CHAPTER 3

Results

3.1 The characteristic and anthropometry of subjects

A total of 78 young adult healthy subjects, ages 19-30 years participated in this current study. A control group of 39 subjects and an OW/OB group of 39 subjects had BMIs of 20.9 ± 0.3 and 31.3 ± 0.5 kg/m^2, respectively. All subject had reported engaging in moderate physical activity. Three subjects are light smokers (<5 cigarettes per day). This was 2 from control group and 1 from OB/OW group, which wasn't considered in result analysis since these numbers were too small. Waist and hip circumferences were measured in centimetres as subjects were told to exhale gently. W/H ratio was calculated by dividing waist circumference into hip circumference. W/H ration in OW/OB group was 0.91 ± 0.01, which was significantly different from the 0.82 ± 0.01 W/H ratio measurements of control group ($p<0.001$). The WC was also significantly different between the two groups (control group = 74.6 ± 1.4, OW/OB group = 112.6 ± 7.4, $p<0.001$*). The HC is also consistent with the results of WC measurements with HC measurements in control group =74.6 ± 1.4 cm. This number is significantly lower in the OW/OB group (112.6 ± 7.4), with $p<0.001$. Independent t-test was used to determine whether there is any significant differences between the two groups with results shown in Table 3.

Table 3 Characteristics and anthropometry of 78 subjects in control group and OW/OB group

	Control group	OW/OB group	P-value
N	39	39	-
Gender (male/female)	12/27	24/15	-
Age	22.3 ± 1.6	22.1 ± 0.3	0.662
BMI (kg/m^2)	20.9 ± 0.3	31.3 ± 0.5	<0.001*
WC (cm)	74.6 ± 1.4	112.6 ± 7.4	<0.001*
HC (cm)	90.7 ± 1.3	122.5 ± 7.5	<0.001*
W/H ratio	0.82 ± 0.01	0.91 ± 0.01	<0.001*

Data expressed as mean ± SD. Independent sample t-test statistic was used in this table. Statistical significant: *p < 0.05.

3.2 ^{1}H MRS LFC assessment and biological analysis

LFC assessment was done on a 1.5 T MRI machine (Achieva, Philips Medical Systems, Best, The Netherlands). Transverse and sagittal T2-weighted TSE images were acquired for voxel localization. Voxels were carefully placed in the right lobe of liver. The entire 78 spectra of liver metabolites were obtained and were then analysed for LFC. The corrected value of liver fat by weight was calculated by a method validated by Longo *et al.* and Szczepaniak *et al* [42, 46]. Representative ^{1}H MRS spectrum from right lobe of liver spectrum is shown in Figure 1.

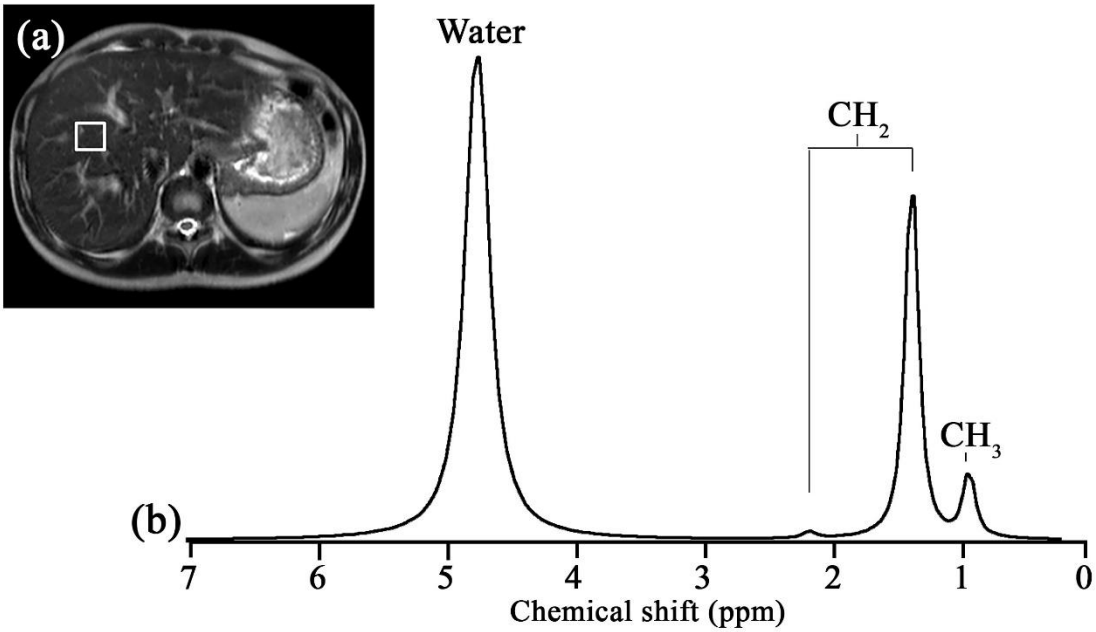

Figure 1. (a) MRI voxel localization in right lobe of liver (b) Typical liver metabolite ^{1}H MRS spectrum shows the water peak occurring at 4.7 ppm, lipid peaks of CH_3 at 0.9 ppm, and CH_2 occuring at 1.3 and 2.1 ppm

