipid metabolism, oxidant-antioxidant status, inflammation and apoptosis in obese male rats supplemented with *Heterotrigona itama* bee bread.
5. To determine liver histopathological changes, NASH scoring, fibrosis and glycogen in obese male rats supplemented with *Heterotrigona itama* bee bread.

1.4 Hypothesis

Heterotrigona itama bee bread significantly improves liver function and histopathological changes in MAFLD rats by improving the hepatic lipid metabolism and reducing oxidative stress, inflammation, and apoptosis.

CHAPTER 2

LITERATURE REVIEW

2.1 Obesity

2.1.1 Overview of obesity

According to the World Health Organization (WHO), overweight and obesity are defined as an aberrant or excessive build-up of fat that presents a risk to health with a BMI equal to or greater than 25 and 30, respectively. Increased intake of highly caloric foods, without an equal increase in energy expenditure, mostly by physical activity, will result in the accumulation of excessive adipose tissue leading to weight gain (World Health Organization, 2021).

A small amount of fat is an essential part of a healthy and balanced diet for energy storage, waterproofing, and thermal insulation. Other than that, the intake of foods rich in essential fatty acids is also important for body cellular functions, signaling and fighting against infections. However, fat becomes problematic when people consume it more than their needs, making them vulnerable to various life-threatening comorbidities such as insulin resistance (IR), cardiovascular disease, T2DM, cancer, neurodegeneration and MAFLD (Saltiel and Olefsky, 2017).

Obesity is a complex, multifactorial and preventable disease consisting some potential contributory factors leading to weight gain. While the major issue is a surplus intake of energy (dietary intake) relative to energy expenditure (energy loss via metabolic and physical activity), other factors such as genetic, physiologic, environmental, psychological, social and economic factors have also been reported to be interrelated in varying degrees to promote the development of obesity (Shukla et al., 2015). The trend analysis of data from surveys performed by the National Health

and Nutrition Examination Survey reported a decreasing trend in energy intake among adults and children in the United States from 2003 to 2016 (Marriott et al., 2019; Ford and Dietz, 2013). Nevertheless, the trends reported in energy expenditure from physical activity remain a concern.

2.1.2 Diagnosis and clinical evaluation

In a practical setting, obesity is typically diagnosed after performing a physical examination and some tests. BMI measurement is usually chosen as the first screening tool used to evaluate obesity, as it correlates with the amount of body fat. BMI is a measure of weight adjusted for height and was invented by a Belgian statistician, mathematician and astronomer named Adolphe Quetelet (Andolfi and Fisichella, 2018). The current guidelines from the United States Centers for Disease Control and Prevention (CDC) and the WHO define the adults' normal BMI range as 18.5 to 24.9 kg/m^2, while a BMI $\geq$ 25 kg/m^2 is considered to be overweight. Moreover, a BMI $\geq$ 30 kg/m^2 is classified as obese, and an individual with a BMI $\geq$ 40 kg/m2 is regarded as severely obese (World Health Organization, 2021).

However, BMI may lack correlation with body fat, especially in people with a high proportion of muscle tissue to fat, such as athletes who have a high BMI yet with a low percentage of body fat. Furthermore, there is also a large inter-individual variability in the percent of body fat, in terms of age, sex, and ethnicity (Gallagher et al., 2000; Gallagher et al., 1996). The Asian populations also have greater body fat in their abdominal cavity than Caucasians, hence increasing their susceptibility towards obesity-related diseases specifically T2DM. Therefore, new BMI cut-off points have been proposed by the WHO as well as increasing public health awareness among Asian populations. The new categories of BMI with 23-27.5 kg/m^2 as overweight and BMI $\geq$ 27.5 kg/m^2 as obese are implemented for Asian populations (Nishida et

al., 2004). However, a few Asian countries adjusted the criteria accordingly; for example, a BMI < 24.0 kg/m^2 is considered normal, whereas BMI ≥ 28.0 kg/m^2 is regarded as obese in China (Zhou, 2002).

