Correlation of Short Chain Fatty Acid (SCFA) Levels with Transient Elastography Values and Controlled Attenuation Parameters in Patients with Non-Alcoholic Fatty Liver Disease (NAFLD)

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ABSTRACT

Background: *The current hypothesis regarding the mechanism of Non-Alcoholic Fatty Liver Disease (NAFLD) is the multiple hit theory, where one of the factors involved is gut microbiota. Short-chain fatty acid (SCFA) is the main metabolite of gut microbiota and is suspected to play a role in the development of NAFLD. This study aims to determine the correlation between SCFA levels (acetate, propionate, butyrate) and the degree of fibrosis and steatosis in patients with NAFLD assessed by controlled attenuation parameter (CAP) and transient elastography (TE).* ***Methods:*** *A cross-sectional study that included 33 consecutively selected patients at Cipto Mangunkusumo Hospital was conducted from January to August 2023. Fecal sample collection was performed for SCFA examination using GC-MS (Gas Chromatography-Mass Spectrometry). Absolute fecal SCFAs were analyzed for correlation with steatosis and fibrosis based on controlled attenuation parameter (CAP) and transient elastography (TE) values.* ***Results:*** *Subjects were predominantly female (51.5%), with an average age of 49 years, an average CAP value of 296 dB/m, and a median transient elastography value of 6.1 kPa. The ratio of acetate, propionate, and butyrate values in the subjects was 59:24:17. A moderate negative correlation was observed between the absolute butyrate and CAP values (r=-0.522; p=0.002).* ***Conclusion:*** *There is no correlation was identified between short-chain fatty acid levels and transient elastography values.*

Keywords: *short chain fatty acid, SCFA, NAFLD, transient elastography, controlled attenuation parameter.*

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as a condition of fat accumulation in the liver in patients who have no history of excessive alcohol consumption. NAFLD covers a wide spectrum of conditions, from simple fat accumulation (fatty liver or steatosis) to non-alcoholic steatohepatitis (NASH), and can progress to fibrosis and cirrhosis with varying clinical consequences. In the majority of cases, NAFLD is asymptomatic and associated with obesity and metabolic syndrome. The estimated prevalence of NAFLD is around 20–30% in adults and is higher in developed countries. And globally, the prevalence of NAFLD ranges from 13.5% to 38.1%.[1,2] The prevalence of NAFLD in Indonesia is higher than the global prevalence, amounting to 30.6% in 2002, where this prevalence has increased to 51% in 2015.[3,4]

In general, the diagnosis of NAFLD is made based on three criteria, i.e., the absence of a history of significant alcohol consumption (< 20 grams or 30 grams per day in both men and women), the presence of hepatic steatosis based on imaging or histology, and the exclusion of other liver diseases.[5] Nowadays, non-invasive diagnostic procedures are such as transient elastography (TE) and controlled attenuated parameter (CAP), have assisted in the diagnosis of NAFLD and further classify the degree of fibrosis and steatosis, respectively.[6]

The pathogenesis of NAFLD is complex and multifactorial. Nowadays, multiple-hit theory is accepted as genetic, diet, and environmental factors are regarded as factors that lead to metabolic derangement, such as insulin resistance, adipocyte proliferation, and changes in gut microbiota.[7] About 90% of the human gut microbiota is dominated by four phyla, i.e., Bacteroidetes, Firmicutes, Proteobacteria (including *E. coli* species), and Actinobacteria. The composition of gut microbiota is influenced by a number of factors, including diet, lifestyle, antibiotics, and genetics.[8] In general, changes in the composition of the gut commensal microbiota compared to healthy individuals are referred to as dysbiosis. Dysbiosis can include a reduction or loss of beneficial microbiota, an increase in harmful microbiota (pathobionts), and a reduction or loss of gut microbiota diversity.[9] This can disrupt metabolism and immune responses, predisposing to obesity and obesity-related comorbidities, including insulin resistance and NAFLD.[10] Dysbiosis also increases intestinal permeability and potentially causes lipopolysaccharidemia.