One interesting result is that the LFC in OB/OW group is almost 3 times higher when compare to control group. The LFC in OB/OW group was 8.071.02 %, while in the control group, LFC was 2.74 ± 0.20%. This was statistically significant with $p < 0.001$*. 19 subject (48.7 %) in OW/OB group had LFC > 5.56 % which is considered to be the cut off point for NAFLD according to previous large cohort studies on ^{1}H MRS liver fat content [42], in which 47.4 % of high LFC had dyslipidaemia and 10.5 % had HbA1c in the pre-diabetic range (5.7-6.4 %). However, only one subject (2.6 %) in control group had an LFC that exceeded the cut-off point.

LFC, anthropometric, and biochemical results were significantly different between the two group except with regards to age and Cho. The OB/OW group reported statistically higher BMI, LFC, WC, HC, FG, Tri, LDL, HbA1c, and statistically lower HDL. Cho was also found to be increasing in OW/OB group, but this tendency was not statistically significant. The prevalence of dyslipidaemia in OB/OW group (69.2%) was higher than in control group (48.7%). Impair fasting glucose indicated pre-diabetes was found in 3% of OW/OB with 3% of OW/OB subjects having HbA1c of more than 6.5%. There were no subjects in control group who exceeded the normal FG and HbA1c range.

Next, an independent t-test was used to determine the statistical differences taking place between the two groups. The mean value with p-value of LFC and biological information are shown in Table 4.

Table 4 LFC and biochemical analysis results of 78 subjects in control group and OW/OB group

	Control group	OW/OB group	P-value
N	39	39	-
Gender (male/female)	12/27	24/15	-
Age	22.3 ± 1.6	22.1 ± 0.3	0.662
LFC (%)	2.74±0.20	8.07±1.02	<0.001*
FG (mg/dl)	83.1 ± 1.1	89.9 ± 1.1	<0.001*
Cho (mg/dl)	187.3 ± 6.8	200.7 ± 6.1	0.147
Tri (mg/dl)	77.8 ± 5.2	117.1 ± 8.8	<0.001*
HDL (mg/dl)	59.3 ± 2.5	47.7 ± 1.4	<0.001*
LDL (mg/dl)	111.1 ± 5.6	130.1 ± 5.1	0.014*
HbA1c (%)	5.06 ± 0.07	5.46 ± 0.07	<0.001*

Data expressed as mean ± SD. Independent sample t-test statistic was used in this table. Statistical significant: *p < 0.05.

A Pearson correlation analysis was conducted as a preliminary analysis for a possible predictor variable using LFC, and is presented in Table 5. Various statically significant correlations of LFC and variables were found, with moderate correlation occurring with BMI ($r = 0.531$, $p < 0.001$) and mild correlation occurring with W/H ratio ($r = 0.388$, $p < 0.001$), HbA1c ($r = 0.345$, $p = 0.002$*), and WC ($r = 0.259$, $p = 0.022$). Among the blood biochemical results, HbA1c showed the highest correlation with LFC, followed by Tri ($r = 0.223$, $p = 0.05$). The Pearson correlations and data distribution by sex in both groups are shown in Figure 2. This indicates that the overall data between male and female in each group are distributed in the same way.

Table 5 Pearson correlation between characteristic and biochemical analysis with LFC

	Correlation with LFC	
	r	P value
Age	-0.058	0.611
BMI (kg/m^2)	0.531	<0.001*
WC (cm)	0.259	0.022*
HC (cm)	0.212	0.062
W/H ratio	0.388	<0.001*
FG (mg/dl)	0.144	0.210
Cho (mg/dl)	0.093	0.419
Tri (mg/dl)	0.223	0.050*
HDL (mg/dl)	-0.185	0.105
LDL (mg/dl)	0.133	0.246
HbA1c (%)	0.345	0.002*

r = Pearson correlation coefficient, Statistical significant: *p < 0.05;

The correlation was then compared between HbA1c and FG to determine the indicator for diabetes. Even if a low positive correlation was found in FG, it was not statistically significant (r = 0.144, p = 0.210), while the HbA1c was shown to be statistically significant with LFC. The correlation of diabetes (HbA1c and FG) markers are compared in Figure 3.