In addition to BMI, the waist-to-hip ratio (WHR), waist circumference (WC), skinfold thickness, and machinery techniques such as computed tomography (CT) scan, dual-energy X-ray absorptiometry (DXA) and magnetic resonance imaging are also increasingly being used to evaluate overweight and obesity, body fat distribution and health risks related to metabolic syndromes (Ashwell et al., 2012; Camhi et al., 2011; Carroll et al., 2008).

2.1.3 Prevalence of obesity

Obesity has been regarded as a major nutritional health problem around the globe. Worldwide obesity has nearly tripled since 1975. In 2016, more than 1.9 billion adults aged 18 years and older were overweight, and of these, over 650 million were obese. Thus, 39% of adults aged 18 years and over were overweight in 2016, and 13% were obese. Furthermore, 39 million children under the age of five were overweight or obese in 2020. Hence, the problem is three-fold important more than in 1975, and about 13% of the population is predicted to be obese (World Health Organization, 2021).

In addition, obesity has reached epidemic levels in many developing countries, including Malaysia, which has been nominated as the highest obesity rate in adults among Southeast Asian Countries (Ng et al., 2014). The findings from The National Health and Morbidity Survey carried out in 2006, 2011 and 2015 revealed that the prevalence of overweight and obesity among the adults in Malaysia has shown an increasing trend with 29.1% and 14.5% in 2006, 29.4% and 15.1% in

2011, 30.0% and 17.7% in 2015, respectively (Chan et al., 2017). Nevertheless, the latest finding in 2019 showed that the current prevalence of overweight and obesity among Malaysian adults has increased to 30.4 % and 19.7 %, respectively (NHMS, 2020).

2.1.4 Animal model of obesity

The incidence of obesity continues to increase worldwide, making it crucial that animal models sharing characteristics of human obesity and its co-morbidities be designed in the quest for prevention and/or treatments against obesity. Two factors that are mainly contributing to the development of obesity in animal models are genetic and environmental controls. Although there is a clear and well-documented genetic component for the tendency to become obese, the majority of human obesity are however considered to be polygenic, which means that numerous genes integrated and performed each risk factor, resulting in obesity. Small rodents such as rats and mice are usually chosen, and the obesity will be developed based on mutations or manipulations of one or a few individual genes (Lutz and Woods, 2012), such as by modulating the leptin signaling pathway (Schwartz et al., 2000). These transgenic models provide ideal models for studying the specific molecular targets or pathways by a prospective therapeutic *in vivo*. However, due to its high cost and slow progress in understanding its energy regulation, diet-induced models of obesity (DIO) are often preferred to study the causes of obesity (Speakman et al., 2007).

DIO animal models are believed to be able to mimic the state of common obesity in humans, such as increased body weight and adiposity, increased circulating leptin and insulin levels, increased TG levels, and decreased insulin sensitivity compared to the genetically modified models, hence DIO has become the

best choice for researching prospective therapeutics (Lutz and Woods, 2012). In the DIO animal studies, the selection of rodent species and strain are important variables where rats and mice represent particular advantages and disadvantages in determining the success of obesity studies. For instance, mice are smaller in size compared to rats and hence need less resources in care such as housing, space, and material requirements (e.g., drugs), however, that also means less body fluids can be sampled from them compared to rats. There are a few strains of mice that are more prone to DIO, such as C57Bl6, AKR, and DBA/2 strains, while other inbred strains such as SWR/J and A/J are more resistant (Bagnol et al., 2012). For rat strains, outbred *Sprague Dawley* rats are most frequently used as a model of obesity as they are more likely to become obese when exposed to a high-energy diet (Lutz and Woods, 2012). A few types of high-energy diets have been introduced to induce obesity, including cafeteria and high-fat diets. The cafeteria diet is a feeding regime in which the animals are supplied with a choice of some palatable food items of varied composition, appearance and texture in addition to their normal non-purified diet. In addition, the animals are allowed concurrent access to both cafeteria diet and standard chow *ad libitum* in order to create more closely parallel human dietary choices. This type of diet managed to induce obesity mainly through hyperphagia and has been used to study the mechanism of energy balance regulation and diet-induced thermogenesis (DIT) due to sympathetic activation of brown fat (Rothwell and Stock, 1988). Nevertheless, the regulation and measurement of macronutrient intake in each animal will be challenging due to the varieties in the cafeteria diet (Bagnol et al., 2012). In addition, the most frequent mode of DIO in rodents is via intake of fat-enriched diets or HFD, whereby either carbohydrate-derived calories are replaced with fat-derived calories, or where saturated fats such as coconut oil, lard or

