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The Differential Gene Expression Analysis Of Hepatic Inflammatory Genes For Diagnosis Of Early Stages Of Fibrosis In Non Alcoholic Fatty Liver Disease (NAFLD)

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Abstract
Nonalcoholic Fatty Liver Disease (NAFLD) is the common liver metabolic disorders, which affects more than 30% of the world's population. If NAFLD is left untreated, it progresses to advance stages such non-alcoholic steatohepatitis (NASH), liver fibrosis, and cirrhosis. Inflammation being an initiator in the progression of NAFLD to advance stages The fundamental purpose of this study was to diagnose the progressive stages of NAFLD by quantitative gene expression of inflammatory biomarkers including IL-6, TNF-α and IL-2R-α on biopsy-proven NAFLD patients as well as based on recommended fibroscan in three groups i.e. No fibrosis, early stages of fibrosis; F1 & F2 and late fibrosis; F3. Liver biopsy is the gold standard for quantifying steatosis, NASH and fibrosis in liver. However, it is an invasive procedure with potential adverse effects and large inter observer variability. Fibroscan is also being used to categorize fibrosis stages, however, it overestimates the fibrosis score in the early stages of fibrosis (F1 & F2) in NAFLD. Hence, various noninvasive markers are need for the diagnosis of early stages of liver fibrosis (F1 & F2). The liver tissue biopsies and blood samples were collected as per defined criteria i.e. deranged Liver Function Tests (LFTs) as well as recommended fibroscan scores. Our results showed that tshe ALT/AST ratio was increased in early (1.17) and late fibrosis (1.41) and the mean fibroscan scores observed for early fibrosis (F1 & F2) and late fibrosis (F3) were 8.64 Median kPA and 12.02 Median kPA respectively. The quantitative differential expression of all three hepatic inflammatory biomarkers including IL-6, TNF-α and IL-2R-α on biopsy-proven NAFLD patients showed significant upregulation in both early and late fibrosis The highest significant quantitative changes in gene expression such as 4.79-fold and 16.45-fold were observed in the case of IL-6 in early and late fibrosis NAFLD patients respectively in comparison to other two inflammatory biomarkers (TNF-α, IL-2R-α). Therefore, the data of this study suggest that the quantitate expression analysis of these three inflammatory genes may be used as biomarkers for noninvasive tests for early dignosis and progression of advance stages of fibrosis in NAFLD patients.

Keywords: Biomarkers, Inflammatory, NAFLD, NASH, Liver Fibrosis

Introduction

The liver is a central organ in the human body, coordinating several key metabolic roles. Due to its unique position in the human body, the liver interacts with components of circulation targeted for the rest of the body and in the process, it is exposed to a vast array of external agents such as dietary metabolites and compounds absorbed through the intestine. Nonalcoholic Fatty Liver Disease (NAFLD) is the most common liver metabolic disorders, affecting 30% of the world's population (Canivet and Boursier, 2022; Younossi et al., 2016). If left untreated, NAFLD progresses to advance stages such non-alcoholic steatohepatitis (NASH), liver fibrosis, and cirrhosis (Maurice and Manousou, 2018). People with NASH are at a high risk of developing liver fibrosis (fibrosis progression proportion ~ 40%), and 15%, of the NASH patient's progresses to cirrhosis (Alkhouri and McCullough, 2012; Loomba et al., 2021). The liver fibrosis has also been reported as the predominant predictor of prognosis in NAFLD patients. Several genetic diseases predispose the liver to fibrosis (Scorza et al., 2014).

Liver biopsy is the gold standard for quantifying steatosis, NASH and fibrosis in liver in NAFLD patients (Thallapureddy et al., 2025). However, the liver biopsy is an invasive procedure with potential adverse effects and large inter- and intra-observer variability (Bhat et al., 2021). Non-Invasive Tests (NITs) are being explored to aid in the early diagnosis of progressive stages of NAFLD. Fibroscan which measures liver stiffness through estimation of the velocity of propagation of a shear wave through liver tissue and the fibrosis score is utilized to categorize liver biopsies into four subgroups such as F1 (perivenular fibrosis), F2 (periportal fibrosis), F3 (bridging fibrosis), and F4 (cirrhosis) (Bhat et al.., 2021). The recommended fibroscan score which is being routinely used for normal subjects (F0-F1) is between 2 and 7 kilopascals (kPA), for early fibrosis (F2) between 7.5 and 10 kPA and above 10 kPA (10-14kPA) for late fibrosis (F3) in the diagnosis of fibrosis stages in NAFLD patients as per