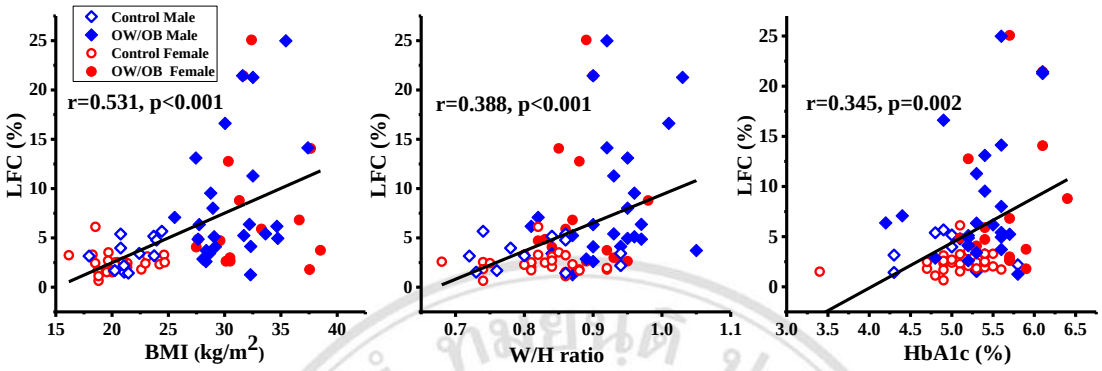

Figure 2. The Pearson correlation coefficient (r) and data distribution by sex in each group between BMI, W/H ratio, HbA1c and LFC measured by ^{1}H MRS

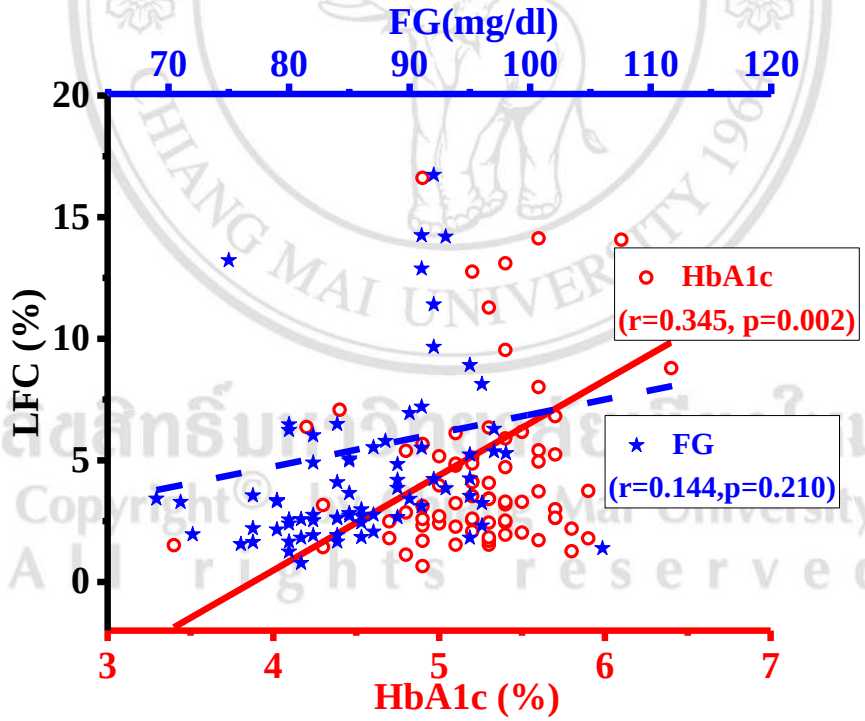

Figure 3. Pearson correlation between HbA1c (circle in red, lower x axis), FG (star in blue, upper x axis) and ^{1}H MRS measured LFC

A multiple linear regression was used to predict the LFC from significantly correlated blood biochemical markers (HbA1c and Tri) and anthropography markers (BMI, W/H ratio, and WC). Standardized coefficients and correlations are presented in Table 2. BMI and HbA1c were found to be significant positive independent predictors for LFC after adjusting for age and sex. However, only BMI remained a statistically significant independent predictor for LFC after adjusting for age, sex, and BMI.

Table 6 Multiple linear regression analysis showing relationship of biochemical marker (HbA1c and, Tri), and anthropometry marker (BMI, WC, and W/H ratio) with LFC as the dependent variable

	Model 1			Model 2			Model 3		
	R^2	β(SE)	p	R^2	β(SE)	p	R^2	β(SE)	p
HbA1c	0.135	0.306 (1.273)	0.002*	0.174	0.339 (1.283)	0.004*	0.298	0.120 (1.379)	0.327
Tri		0.131 (0.012)	0.247		0.065 (0.013)	0.590		-0.029 (0.012)	0.590
	Model 1			Model 2					
	R^2	β(SE)	p	R^2	β(SE)	p			
BMI	0.295	0.463 (0.109)	<0.001*	0.299	0.459 (0.111)	<0.001*			
WC		-0.026 (0.016)	0.824		-0.034 (0.017)	0.774			
W/H ratio		0.145 (8.768)	0.247		0.136 (9.018)	0.288			

Model 1 is unadjusted model. Model 2 is model 1 adjusted for sex, age. Model 3 is Model 2 adjust for BMI. Statistical significant: *p<0.05. Abbreviators; β = standardized coefficient; SE = estimated error; R^2 = correlation coefficient.

3.3 NMR measurement and serum analysis

46 NMR serum metabolites spectra were acquired from 23 subjects in the control group, and also from 23 subjects in the OW/OB group. NMR spectrum showed 9 distinguishing metabolites: lipids CH_3, lipids CH_2, lactate, alanine, $C\underline{H}_2$-CH= bond of lipids, Creatine, Choline, α-glucose, β-glucose. Chemical shift assignment of metabolite peaks is based on previously published research [41, 51], and listed in Table 6. Representative NMR serum metabolites spectra of control group and OW/OB group were shown in Figure 4 and Figure 5 respectively.

