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The Role of Gut Microflora Dysbiosis in Clinical Manifestation of Patients with Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis.

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Background and aim: The aim of this study was to evaluate the role of gut microbiota in a wide variety of clinical manifestations of patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).

Methods: The study enrolled 133 cases of patients with NAFLD/NASH who were diagnosed at Enmedic Clinic, Tbilisi/Georgia, and carried out between May 2017 and May 2021. Patients were 21-65 years of age attending our clinic. Patients were diagnosed with NASH/NAFLD based on a fibro scan of the liver and ultrasound investigation, and additionally for NASH with raised serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels greater than the upper limit of normal (40 IU/ ml). The trial profile of patients is shown in figure 1.

10 patients' loss follow up The 123 patients were divided into three groups. Group A (61 patients) was diagnosed with NAFLD, the 42 patients of group B were diagnosed with NASH and group C (control) were 20 healthy volunteers.

Results: Family Enterobacteriaceae, family Lactobacillaceae, and genus Bacteroides like Streptococcus were increased in patients with NAFLD/NASH compared with controls, also uncultured Clostridiales, as well as entero-hemolytic Escherichia Coli, were increased, whereas genus Faecalibacterium, and genus Bifidobacterium, as well as hemolytic Enterococcus faecalis, were decreased in patients with NAFLD/NASH. Significant loss of beneficial bacteria for intestinal barrier function like Faecalibacterium was observed. The diversity of the microbiota was decreased in patients compared with controls.

Conclusions: This study found that the gut microbiota plays a large role in the development of NAFLD/NASH. NAFLD initially developed in patients with significant dysbiosis. It suggests also that the gut microbiome correction may be beneficial for the treatment of patients with NAFLD/NASH.