criteria given by Memorial Sloan Kettering Cancer Center. However, it has been also revealed that the studies addressing the accuracy of fibroscan versus liver biopsy are at scarce. A recent study has suggested that fibroscan overestimates the fibrosis score in the early stages of fibrosis (F1 & F2) in NAFLD, whereas, it has higher accuracy in detecting advanced stages of fibrosis (F3) and cirrhosis (Gomez-Dominguez et al., 2006). In particular examples, the study has reported that the subjects who had F1 fibrosis on liver biopsy had a mean fibroscan value of 11.8 Kpa, which fits the norms of F3 fibrosis, similarly, patients with F2 fibrosis on liver biopsy, the mean fibroscan value was 16.96 Kpa which depicts an overestimation of the fibrosis score (Bhat et al., 2021). Hence, various noninvasive markers are being explored to find out more accurate biomarkers for the early diagnosis of progression of fibrosis, especially early stages of fibrosis (F1 & F2) in NAFLD patients.

In NAFLD and NASH, impaired gluconeogenesis dysregulates the metabolic function and increased liver fat, which increases inflammation by activating both innate and adaptive immune cells. The liver maintains glucose homeostasis, and its dysregulation usually result in elevated blood glucose levels. Furthermore, insulin resistance from impaired glucose metabolism elevates gluconeogenesis and blood glucose, creating a vicious cycle that worsens the hepatic inflammation and fibrosis, a hallmark of NASH and early stages of fibrosis (Oladipupo et al., 2025). The inflammation, ballooning, and presence of Mallory hyaline are characteristic histological features in liver biopsy that along with fat (steatosis), indicate NASH. NASH has been reported to be directly involved in increasing a patient's risk for liver-related mortality and the advance stages of fibrosis is the main predictor of liver-related mortality (Bril et al., 2016). Some proportions of NAFLD patients, not all, develop progression to NASH (Hagstrom et al. 2017) and the transition to an inflammatory stage is the key mechanism of pathogenesis to NASH (Parthasarathy et al. 2020). The initial trigger of pathogenesis is metabolic injury, which occurs both intra- and extrahepatic ally and the injury typically lead to the activation of different immune cells (Schuster et al. 2018). The different immune cells, both of the innate and the acquired immune system, have been reported to be involved in this process (Huby and Gautier 2022; Peiseler et al. 2022). Under physiological conditions, constitutive cytokine generation is absent or minimal in the liver. Nevertheless, pathological stimuli like lipid accumulation induces hepatic cells such as Kupffer cells, i.e. liver-resident macrophages, can adopt an inflammatory phenotype, release inflammatory cytokines and activate other immune cells (Tilg and Diehl, 2000). As a result of progressive inflammation, Kupffer cells are increasingly replaced by monocyte-derived macrophages having a distinct lipid-associated and scar-associated phenotype. Many other immune cells are involved in balancing the progression and regression of inflammation and subsequent fibrosis which includes neutrophils, T lymphocytes – such as auto-aggressive cytotoxic as well as regulatory T cells – and innate lymphoid cells (Wiering and Tacke, 2023). The sustained inflammation and increased immune cell activity have emerged as key drivers of NASH to progressive stages of fibrosis (F1 & F2) through the activation of hepatic stellate cells (HSCs) (Carter and Friedman 2022). Inflammation has revealed as a major determinant in the pathogenesis of NASH and fibrosis (Wiering and Tacke, 2023), the mechanisms involved in the onset of inflammation in NAFLD may contribute and aid in developing possible non-invasive tests for the precision diagnostics of progressive stages of fibrosis in NAFLD.