Based on previous research, it seems that the composition of the intestinal microbiota does not have much influence on the development of NAFLD,[11] and it is suspected that metabolites from the intestinal microbiota have a greater influence on NAFLD and its development. The main metabolite of the gut microbiota is SCFA, of which most (90–95%) are in the form of acetate, propionate, and butyrate. SCFA can induce DNL (de novo lipogenesis) in the liver, modulation of the endocannabinoid system, modulation of choline metabolism needed for VLDL (Very Low-Density Lipoprotein) synthesis and lipid transport, modulation of bile acid homeostasis, endogenous ethanol formation, and an increase in LPS (lipopolysaccharide), which can activate pro-inflammatory cytokines in the liver.[12]

It was reported that patients with NAFLD have higher levels of SCFA and SCFA-producing bacteria, where dysbiosis of the gut microbiota can cause abnormalities of SCFA components.[13] However, other studies reported different trends and associations between SCFA levels and the development of NAFLD.[14,15] This study aims to investigate the relationship between the gut microbiota metabolites, specifically SCFA, and the degree of fibrosis and steatosis in patients with NAFLD.

METHODS

This study used a cross-sectional design to determine the correlation between levels of short-chain fatty acids (acetate, propionate, and butyrate), transient elastography (TE), and controlled attenuation parameter (CAP) values in patients with NAFLD. This research was carried out at Cipto Mangunkusumo Hospital (RSCM) from January to September 2023. Samples were selected randomly using the consecutive sampling method. Minimal sample

size was determined to be 33 patients. The inclusion criteria was patients with NAFLD who are more than 18 years old and willing to participate in the research, and the exclusion criteria were patients who had other chronic liver diseases, such as hepatitis B, hepatitis C, and autoimmune hepatitis; patients who were on antibiotic treatment or had a history of taking antibiotics for at least the last month; patients with a history of significant alcohol consumption (women > 20 g/day and men > 30 g/day); patient with a history of gastrointestinal surgery or suffers from inflammatory bowel disease; and/or the patient is pregnant or breastfeeding.

Ethical Clearance

All patients who take part in this research have received a verbal and written explanation, followed by written consent. This research has passed the review of the Health Research Ethics Committee - Faculty of Medicine Universitas Indonesia and Dr. Cipto Mangunkusumo National Hospital and was granted the ethical clearance number KET-1349/UN2.F1/ETIK/PPM.00.02/2022.

Clinical and Laboratory Parameters

Recorded clinical parameters were body weight (kg), height (cm), body mass index (BMI), and abdominal circumference. All patients underwent fatty liver ultrasound and FibroScan® to measure fibrosis (kPa) and steatosis (dB/m). The tested laboratory parameters were AST, ALT, triglyceride, total cholesterol, LDL, HDL, fasting blood glucose (FBG), 2-hour post-prandial glucose (2PPG), and HbA1c.

Short Chain Fatty Acids (SCFA) Analysis

All participants provided feces samples, which were then collected, preserved in sterile plastic containers, and quickly frozen at 20°C to be tested quantitatively for the levels of short-chain fatty acids, such as acetate, propionate, and butyrate, using the GC-MS (gas chromatography-mass spectrometry) examination method.

The sample was thawed, then homogenized with an aliquot and a spatula. It was then weighed in a vial and prepared in six concentration levels ranging from 8 to 0.25 µmol/L. The standard diluent was then prepared. As soon as the mixture is homogenous, mix in 300 µL of H_2O and 100 µL of supernatant. Then, mix in 425 µL of isopropanol alcohol (IPA) and 75 µL of 1.5 N HCl. Following that, the sample was removed, sealed, and injected into the GC-MS system with 1.2 µL of the solution into a yellow gas chromatography (GC) tube. 200 mg of the stool sample was centrifuged at 10,000× g for 5 minutes, then sonication for 20 minutes was completed with the addition of 1 mL of standard diluent. According to the guidelines and standards provided by the manufacturer, Prodia Laboratories Indonesia prepared all the sample and control preparations.