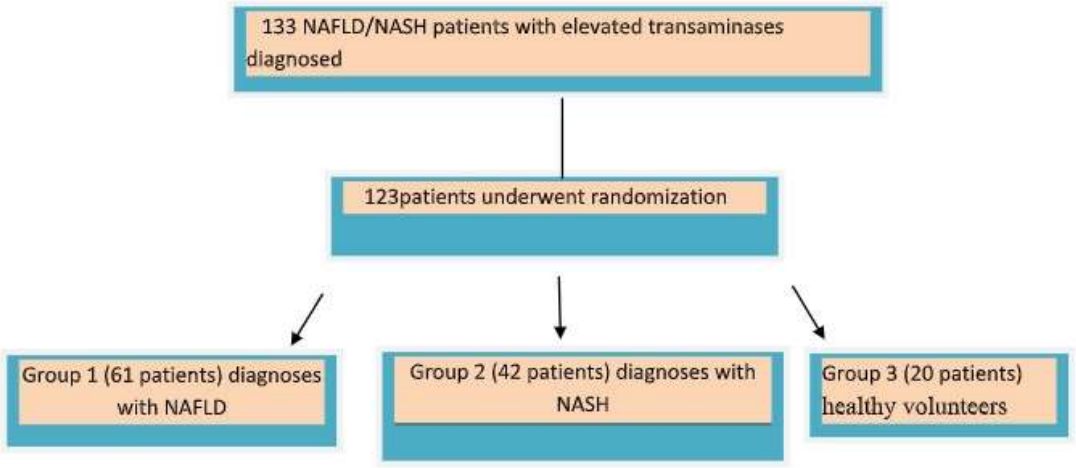

Figure1. The trial profile of patients

Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent chronic liver disease in the world; an estimated 6–35% of the worldwide are affected and the prevalence of NAFLD has been increasing in recent years due to the improvement of living standards [2, 14]. It is estimated that NAFLD affects one-third of all adults in the US, and attention to NAFLD in Asian populations has also been paid over the past decades [3, 5]. NAFLD is characterized by the accumulation of hepatic triglyceride (TG) that exceeds 5% of the total liver weight (5-6). Hepatic steatosis is regarded as the benign beginning of NAFLD, as no severe liver injury is observed during this stage, but certain patients with hepatic steatosis progress to non-alcoholic steatohepatitis, NASH-associated cirrhosis and hepatocellular carcinoma (HCC) [6, 35]. Liver biopsy remains the gold standard for the diagnosis of NAFLD/NASH, although ultrasonography which is a non-invasive, simple tool, can be used for the early detection of fat accumulation in asymptomatic patients. And in addition, various non-invasive diagnostic methods, including computed tomography, and magnetic resonance imaging have been used for detecting hepatic fatty infiltration. Furthermore, magnetic resonance elastography and transient elastography have been frequently used for diagnosing NAFLD with advanced fibrosis [8, 28].

Both NAFLD, as well as NASH, are complex diseases that may be attributed to a combination of factors, including genetics, diet and gut microbiota [8, 37]; however, the detailed pathogenesis of NAFLD/NASH has remained to be fully elucidated. Common risk factors of them include aging, lifestyle, central obesity-associated insulin resistance (IR) and the development of metabolic syndrome [11, 36]. In 1998, Day and James published a theory on the pathogenesis of NAFLD called the 'two hits' hypothesis, according to which lipid deposition in the liver was the first hit, while the subsequent second hit includes oxidative stress, lipid peroxidation, lipotoxicity and mitochondrial dysfunction. It was the second hit that contributed to the development of NASH. The more recently postulated "multiple parallels" hits hypothesis suggested that inflammatory mediators from various tissues, particularly the adipose and gut tissues, participate in the activation of inflammation, progression of fibrosis and tumorigenesis. There is a clear understanding that carriers of certain common genetic variants, including patatin - like phospholipase domain-containing 3, transmembrane 6 superfamily member 2, membrane-bound O-acyltransferase domain-containing 7 and microsomal TG transfer protein, increase the risk of developing severe forms of NAFLD.

The major NAFLD risk factors (ie, diet, obesity and insulin resistance) are closely connected with the gut microbiome [16, 27]. It is reasonable to speculate that the gut microbiota and the pathophysiology of NASH are closely intertwined. In humans, characterization of the faecal microbiomes of 86 patients with NAFLD (n = 72, stages 0-2 fibrosis; n = 14, stages 3-4 [advanced] fibrosis according to the liver elastography) revealed that patients with NAFLD and advanced fibrosis had increased levels of Proteobacteria, whereas patients with mild fibrosis had increased levels of Firmicutes. In addition, features that were predictors of advanced fibrosis in patients with NAFLD were related to the gut microbiota. NAFLD is also a poorly

recognized complication of PEI. The mechanisms underlying NAFLD in PEI are different from NAFLD associated with metabolic syndrome because it is mainly due to malabsorption of essential amino acids such as choline which leads to a decrease in plasma concentrations of apoprotein B, a major component of very-low-density lipoprotein.

Gut microbiota is a highly dynamic entity and presents a constant flow in its composition. These variations in the percentages of different bacterial species depend upon several environmental factors with different impacts on the gut microbiota composition. Among others, these environmental factors include the intestinal mucosa state (which directly affects the degree of permeability of the gut barrier), the immune system health of the host (which promotes an increased proliferation of particular and hazardous species in case of immune deficits), drugs presence (because some bacteria are more sensitive to particular medicines which allow proliferation of other species to occupy the empty niche), the type of diet (food rich in fats, fiber or some phytochemicals directly affects the proliferation of specific bacteria) and even other microbiota members [9]. Therefore, these environmental factors might produce stressful culture conditions that can alter the natural composition of the gut microbiota by decreasing microbial diversity, known as dysbiosis, and they may be the cause of increased risk to develop some diseases [15]. Indeed, dysbiosis is directly related to an increased intestinal permeability as a consequence of some aspects, including the epithelial barrier deterioration, small intestinal bacterial overgrowth, tight junctions' alteration, and even the whole bacterial translocation, causing endotoxemia, which might reach and damage the liver through the portal vein [8, 12, and 17].