Table 7 Assignment of serum metabolites of serum spectra obtained from 400 MHz NMR

Metabolites	Chemical Shift (ppm, δ)
Lipids CH_3 (VLDL+LDL)	0.9
Lipids CH_2 (VLDL+LDL)	1.3
Lactate	1.33, 4.12
Alanine	1.48
$C\underline{H}_2$-CH= bonds of lipids	2.0
Creatine	3.03
Choline	3.19
α-glucose	5.22
β-glucose	4.63

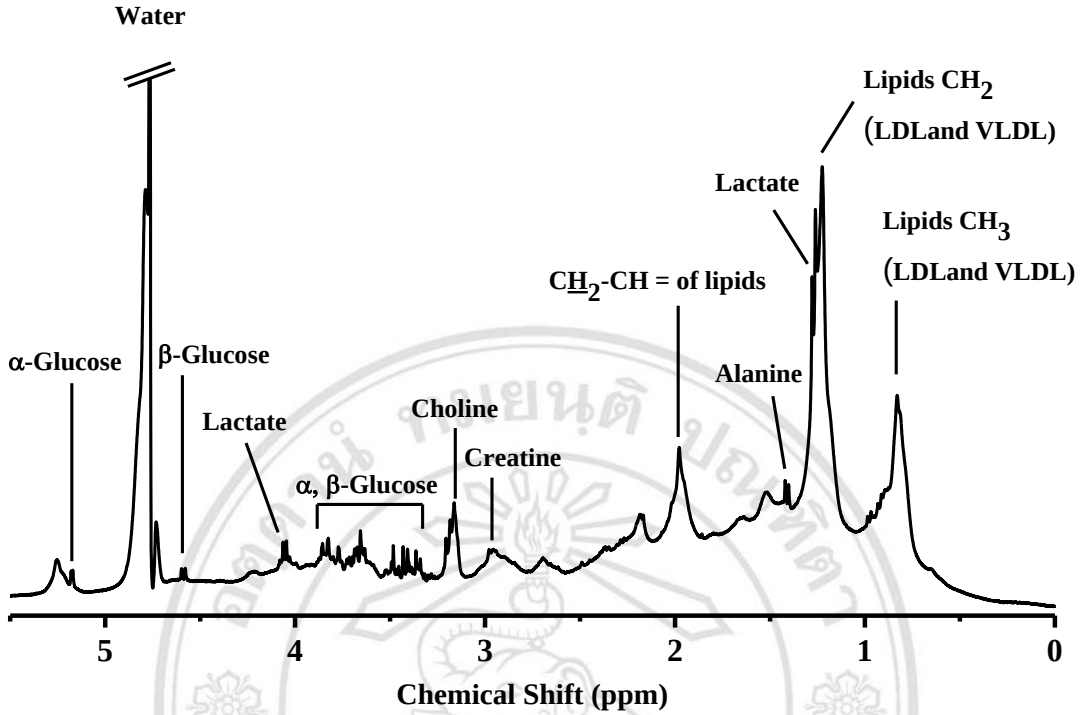

Figure 4. Representative NMR serum metabolites spectra of control group

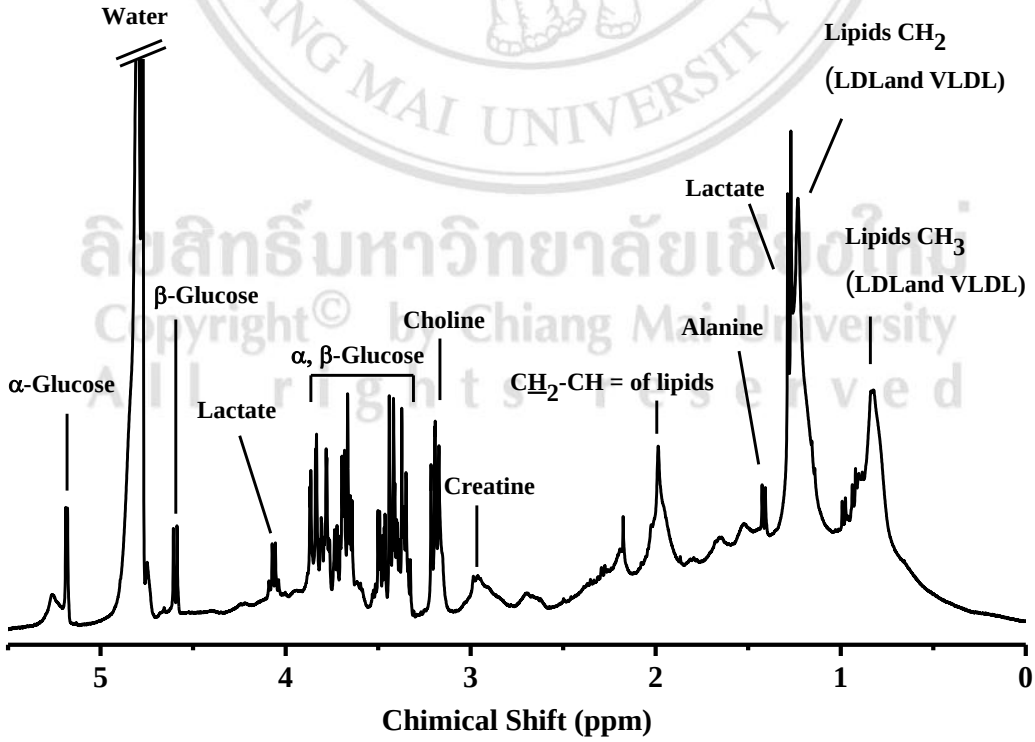

Figure 5. Representative NMR serum metabolites spectra of OW/OB group