beef tallow are added to the standard chow (Bagnol et al., 2012). This diet provides a well-controlled and well-defined composition of macronutrients to the experimental animals. Previous study reported the ability of this diet to reduce the central actions of insulin and leptin, hence increasing the body fat composition effectively (Suleiman et al., 2021a).

2.2 Lipid metabolism

Lipid metabolism is a process that encompasses the synthesis and degradation of lipids in cells. It involves the breakdown or storage of fats for energy generation and the synthesis of structural and functional lipids (i.e., phospholipids, glycolipids, sphingolipids, cholesterol, prostaglandins, etc.) for the requirement of cell membrane development. In order to maintain a dynamic equilibrium state of lipid metabolism, the body constantly oxidises the lipids to meet the metabolic needs, meanwhile, other lipids are being synthesised and stored (Gyamfi et al., 2019).

2.2.1 Lipid digestion and absorption

Digestion is recognised as the first step in lipid metabolism, as it involves the process of breaking the TGs down into smaller monoglyceride units with the aid of lipase enzyme. Most of the lipid digestion takes place in the small intestine, even though some are initially digested in the mouth and stomach through the actions of salivary lipase and gastric lipase, respectively (Mu and Høy, 2004). Bile is crucial for further lipid digestion and absorption in the small intestine. The main components of bile required for lipid digestion are the bile salts and phospholipids. Bile salts are synthesised from cholesterol in the liver and are accountable for the emulsification of lipid droplets, causing them to be subdivided into smaller droplets, hence increasing the surface area of lipids being exposed to the pancreatic lipase (Xiccato, 2010). Pancreatic secretions into the small intestine are also crucial for lipid digestion and

absorption. The action of pancreatic lipase on the TG results in the formation of 2-monoacylglycerols and fatty acids.

Fatty acids and monoacylglycerols can enter the intestinal cells by simple diffusion into the lipid membrane, although some studies also reported their transportation are via the assistance of transmembrane carrier proteins (Glatz et al., 2010). The fatty acids enter the epithelial cells of the intestinal cells and into the blood capillary of a villus. Some of the bile salts are returned to the liver via the portal vein to be reincorporated into bile and recycled, meanwhile, other small quantities of bile salts are not reabsorbed and enter the large intestine to be removed from the body. Loss of this quantity of bile salts in the faeces is the only route for cholesterol excretion from the body (Glatz et al., 2010). A previous study reported that anti-obesity treatment such as orlistat inhibits the absorption of fatty acids into circulation via the suppression of pancreatic lipase in the small intestine, hence excreting the fatty acids together with the bile acid into the faeces. This condition will also increase the bile acid synthesis in the liver in order to replace the loss of bile through the faeces (Gades and Stern, 2002).

2.2.2 The pathways of lipid transport

Due to the hydrophobic nature of membrane lipids, TGs, and cholesterols, special transport proteins identified as lipoproteins are required. There are three major pathways of lipid transportation regulated in the body, including via the exogenous pathway, endogenous pathway, and reverse cholesterol transport (Havel, 2010).