The T cells are a crucial component of the adaptive immune response. Upon activation, naive CD4+ T cells differentiate into a variety of effector Th subsets, each with its unique cytokine profile and functions. The Th subsets were initially classified as Th1 and Th2 cells based on their ability to produce different patterns of cytokines and perform different effector functions. Recently, a new subset of Th cells, termed Th17 cells have been identified, which play critical roles in infection, autoimmunity, and inflammation, particularly in mucosal surfaces of the lungs, skin, and gut.

Inclusion criteria included male and female non-diabetic NASH patients 21-65 years of age, diagnosed with NAFLD/NASH on fibroscan of the liver with raised serum ALT level, absence of significant alcohol consumption (weekly ethanol consumption of <40g) and ability to provide informed consent.

Exclusion criteria included patients having a history of alcohol consumption (more than 20g/day), diabetes, presence of any other form of liver disease, positive screening of viral hepatitis B and C, other hepatic diseases including autoimmune hepatitis, Wilsons's disease, hemochromatosis and alpha-1 antitrypsin deficiency. Pregnancy or lactation, decompensated cirrhosis, use of drugs such as metformin, pentoxifylline or gemfibrozil. Use of cholesterol lowering drugs (statins or fibrates), severe or morbid obesity (body mass index±35 kg/m^2), refusal to participate in the study, concomitant disease with reduced life expectancy, severe psychiatric condition and drug dependence.

Anthropometric and biochemical measurements: Complete physical examination and anthropometric parameters were obtained including height, weight, complete blood count, serum transaminases, prothrombin time, serum creatinine, fasting and 2-hour postprandial blood sugar levels (PPBS), complete lipid profile, antinuclear antibody, HBsAg, Anti-HCV, HIV, serum ceruloplasmin, serum ferritin, abdominal ultrasound, fibroscan and upper gastrointestinal endoscopy. Aspartate aminotransferase to platelet ratio (APRI), AST/ALT ratio (AAR), and NAFLD Fibrosis score (NFS) was calculated. Improvement of histological features of NASH (steatosis and fibrosis score) was assessed using Fibroscan in all groups before starting the treatment (baseline) and after 12 months of treatment. The standard fibroscan scoring system to evaluate the changes in histologic features like liver stiffness (fibrosis) and steatosis is defined and shown in *Table 2*. Fibroscan was done in Enmedic Clinic, Tbilisi under the supervision of hepatologist.

***Table 2*.** The baseline characteristic

Variable	Group A	Group B	Control
Weight (kg)	80.5±2.8	80.28±2.21	79.6±2.30
BMI (kg/m2)	30.35±3.31	30.16±2.34	29.24±2.25
ALT (U/L)	89.9±46.7	100±24.9	106±14.9
AST (U/L)	74.5±16.5	78±15.2	78±12.9
GGT (U/L)	77.9±76.8	70.1±45.9	75.5±72.9
Triglyceride (mg/dl)	202.3±25.5	219.3±26.8	200.11±22.7
Total Cholesterol (g/dL)	195.8±16.2	194.73±15	197.11±14.4
HDL-cholesterol (g/dL)	41.22±2.47	40.51±2.5	39.89±2.1
LDL-cholesterol (o/d')	111.77±19.85	109.72±19.1	113.12±17.8
Steatosis score (dB/m)	276±14.9	281±12.6	279±14.9
Fibrosis score (kP/a)	7.8±0.4	7±0.36	7±0.8

The mean age of patients with fatty liver in males was 44.3 years and in females was 51.9 years. 22.9% of patients with NAFLD had increased liver size. Significant association with increasing grades of fatty liver was found with increasing levels of TC ($p = 0.028$), LDL ($p = 0.017$), liver size ($p = 0.001$), and body mass index (BMI) ($p = 0.045$) in patients diagnosed with NAFLD. No significant association with increasing grades of FLD was found with increasing levels of TG ($p = 0.32$) and high density lipoprotein ($p = 0.25$). (14) (15) In contrast our study has shown a strong association with median HDL level and there was no significant association with TG, TC and LDL. This difference in the lipid abnormality spectrum might have arisen due to the wide distribution of lipid level values and patterns of alcohol consumption and USG grading of fatty liver.