GC-MS was the technology used to analyze SCFA. In this test, hydrogen is the gas medium, and the flow rate is 3.70 mL/min. By comparing the time and mass spectrum of a genuine standard with an internal calibration method, mass spectroscopy was used to quantitatively analyze the identity of SCFA found in the original sample. By building a calibration curve using the area under the analyte curve on the standard analyte at each level, the concentration of SCFA may be determined.

Statistical Analysis

Categorical and numerical data were presented in percentage and mean ± standard deviation (SD) or median and interquartile range (IQR). The normality of numerical data was assessed using the Shapiro-Wilk test. When data is not normally distributed, a normalization attempt was made by excluding outliers and/or using a logarithmic transformation. Correlation analysis was done for absolute SCFA levels and TE values, and absolute SCFA levels and CAP using Pearson's or Spearman's test, where appropriate. The significance limit value used is α=0.05, and the correlation is considered significant if the p value is <0.05. The correlation result of 0-0.19 is interpreted as very weak, 0.2-0.39 is weak, 0.4-0.59 is moderate, 0.6-0.79 is strong, and 0.8-1 is very strong. The data obtained was analyzed using SPSS v.22.

RESULTS

Research Subjects Characteristics

The characteristics of the subjects are presented in **Table 1**. In this study, the research subjects were dominated by females (51.5%), with an average age of 49 years and an average body mass index of 30.1 kg/m^2. Of the patients with NAFLD in this study, 78.8% were obese, and 97% had central obesity with an average abdominal circumference of 101 cm. In addition, 75.8% of research subjects experienced dyslipidemia, and 45.5% had diabetes mellitus.

Profile of Steatosis, Fibrosis, and SCFA

Steatosis, fibrosis, and SCFA profile are portrayed in **Table 2**. The mean steatosis or CAP value in this study was 296 dB/m, and 23 subjects experienced significant steatosis (69.7%). In terms of fibrosis, or TE value, the median result in this study was 6.1 kPa, with six subjects experiencing significant fibrosis. Based on the SCFA profile of NAFLD patients in this study, it was found that the median value of absolute acetate and propionate were 3.77 and 1.52, respectively, while the median value of absolute butyrate was 1.1. Meanwhile, the absolute median total value in this study was 8.0. The overall ratio of acetate:propionate: butyrate was 59:24:17. **Table 3** shows the SCFA profile based on the degree of steatosis and fibrosis. Lower absolute acetate, propionate, and butyrate levels were found in patients with significant steatosis, while in patients with significant fibrosis, it was only the absolute butyrate level that was lower (**Figure 1**), although no statistical analysis was conducted.

Table 1. Research Subjects Characteristics

Characteristic	Total (n=33)
Gender, n (%)	
Female	17 (51.5%)
Male	16 (48.5%)
Age (years), mean (SD)	49 (15.93)
Body Mass Index, mean (SD)	30.13 (5.25)
Obesity, n (%)	
No	7 (21.2%)
Yes	26 (78.8%)
Abdominal Circumference (cm), median (IQR)	101 (10.21)
Central obesity, n (%)	
No	1 (3.0%)
Yes	32 (97.0%)
Dyslipidemia, n (%)	
No	8 (24.2%)
Yes	25 (75.8%)
Diabetes mellitus, n (%)	
No	18 (54.5%)
Yes	15 (45.5%)
AST (U/L), median (IQR)	26 (17–41)
ALT (U/L), median (IQR)	29 (18–49)
Triglyceride (mg/dL), mean (SD)	131 (54.67)
Cholesterol total (mg/dL), median (IQR)	186 (157–199)
HDL (mg/dL), median (IQR)	43 (40–52)
LDL (mg/dL), median (IQR)	123 (82–139)
FBG (mg/dL), mean (SD)	107 (18.69)
PPG (mg/dL), median (IQR)	132 (105–170)

SD: standard deviation; IQR: interquartile range; AST: aspartate aminotransferase; ALT: alanine aminotransferase; HDL: high density lipoprotein; LDL: low density lipoprotein; FBG: fasting plasma glucose; PPG: postprandial glucose