2.2.2(a) Exogenous (dietary) lipid pathway: Metabolism of chylomicrons

Delivery of TGs from the intestine to other organs requires transport proteins in which these highly non-polar lipids are packaged into a stable form in an aqueous environment. To ensure this transportation process is being carried out smoothly, the non-polar lipids (i.e., TGs, cholesterol ester, fat-soluble vitamins) are enclosed by amphipathic compounds for examples, free cholesterol, phospholipids and specific proteins that are known as apoproteins. The primary apoprotein synthesised by intestinal cells of most species is ApoB-48, and the resulting particle is called chylomicrons. The TGs, dietary fats, and cholesterol which are inside the chylomicrons, then enter the lacteals of the lymphatic system, which later drains into the venous blood at the thoracic duct. Once they are in the bloodstream, the reaction of chylomicrons at the capillaries of adipose tissue and muscle cells releases TGs to the adipose tissue and muscle to be stored and available for the body's energy needs. The lipoprotein lipase (LPL) hydrolyses the TGs in the chylomicrons into fatty acids and glycerol. The other components of the chylomicrons, such as apolipoproteins are transferred to high-density lipoprotein (HDL) meanwhile the remaining chylomicrons remnant particles are removed from the plasma by the way of chylomicrons remnant receptors present in the liver (Xiccato, 2010). The exogenous lipid pathway is summarised in Figure 2.1.

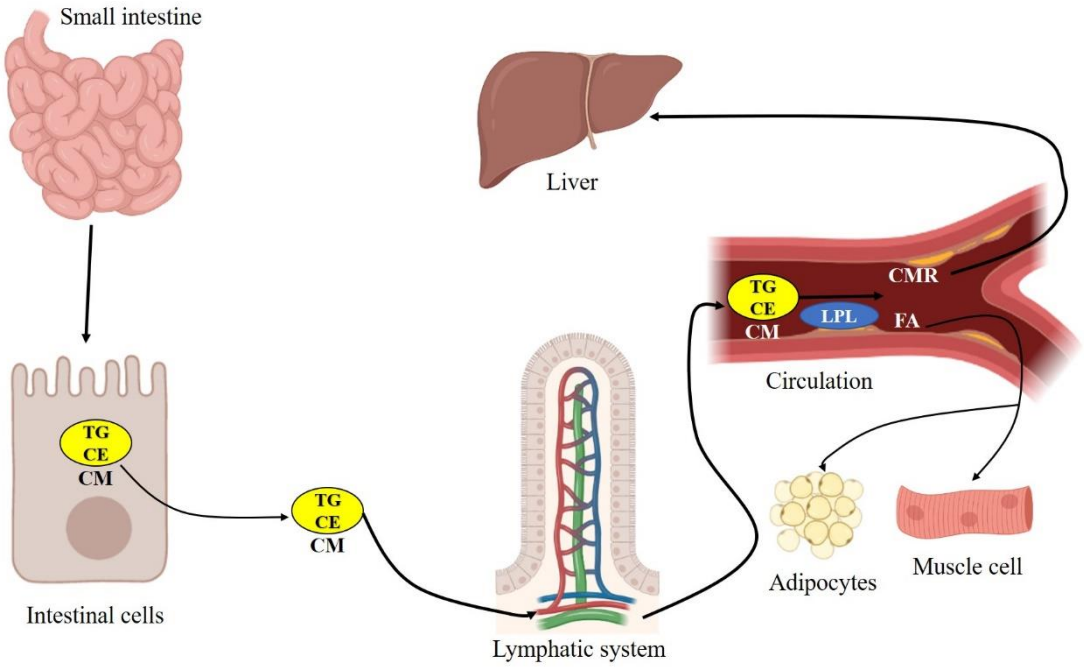

Figure 2.1 Exogenous lipid pathway. CE, cholesterol ester; CM, chylomicron; CMR, chylomicron remnant; FA, fatty acid; LPL, lipoprotein lipase; TG, triglyceride.