Serum was separated from the blood sample of the patients diagnosed with fatty liver. Within two days, laboratory investigations were performed. A lipid profile test was done which included TG by Glycerol Phosphate Oxidase- Phenol Antipyrine (GPO/PAP method), TC and HDL by Cholesterol Oxidase-Phenol Antipyrine (CHOD-PAP), and calculated LDL by Friedwald's equation. The biochemical tests were carried out on fully automated analyzer, Siemens (Germany). Stool samples were collected from patients and also from healthy volunteers.

Data Analysis: All the data from cases were fed in Microsoft office (MS Excel) and then analyzed by Statistical Package for Social Service (SPSS) for window version; all the data were expressed in terms of percentage frequency, median and compared by non-parametric test. P-value < 0.05 was considered to be statistically significant.

Results: In our study, we observed raised serum triglycerides (TG), total cholesterol (TC), and low density lipoprotein (LDL) in 82.67%, 60% and 65.33% cases respectively and significantly low HDL in 65.33% of NAFLD patients. Both NASH and NAFLD are frequently accompanied by extrahepatic complications, including cardiovascular disease and malignancy. The survival of patients with NAFLD/NASH depends on various disease-associated conditions. Elevated LDL or TG or low HDL pattern is associated with NAFLD. Our data also confirms the association between fatty liver and albumin with lipid profile.

In parallel, given that Th17 cells play a predominant role in mucosal cell integrity and defense, the hypothesis that altered gut permeability may play a role in the development of NASH/NAFLD takes on another dimension. This may be putatively related to the impairment of the gut's ability to block uptake of exogenous psychotomimetic compounds or may be related to commensal bacterial translocation and the development of autoimmunity. Although these hypotheses remain tentative and require further investigation, emerging research suggests that immunomodulatory properties of gut microbiota extend to the brain. Some specific intestinal microbial species known to induce Th17 cells can initiate the inflammatory cascade in the CNS. Germ-free mice colonized with segmented filamentous bacteria were found to have increased Th17

cells in both the colon and small intestine, as well as within the spinal cord and developed experimental autoimmune disorders. Th17 cells demonstrate high-grade plasticity, and such plasticity allows Th17 cells a functional adaptation to various physiological situations during immune responses. Th17 cells display enhanced antitumor immunity and play important roles in transplant rejection. Th17 cells are more effective in host defense against microbes, especially bacteria and some fungi. Th17 cells regulate innate immune responses and participate in bacterial clearance during NAFLD. In addition, Th17 cells play an important role in gastrointestinal tract function. Th17 cells bridge innate and adaptive immunity and mount robust antimicrobial inflammatory responses. Although the functional plasticity of Th17 cells provides protection against microbes, they also mediate pathological inflammation. In a recent study, Th17 plasticity in autoimmune arthritis was shown to be driven by the inflammatory environment.

Our data showed that family of Enterobacteriaceae, family Lactobacillaceae, and genus Bacteroides *like* Streptococcus were increased in patients with NAFLD/NASH compared with controls, also uncultured Clostridiales, as well as entero-hemolytic Escherichia Coli, were increased, whereas genus Faecalibacterium, and genus Bifidobacterium, as well as hemolytic Enterococcus faecalis, were decreased in patients with NAFLD/NASH. Loss of beneficial bacteria for intestinal barrier function like Faecalibacterium was observed. The diversity of the microbiota was decreased in patients compared with controls. The Fungal community of patients was characterized by an increase in different *Candida* species and a reduction in Saccharomyces cerevisiae compared with the control group.