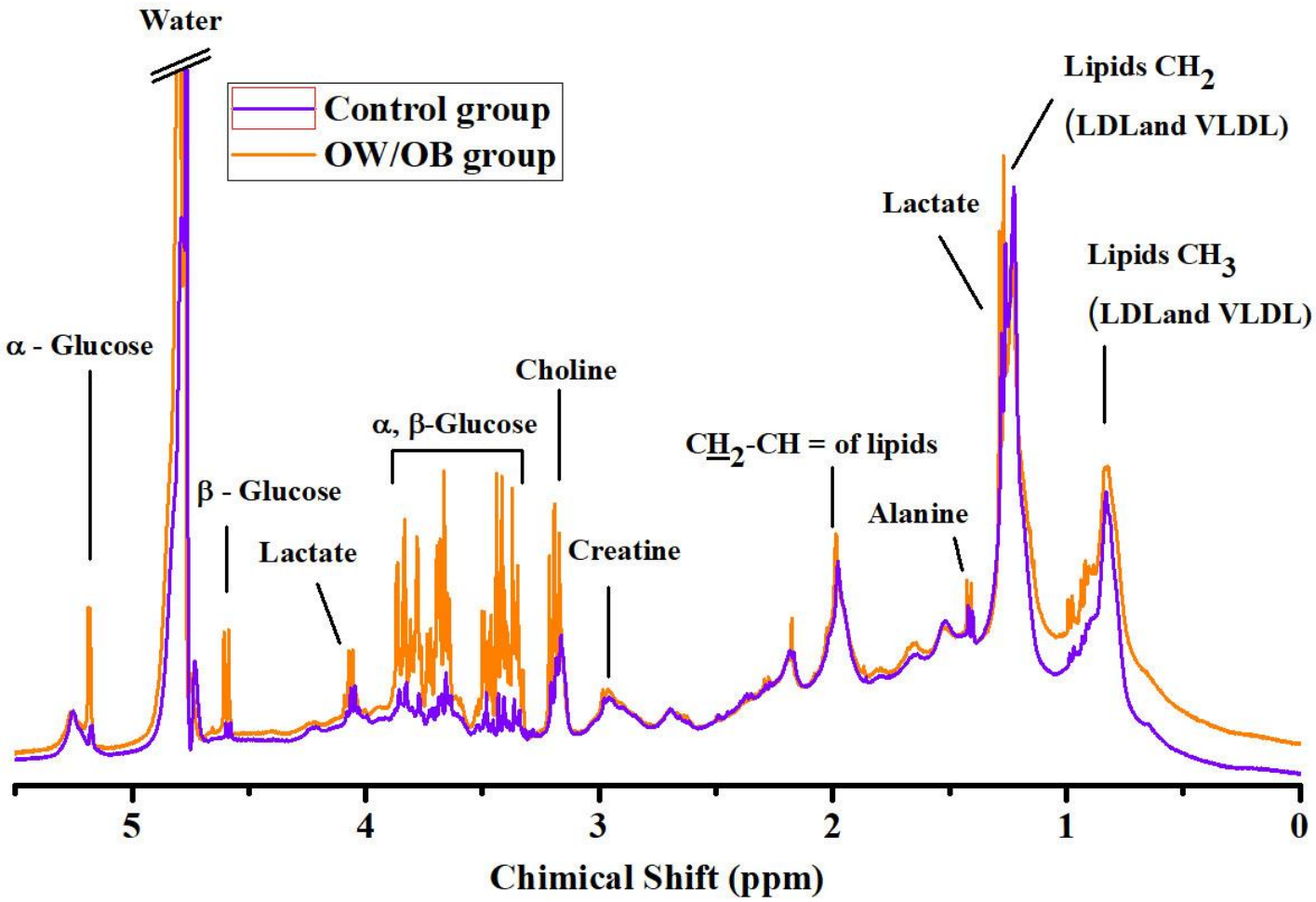

Figure 6. Overlay representative NMR serum metabolites spectra of control group (purple line) and OW/OB group (orange line)

Top spin software was used to analyze every spectrum, and each metabolite was manually identified by its unique chemical shift. Metabolite quantification was done by the integration of area under the peak of each interested metabolite. All serum NMR metabolites quantification were referenced to lactate at 4.1 ppm.