2.2.2(b) Endogenous lipid pathway: VLDL metabolism and transport

The endogenous lipid pathway comprises the liver synthesising lipoproteins. The TGs and cholesterol, and along with Apolipoprotein (Apo) B-100 form a lipoprotein complex in the liver known as nascent very low-density lipoprotein (VLDL). Upon secretion of nascent VLDL into the circulation, it acquires Apo-C and Apo-E from the circulating high-density lipoprotein (HDL) to become fully-fledged VLDL. The TGs inside the VLDL are then hydrolysed by the LPL, which is found primarily on the surface of blood capillaries within muscles and in adipocytes, and needs the presence of Apo-C as a cofactor. The hydrolysation of TGs in the VLDL produces fatty acid and glycerol molecules. The released fatty acid is then taken up by the peripheral tissues. During the hydrolysis of TGs, the Apo-C is transferred back to the circulating HDL. After the hydrolysis of TGs, the VLDL remnant becomes smaller and denser, which is known as intermediate-density lipoprotein (IDL). Moreover, during the hydrolysis of TGs inside the VLDL, the cholesterol is also transferred to the circulating HDL and esterified before being transferred to IDL in the exchange of TGs inside the IDL particle by the enzyme cholesterol ester transfer protein (CETP), a plasma protein that helps the transport of TGs between the lipoproteins. The IDL has dual fates, some of the IDL particles are taken up by the liver via low-density lipoprotein (LDL) receptor on its surface that recognise the presence of ApoB-100 and Apo-E on the IDL particles, and the majority of the IDL particles are converted to LDL in the circulation. The newly converted LDL particles are transferred into the circulation and peripheral tissues (Danesh et al., 2000). The endogenous lipoprotein pathway and VLDL metabolism and transport are summarised in Figure 2.2.

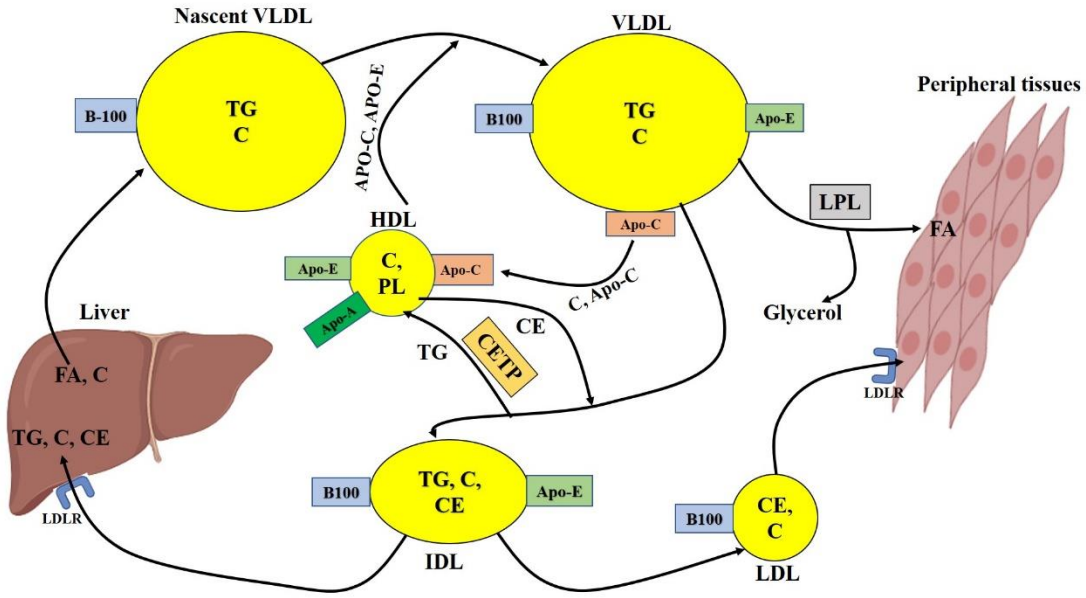

Figure 2.2 Endogenous lipid pathway, and VLDL metabolism and transport. Apo-A, Apolipoprotein A; Apo-E, Apolipoprotein E; Apo-C, Apolipoprotein C; B100, Apolipoprotein B-100; C, cholesterol; CE, cholesterol ester; CETP, cholesterol ester transfer protein; FA, fatty acid; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; PL, phospholipid; TG, triglyceride; VLDL, very low-density lipoprotein.