Table 2. Profile of Steatosis, Fibrosis, and SCFA

Variable	Total (n=33)
Steatosis (CAP) and Fibrosis (TE) Profile	
CAP value (dB/m), mean (SD)	296 (37.72)
Significant Steatosis, n (%)	
No	10 (30.3%)
Yes	23 (69.7%)
Fibrosis or TE value (kPa), median (IQR)	6.1 (5.3 – 7.6)
Significant Fibrosis, n (%)	
No	27 (81.8%)
Yes	6 (18.2%)
SCFA Profile	
Absolute Acetate (mg/mL), median (IQR)	3.77 (2.62 – 5.24)
Acetate (%), median (IQR)	59 (55-65)
Absolute Propionate (mg/mL), median (IQR)	1.52 (1.10 – 2.29)
Propionate (%), median (IQR)	20 (16.5 – 24.5)
Absolute Butyrate (mg/mL), median (IQR)	1.10 (0.80 – 1.90)
Butyrate (%), median (IQR)	14 (7.5 – 16.5)
Absolute Total (mg/mL), median (IQR)	8 (5 – 10)

SCFA: short-chain fatty acids; CAP: controlled attenuation parameter; TE: transient elastography; IQR: interquartile range

Table 3. SCFA profile based on the degree of steatosis and fibrosis

SCFA level	CAP (dB/m)		TE (kPa)	
	<280 (n=10)	>=280 (n=23)	<8 (n=27)	>=8 (n=6)
Absolute Acetate (mg/mL), median (IQR)	4.06 (2.93 – 5.32)	3.77 (2.26 – 5.09)	3.54 (2.62 – 5.24)	4.61 (3.77 – 5.02)
Acetate (%), median (IQR)	56 (53 – 61)	62 (57 – 66)	59 (55 – 64)	61 (55 – 67)
Absolute Propionate (mg/mL), median (IQR)	1.69 (1.17 – 2.53)	1.50 (1.01 – 2.26)	1.47 (1.01 – 2.09)	2.38 (1.89 – 2.60)
Propionate (%), median (IQR)	18 (15 – 26)	21 (17 – 23)	19 (16 – 22)	26 (21 – 27)
Absolute Butyrate (mg/mL), median (IQR)	1.85 (1.10 – 2.00)	1.0 (0.80 – 1.45)	1.2 (0.90 – 1.85)	0.85 (0.80 – 1.90)
Butyrate (%), median (IQR)	16 (14 – 17)	12 (7 – 15.5)	14 (11 – 17)	7 (7 -16)
Absolute Total (mg/mL), median (IQR)	9 (6 – 11)	7 (4.5 – 10)	7 (5 – 10)	9 (8 – 10)

CAP: controlled attenuation parameter; TE: transient elastography; IQR: interquartile range