Lipids were calculated into total lipid levels by adding up the relative concentration of lipids at 0.9, 1.3, and 2.0 ppm. Additionally, α-glucose and β-glucose were calculated into total glucose, and the results demonstrated that both total lipid and total glucose were higher in OW/OB group. Statistical analysis indicates significantly different of lipids CH_3 (p-value = 0.016), lipids CH_2 (p-value = <0.001), $C\underline{H_2}$-CH= bond of lipids (p-value = 0.049) and total lipid (p-value = 0.005). The metabolite quantification results of OW/OB group showed higher α, β-glucose at 5.22 ppm, and 4.63 ppm with higher level lipids at 0.9, 1.3, 2.0 ppm. Other metabolites were altered when compared to control group, but did not do so in significantly different ways. However, creatine at 3.03 ppm was not found in all cases, therefore creatine was excluded from any further statistical analysis. The control group of metabolites were set to be a reference at 100% for normalization. Afterward, the percentage change in OW/OB group was then compared to control group and calculations were maded (Table 7). The significantly higher CH_2 lipids, CH_3 lipids and total lipid were consistent with biochemical analysis of blood that also showed significantly higher LDL in OW/OB group. Boxplots of relative quantity to lactate of significantly different metabolites are show in Figure7.

Table 8 The percentage change and trends of metabolite levels obtained in OW/OB group when compared to control group, and significant levels obtained from Mann-Whitney U test

Metabolite	Chemical Shift (ppm, δ)	Trends	Percentage change (%)	P-value
Lipids CH_3 (VLDL+LDL)	0.9	↑	26.49	0.016*
Lipids CH_2 (VLDL+LDL)	1.3	↑	47.98	<0.001*
Lactate	1.33	↑	13.27	0.956
Alanine	1.48	↓	0.37	0.684
$C\underline{H_2}$-CH= bond of lipids	2.0	↑	16.15	0.049*
Choline	3.19	↓	8.20	0.750
β-glucose	4.63	↑	6.92	0.974
α-glucose	5.22	↑	6.48	0.956
Total glucose	-	↑	6.74	0.974
Total lipid	-	↑	34.10	<0.001*

Arrows ↑ and ↓ represent the increasing or decreasing levels of metabolite for OW/OB compared to control group. * indicate significant difference at p-value < 0.05.