2.2.2(c) Reverse cholesterol transport: HDL metabolism and transport

Reverse cholesterol transport (RCT) is a mechanism by which the body eliminates excess cholesterol from peripheral tissues and delivers them to the liver, where it will be redistributed to other tissues or removed from the body. The major lipoprotein that plays a role in this process is the HDL. Despite being the smallest lipoproteins, HDL has the highest relative density in comparison with other lipoproteins. HDL is primarily produced by the liver and small intestines. Almost 80 % of the HDL in the circulation is secreted by the liver (Zhou et al., 2015).

In this process, the intestine and liver synthesised the protein Apo-A1, which is the most protein content of the HDL. This Apo A-1 enters the circulation and travels to the peripheral tissues. In addition, once the Apo A-1 enters the veins and arteries, it also interacts with the receptors in numerous cell types (i.e., hepatocytes, erythrocytes, and macrophages) known as ATP-Binding Cassette, Sub-Family A (ABC1), Member 1 (ABCA1) (Chapman et al., 2010; Leaf, 2003). The interaction between Apo A-1 and the macrophages attracts more accumulations of cholesterol and some lipids (phospholipids) towards the Apo A-1. This process leads to the formation of nascent HDL particles, which are later interrelated with Scavenger receptor class B member 1 (SR-B1) and ATP-binding cassette, sub-family G, member 1 (ABCG1), in order to produce more cholesterol, forming a mature molecule of HDL. These processes are catalysed by the enzyme Lecithin-cholesterol acyltransferase (LCAT).

The HDL particles then deliver their cholesterol to the liver by two pathways: direct and indirect pathways. In the direct pathway, the mature HDL molecules interact with SR-B1 in the liver, hence allowing the transfer of its cholesterol content. The remnant HDL molecule can resume circulation and repeat the RCT

process again. Meanwhile, the indirect pathway consists of the interaction of mature HDL molecules with the LDL, as explained before in the endogenous lipid pathway. In this process, HDL transfers its cholesterol content to the LDL in exchange for TGs molecules via the enzyme CETP (Cavelier et al., 2006; Honey, 2007). Reverse cholesterol transport and metabolism of HDL are summarised in Figure 2.3.