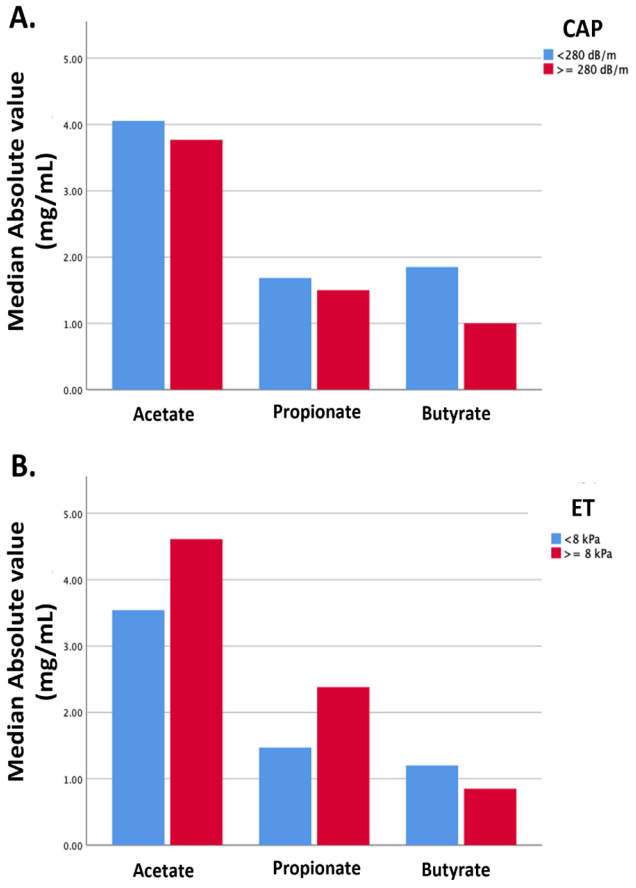

Figure 1. SCFA profile based on degree of steatosis (A) and fibrosis (B)

SCFA-steatosis and SCFA-fibrosis Correlation

Only absolute butyrate level demonstrated a significant correlation with CAP value (r=-0.522; p=0.002). Meanwhile, no correlations were found between any absolute SCFA levels and transient elastography value. A detailed correlation between SCFA level and CAP and TE value was given in **Table 4**. The scatter plots of different SCFA components and steatosis and fibrosis values are on **Figure 2**.

Table 4. SCFA-CAP and SCFA-TE correlation

SCFA level	CAP		TE	
	r	p value	r	p value
Absolute Acetate	-0.219	0.220	-0.061	0.735
Absolute Propionate	0.028	0.877	0.024	0.894
Absolute Butyrate	-0.522	0.002*	-0.245	0.169
Absolute Total	-0.261	0.143	-0.089	0.624

SCFA: short-chain fatty acids; CAP: controlled attenuation parameter; TE: transient elastography. Data are presented as correlation coefficients (with an r value of 0.4-0.59 indicating moderate correlation, 0.6-0.79 indicating strong correlation, and 0.8-1 indicating very strong correlation) and considered significant (p<0.05). Note: all analyses were done using Pearson's correlation

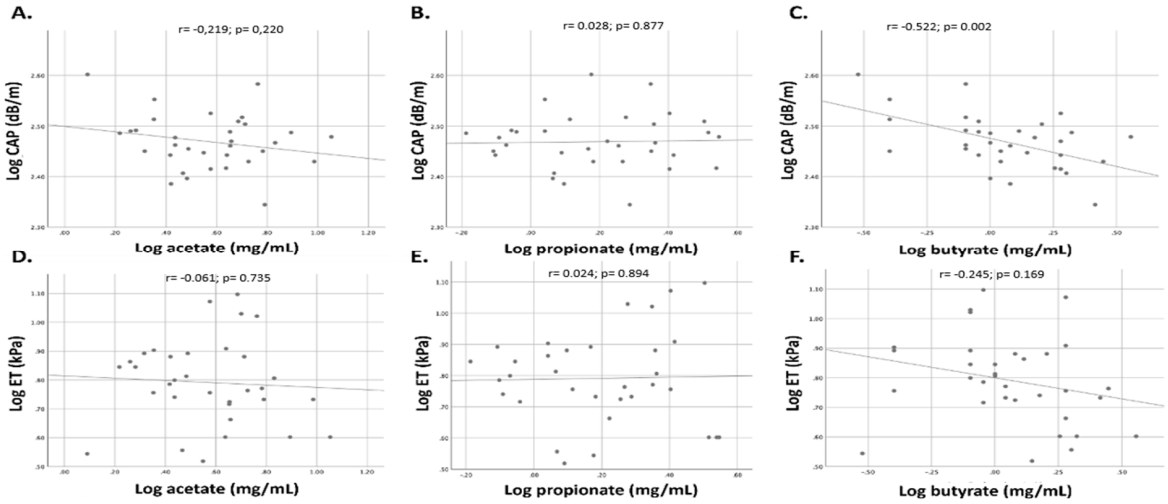

Figure 2. Scatter plots of SCFA-CAP (A, B, C) and SCFA-TE (D, E, F)