Table no 3 shows the association of lipid profile variables with different types and grades of NAFLD/NASH. The NAFLD was found maximum with increased TG in 32 (72.72%), decreased HDL 30 (68.18%), increased TC in 14 (31.81%), increased LDL in 9 (20.45%) as compared to NASH with TG in 32 (57.14%), decreased HDL in 30 (68.18%), increased LDL in 50 (10.71%) respectively.

Table 3

Comparison between NAFLD and Serum Lipid Profile

Ultrasound Grades	Grade I		Grade II		Grade III		
Serum Lipid Profile (mg/dl)	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	*P* value
Triglyceride	162.7	52.49	220.53	119.41	276.71	119.76	0.0
Total Cholesterol	185.20	36.50	214.29	47.68	251.40	53.91	0.00
HDL	45.38	5.57	41.61	4.81	32.71	3.63	0.00
LDL	104.88	25.18	124.72	30.11	155.88	44.45	0.00
VLDL	25.55	5.77	27.75	3.70	35.42	16.42	0.00

On statistical analysis using Analysis of Variance Test (ANOVA), comparison of lipid changes in different grades of NAFLD was made and *P* values <0.05 was considered significant. It was observed that increasing grades of NAFLD were significantly associated with increasing levels of serum total cholesterol (*P* value-0.001), LDL (*P* value-0.000) and VLDL (*P* value-0.003) and decreasing HDL (*P* value-0.000). No significant association was found between serum triglyceride levels (*P* value-0.05) and increasing grades of sonographically diagnosed NAFLD.

Additionally, significantly higher individual variability was observed among diagnosis-based groups of patients as compared to the patients of the control group in both bacterial and fungal community (Figure 4).

TABLE 4. Mean Abundance of Gut Microbiome Taxa in Patients With No/Mild Fibrosis (F0/F1 Stage) or Significant F≥2 Fibrosis

Bacteria	F0/F1 (n = 30)	F≥2 (n = 27)	*P* Value*
Actinobacteria	**0.9**	**1.8**	**0.987**
Bifidobacteriaceae	0.9	1.8	0.949
Bifidobacterium	*0.9*	*1.8*	*0.949*
Bacteroidetes	**66.2**	**69.6**	**0.388**
Bacteroidaceae	42.4	57.8	0.018
Bacteroides	*42.4*	*57.8*	*0.018*
Porphyromonadaceae	1.9	1.0	0.231
Parabacteroides	*1.9*	*1.0*	*0.231*
Prevotellaceae	16.2	6.8	0.017
Prevotella	*16.2*	*6.8*	*0.017*
Rikenellaceae	2.0	1.6	0.949
Paraprevotellaceae	2.8	0.8	0.386
Firmicutes	**26.7**	**25.4**	**0.798**
Clostridiales, unknown†	1.7	1.4	0.270
Lachnospiraceae	10.9	11.3	0.774
Blautia	*1.9*	*1.6*	*0.975*
Unknown†	*4.9*	*5.9*	*0.397*
Ruminococcaceae	8.6	7.5	0.576
Ruminococcus	*0.7*	*1.7*	*0.037*
Unknown†	*7.2*	*5.1*	*0.250*
Veillonellaceae	2.9	2.8	0.620
Megasphaera	*1.2*	*1.9*	*0.891*
Erysipelotrichaceae	1.6	0.7	0.010
Proteobacteria	**3.8**	**2.1**	**0.129**
Alcaligenaceae	1.4	0.8	0.482
Sutterella	*1.4*	*0.8*	*0.482*
Enterobacteriaceae	1.9	1.0	0.099
Unknown†	*1.5*	*0.7*	*0.128*

Phyla, *families, and genera* with >1% occurrence in the whole population are presented.
*By Mann-Whitney's test.
†16S rRNA sequence distinct from any known genera in this family/genus.