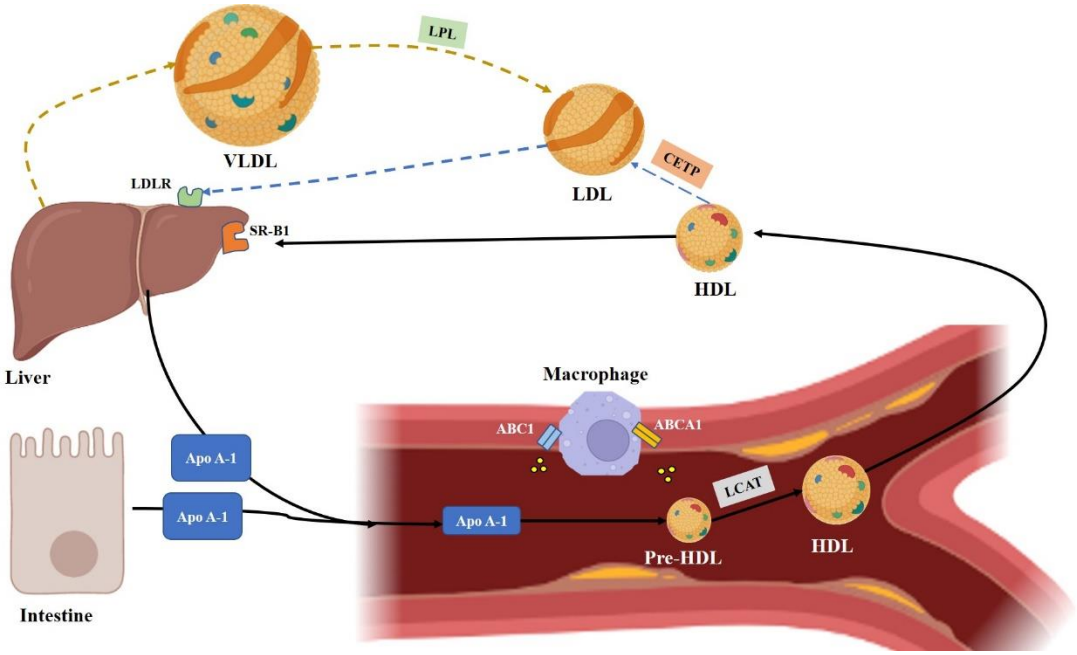

Figure 2.3 Reverse cholesterol transport, and HDL metabolism and transport. Apo A-1, Apolipoprotein A-1; ABCA1, ATP-Binding Cassette A1; ABC1, ATP-Binding Cassette 1; CETP, cholesterol ester transfer protein; HDL, high-density lipoprotein; LCAT, Lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; VLDL, very low-density lipoprotein.

2.2.3 Lipogenesis: Role of fatty acid synthase

The fatty acid synthase (FAS) enzyme is a very large protein composed of two multifunctional polypeptide chains, each consisting of seven distinct enzyme activities required to elongate a growing fatty acid. The two polypeptide chains are organised head-to-tail, resulting in two separate sites for the synthesis of fatty acids, hence each enzyme complex can assemble two fatty acids concomitantly. FAS catalyses the condensation of acetyl-CoA and malonyl-CoA in order to generate long-chain fatty acids in the cytoplasm (Smith, 2003).

FAS is commonly found in tissues, including lactating mammary glands, adipose and liver. FAS is transcriptionally regulated by SREBP-1 and carbohydrate response element binding protein (ChREBP), which are stimulated by insulin and glucose, respectively (Ferre and Foufelle, 2010). It is required for the conversion of dietary carbohydrates into fat (*de novo* lipogenesis) and for supplying fatty acids during fatty acid β-oxidation in the mitochondria for ATP generation to provide energy (Hodson and Gunn, 2019).

2.3 Liver

2.3.1 Structure and function

The liver is one of the major organs and accounts for approximately 2% to 3% of average body weight in vertebrates. The liver is located in the right upper quadrant of the abdominal cavity beneath the right hemidiaphragm and protected by the rib cage. It executes numerous biological functions, including detoxification of the organism, and the synthesis of proteins and biochemicals needed for digestion and growth (Abdel-Misih and Bloomston, 2010). The liver also involves in metabolism such as the regulation of glycogen storage, decomposition of red blood

cells, and the production of hormones (Abdel-Misih and Bloomston, 2010). Bile is an alkaline fluid produced by the liver, which consists of cholesterol and bile acids that are needed for lipid breakdown. In humans, the bile is stored inside a small pouch under the liver, known as the gallbladder, which later is secreted to the small intestine to complete digestion. Meanwhile, in rats, the gallbladder is loss hence, the bile is passed directly into the small intestine via the bile duct (Tortora and Derrickson, 2017). Almost 80% of the mass of the liver is contributed by the hepatocytes which are the chief functional cells of the liver and carry out various metabolic, endocrine and secretory functions (Matont et al., 1993). The liver is connected to two large blood vessels: (i) the hepatic artery which carries oxygenated blood from the aorta, and (ii) the portal vein which carries blood rich in digested nutrients from the entire gastrointestinal tract as well as from the pancreas and spleen. These blood vessels are further divided into small capillaries recognised as liver sinusoids, which then lead to lobules, the functional units of the liver. Each of the lobule is made up of millions of hepatocytes. The anatomies of rat and human liver, and the structure of hepatocytes are shown in Figure 2.4 and Figure 2.5, respectively.