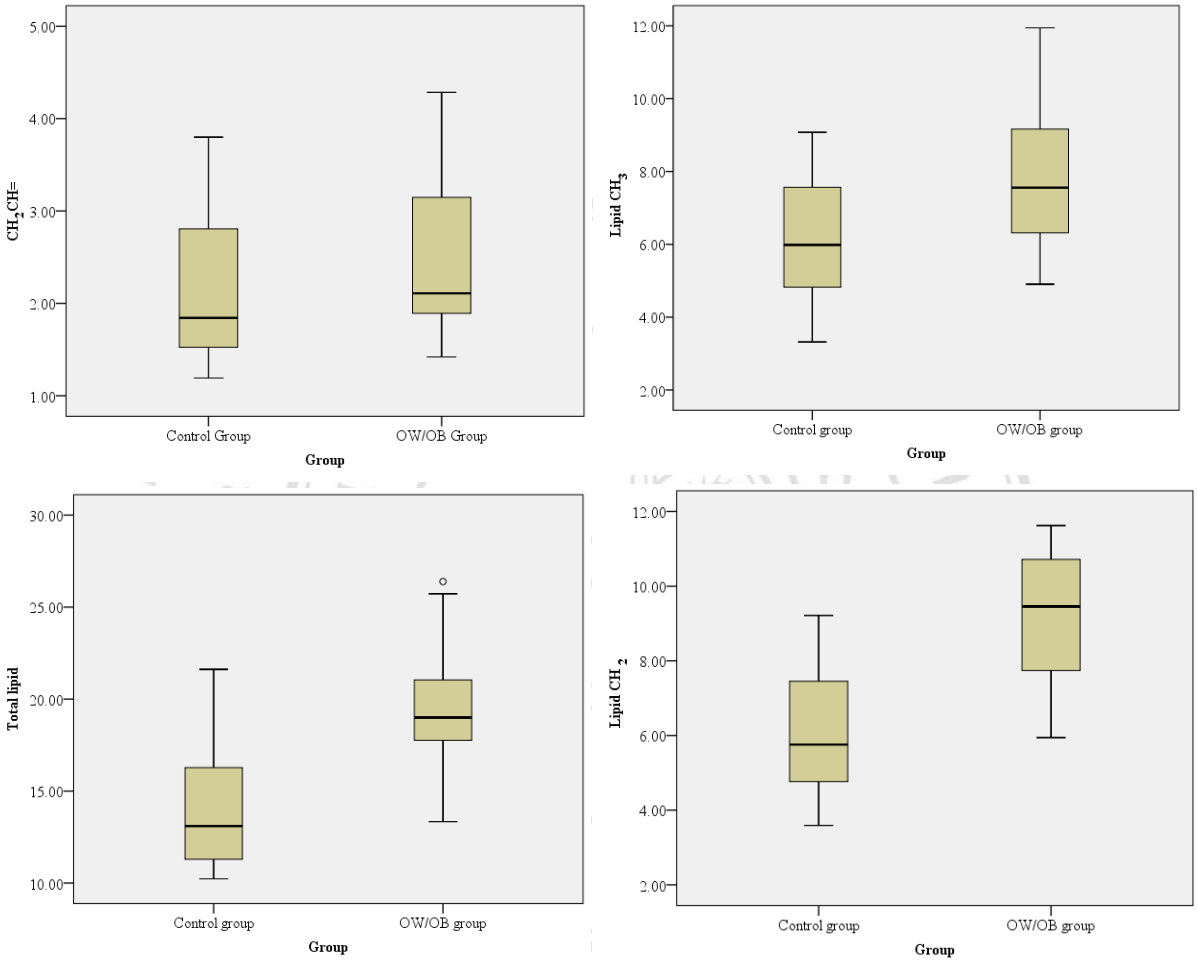

Figure 7. Boxplots show relative quantity to lactate of the significant altered metabolite between control group and OW/OB group

Partial least squares discriminant analysis (PLS-DA) was used for analyzing serum metabolites between control group and OW/OB group. The PLS-DA scores plot reveals the distinctive separation between the two groups (Figure 8).

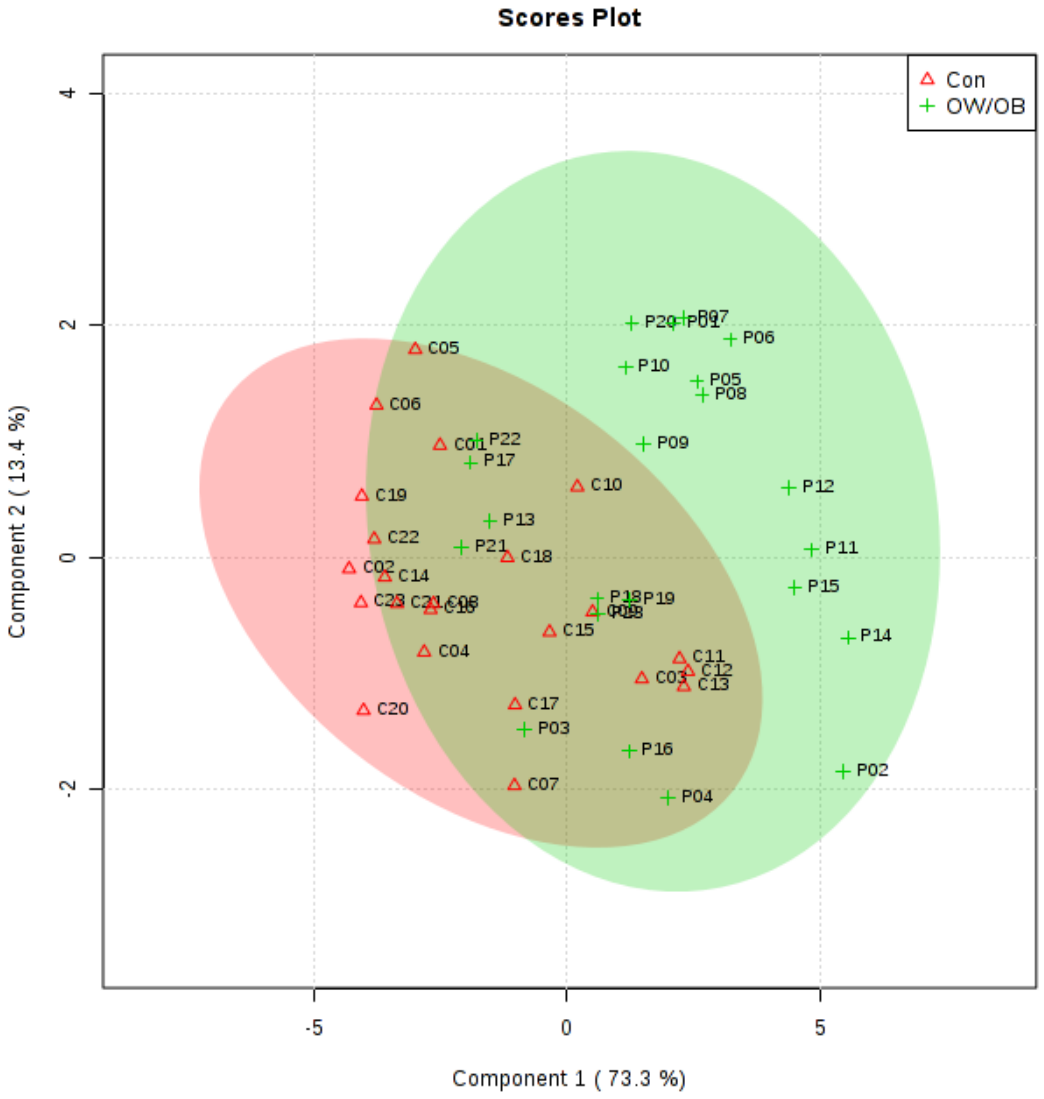

Figure 8. PLS-DA score plot comparing control group (Con, red triangle) and OW/OB group (OW/OB, green cross)