This research suggests that hepatic damage in NAFLD/NASH patients could be due to the summation of oxidative stress and systemic inflammation. Also, the immune tolerance of the liver in these patients is quite impaired. The systemic inflammation results from hyperactivated immune responses that lead to the occurrence of a cytokine storm, which ultimately damages multiple organ systems, but first of all intestinal microbiota.

Current clinical study shows that the gut microbiota is one of the most important pathogenic factors in NAFLD/NASH patients. They are often accompanied by an imbalance of the gut microbiota, inducing a low-grade inflammatory response in the body by destroying the gut barrier, producing insulin resistance through metabolites affecting host metabolism and hormone release, forming a vicious circle that promotes the continuous progress of the disease. Therefore, gut microbiota may be a potential target for the treatment of the NAFLD/NASH. However, further research is needed to deepen understanding of the role of gut microbiota in the prevention and treatment of these diseases.

რეზიუმე

ნაწლავის მიკროფლორის დისბიოზის როლი ღვიძლის არაალკოჰოლური ცხიმოვანი დაავადების და არაალკოჰოლური სტეატოჰეპატიტის კლინიკურ გამოვლინებაში

ღვიძლის არაალკოჰოლური ცხიმოვანი დაავადება და არაალკოჰოლური სტეატოჰეპატიტი წარმოადგენენ ნოზოლოგიებს, რომლებიც ხშირად პროგრესირებს ღვიძლის ციროზში. დღეისათვის არ არსებობს ამ ნოზოლოგიების აღიარებული მექანიზმი და მკურნალობა. მცირდება. ღვიძლი და ნაწლავი ემბრიონის განვითარების პროცესში წარმოქმნილია ვენტრალური ფრაგმენტის ენდოდერმიდან, შესაბამისად არსებობს შინაგანი კავშირი ღვიძლისა და ნაწლავის ანატომიურ და ბიოლოგიურ ფუნქციებს შორის. ნაშრომის მიზანია ნაწლავის

მიკროფლორის დისბიოზის როლის შესწავლა ამ დაავადებების კლინიკურ გამოვლინების სხვადასხვა ეტაპზე.

შესწავლილი 104 პაციენტიდან 62 იყო ღვიძლის არაალკოჰოლური ცხიმოვანი დაავადებით, 42-არაალკოჰოლური სტეატოჰეპატიტით, ხოლო 20 ჯანმრთელი პირი წარმოადგენდა საკონტროლო ჯგუფს.

კვლევამ აჩვენა, რომ ნაწლავის მიკროფლორის დიაპაზონის ცვლილება გავლენას ახდენს ღვიძლის არაალკოჰოლური ცხიმოვანი დაავადების და არაალკოჰოლური სტეატოჰეპატიტის კლინიკურ მიმდინარეობაზე. მეტიც, ამ დისბიოზის კორექცია შესაძლოა გამოყენებულ იქნას ამ დაავადებების სამკურნალოდ.

РЕЗЮМЕ

Роль дисбиоэа микрофлоры кишечника на клинические стадии у больных с неалкогольной жировой болеэнью печени (НАЖБП) и с неалкогольным стеатогепатитом (НАСГ)

НАЖБП и НАСГ приводят к прогрессированию фиброза ткани печени, некрозу гепатоцитов, предрасполагают к развитию цирроза печени, гепатоцеллюлярной карциномы, могут приводить к развитию тяжелой печеночной недостаточности. Целью исследования явилось изучение роли дисбиоэа микрофлоры кишечника на клиническое проявление у больных с НАЖБП и с НАСГ.

Исследование провелено среди 104 пациентов: из них 62 были с НАЖБП, 42 – с НАСГ, а 20 здоровых лиц составили контрольную группу.

Исследование показало, что изменения дисбиоэа микрофлоры кишечника влияет на клиническое проявление у больных с НАЖБП и с НАСГ. Кроме того, коррекция этого дисбиоза приводит к выздоровпению больных.

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