DISCUSSION

In this cross-sectional study, we found that patients with NAFLD were predominantly female (51.5%), which is similar to a previous study in Indonesia (62-67.5%).[11,16] Most of our patients were obese (78.8%), which is consistent with the prevalence of lean NAFLD, which only poses a minor proportion of NAFLD.[17] The proportion of significant steatosis and fibrosis regarding the SCFA profile, our study found a trend of lower absolute acetate, propionate, butyrate, and total SCFA in significant steatosis. Only the butyrate level appears to be lower in significant fibrosis, while the other components were higher. The ratio of acetate:propionate: butyrate in this study is 59:24:17, which is similar to the study by Da Silva et al.,[18] that found the ratio in NAFLD patients is 61.5:22.5:16. This finding supports that the butyrate ratio is lower in NAFLD. Furthermore, in the aforementioned study, Da Silva et al. observed a significant increase in propionate levels in patients with NAFLD compared to the control group.[18] This indicates that patients with NAFLD tend to exhibit a lower butyrate-to-propionate ratio, with an elevation in propionate levels. A study conducted by Iwaki et al. also reported a significant reduction in butyrate levels in non-obese NAFLD patients.[19] This is thought to be attributed to the potential of butyrate in reducing hepatic fat accumulation and ameliorating insulin resistance, while propionate plays a role in lipogenesis and the occurrence of low-grade chronic inflammation in the liver.[20] Meanwhile, there have been no reports on the SCFA ratios in fecal samples of healthy adult subjects in Indonesia. In connection with research conducted by Huda-Faujan et al. in Malaysia[21], assuming that the characteristics of healthy subjects in Malaysia are similar to those in Indonesia, the study revealed a SCFA ratio

of acetate, propionate, and butyrate in healthy subjects as 45:20:38. Conversely, in patients with NAFLD in this study, the ratio was found to be 59:24:17. This indicates an inverse relationship in propionate and butyrate ratios between healthy subjects and those with NAFLD, signifying a decrease in the butyrate ratio and an increase in the propionate ratio in NAFLD patients compared to healthy subjects.

Further correlation analysis revealed a moderately negative correlation between butyrate level and CAP value (r=-0.522; p=0.002). Concordant results were also obtained in a study conducted by Xiong et al., which assessed the relationship between SCFA and the progression of NAFLD.[14] The study revealed a significant reduction in butyrate levels in patients with NAFLD-cirrhosis compared to patients with NASH.[14] Additionally, other studies by Endo et al.[22] and Amiri et al.[23] in animal models also demonstrate the protective effects of butyrate supplementation against the progression of NAFLD. Butyrate is recognized as a primary source of energy for intestinal cells and plays a role in energy metabolism, ameliorating inflammation in the liver, reinforcing intestinal barriers, and also contributing to the improvement of insulin resistance.[12,24] From the currently evolving multiple-hit theory, it is understood that insulin resistance plays a role in the pathogenesis of NAFLD by increasing de novo lipogenesis, which in turn elevates the production of free fatty acids and leads to the accumulation of fats, particularly triglycerides, in the liver. Meanwhile, the absence of a correlation between absolute SCFAs and transient elastography values is presumed to be due to the process of transitioning from steatosis to fibrosis, which requires a more complex pathway and involves numerous factors beyond gut microbiota.[11]

CONCLUSION

The complex relationship between gut microbiota and NAFLD still necessitates further investigation. However, the findings in this study contribute to our understanding of the association between gut microbiota, particularly SCFA, and the development of NAFLD. The results align with prior research by Xiong et al.[14], aiming to elucidate the connection between SCFA and the progression of NAFLD stages, revealing a significant reduction in butyrate levels in patients with NAFLD as the severity of the disease increases. This could serve as a foundation for future studies exploring the potential of SCFA-based therapies for patients with NAFLD.

Our study has several limitations. The cross-sectional design made us unable to observe the SCFA changes related to NAFLD progression in detail and limits causality determination. We also did not include a healthy control to get the "baseline" or normal reference values. Furthermore, we did not have any detailed information about the diet of the subjects.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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