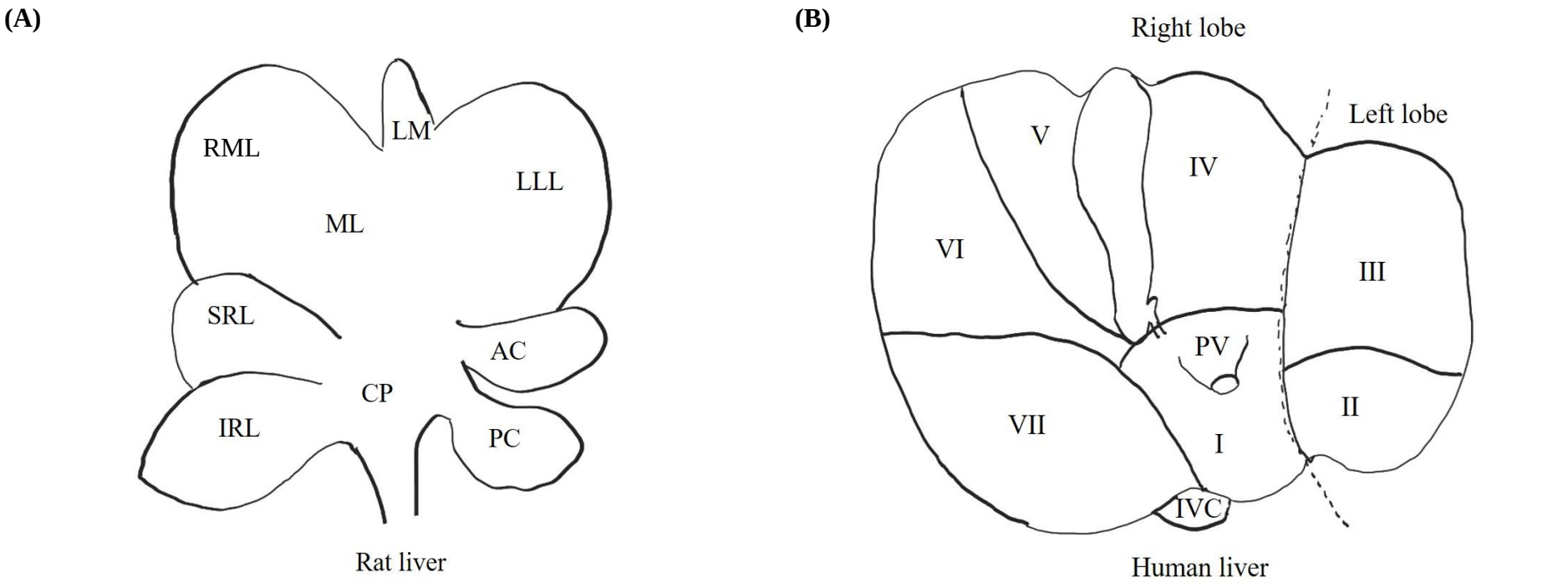

Figure 2.4 **(A)** Visceral surface of the rat liver showing lobes. **(B)** Visceral surface of a human liver showing division into segments. CP, caudate process; AC, anterior caudate lobe: PC, posterior caudate lobe; SRL, superior right lateral lobe; IRL, inferior right lateral lobe; ML, median lobe; RML, right portion of the medial lobe; LML, left portion of the medial lobe; LLL, left lateral lobe; PV, portal vein and IVC, inferior vena cava. (Adapted from Martins and Neuhaus, (2007)).