The measurement of variance importance of interested metabolites was done by Variable importance in projection (VIP) score to identify the potential biomarker that contributed to the model (Figure 9). The red and green colored boxes on the right side of VIP scores plot indicated a high or low relative concentration of metabolite in each group. The threshold for relevant metabolites selection is VIP equals to one or higher. Any metabolite

with VIP score close to, or higher than one (1) can be considered to have significant effect on the given model. VIP scores, lipids CH_2 and lipids CH_3 were considered to be potential biomarkers that distinguished the OW/OB group from the control group, respectively. These result were in agreement with the statistic test by Man-Whitney U test. However, CH2-CH= bond of lipid that was previously shown as statistically significant was not determined to be an importance variable based on the PLS-DA analysis.

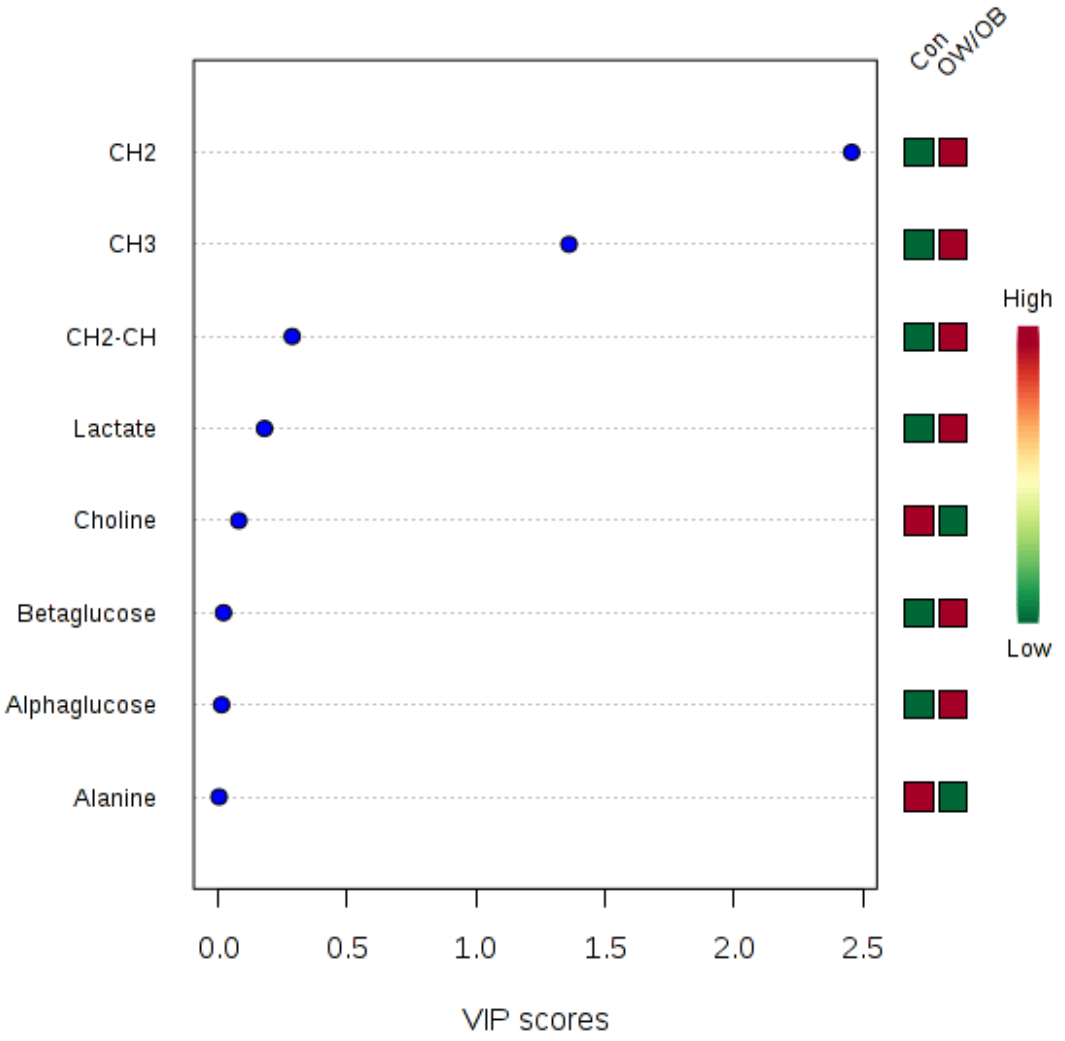

Figure 9. The variable importance in projection (VIP) scores plot from PLS-DA analysis of interested metabolites from control group (Con) and OW/OB group (OW/OB)

In conclusion, NMR metabolites analysis of sera from control group and OW/OB group with 9 metabolites were detected and identified. PLS-DA analysis has found that lipid CH_2 and lipid CH_3 were potential biomarkers to distinguish the OW/OB group from the control group, which is correlated with the results obtained from Mann-Whitney U test. These results are in agreement with the biochemical analysis that reveals significantly higher LDL and Tri from venous blood of OW/OB group.