The sustained inflammation in liver is characterized by an increased expression of various pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 (Moschen et al., 2010). A recent meta-analysis study of 36,074 NAFLD patients and 47,052 controls examining a total of 19 pro-inflammatory cytokines has reported significant association for IL-1β, IL-6, C-reactive protein (CRP), and TNF-α with NAFLD and suggested that such inflammatory mediators may serve as potential biomarkers for early diagnosis and intervention NAFLD (Duan, 2022. Moreover, proinflammatory cytokines like TNF-α, IL-1β, IL-6, and IFNγ are upregulated and have been reported to contribute to liver cell necrosis, which in turn develops liver fibrosis (Das and Medhi, 2023). Importantly, all these inflammatory cytokines exert their effects via specific receptors (IL-1 receptor (IL-1R), TNF receptor (TNFR), IL-6 receptor (IL-6R) in liver fibrosis (Niederreiter and Tilg, 2018). Two recent studies in the adult and pediatric NASH-Clinical Research Network (NASH-CRN) cohorts have reported the association between the plasma levels of 32 cytokines as biomarkers and features of NAFLD histology (Ajmera et al., 2017), (Perito et al., 2017). Furthermore, this study is the first to document the association soluble interleukin-2 receptor alpha (IL2R-α) and fibrosis severity. The soluble IL2R-α levels was significantly higher in the severity of liver fibrosis (Ajmera et al., 2017). Another recent study also suggested that IL2R-α would be a useful single biomarker for early diagnosis of NASH and liver fibrosis by providing evidence of significant higher IHC of IL2R-α in NASH group than the non-NASH group in Taiwanese (Kao et al., 2021). Hence, as evidenced in recent studies, these three inflammatory biomarkers including IL-6, TNF-α and IL-2R-α may be considered for noninvasive tests for early dignosis and progression of fibrosis in NAFLD patients.

This study investigated the relationship of quantitative differential expression of selected hepatic inflammatory genes as biomarkers for progression of NAFLD using biopsy-proven NAFLD patients in three groups i.e. Group 1 (no fibrosis), Group 2 (early fibrosis: F1 & F2) and Group 3 (late fibrosis (F3). The fundamental purpose of this study was to assist the diagnosis of the early stages of fibrosis in NAFLD patients by non-invasive testing of quantitative gene expression of inflammatory biomarkers including IL-6, TNF-α and IL-2R-α on biopsy-proven NAFLD patients.

Methodology

Patients selection:

The fibrosis stage identified NAFLD patients based on Fibroscan score along with age-matched normal healthy control subjects were selected for this study. The fibroscan score considered for normal subjects was between 2 and 7 kilopascals (kPA), for early fibrosis (F1& F2) between 7.5 and 10 kPA and above 10 kPA (10-14kPA) for late fibrosis (F3) of NAFLD patients as per criteria given by Memorial Sloan Kettering Cancer Center. The following sampling criteria for collection of blood samples and acquisition of liver tissues from NAFLD patients and healthy controls were utilized for the screening of subjects.

Controls/Group 1:
n=10 (Age group 30-60 Years)
- Non-Alcoholic
- Non-Hepatitis B&C
- Rule out other diseases (Wilson's disease, drug-induced disease, galactosemia and autoimmune liver disease.)
- USG shows fatty liver
- LFT's Deranged
- Fibro-Scan (F0) No Fibrosis
- Liver Biopsy (No Fibrosis)

Group 2:
n=7 (Age group 30-60 Years)
- Non-Alcoholic
- Non-Hepatitis B&C
- Rule out other diseases (Wilson's disease, drug-induced disease, galactosemia and autoimmune liver disease.)
- USG shows fatty liver
- LFT's Deranged
- Fibro-Scan (F1 or F2) Early Fibrosis
- Liver Biopsy (Early Fibrosis)

Group 3:
n=6 (Age group 30-60 Years)
- Non-Alcoholic
- Non-Hepatitis B&C
- Rule out other diseases (Wilson's disease, drug-induced disease, galactosemia and autoimmune liver disease.)
- USG shows fatty liver
- LFT's Deranged
- Fibro-Scan (F3) Late Fibrosis
- Liver Biopsy (Late Fibrosis)

Liver Tissue Acquisition:
Tissue samples were obtained from diagnostic needle biopsies of the liver from selected NAFLD patients based on criteria of fibroscan score (Nishtar Hospital, Multan) as well as healthy control subjects. Tissue samples of NAFLD patients were fixed in 10% buffered formalin for 24 h, prior to embedding in paraffin. The sections were cut and stained for routine histopathological examination after fixation. The progressive stages of NAFLD were identified using histological staging system, clinical biochemistry assays and National Institutes of Health Nonalcoholic Steatohepatitis Clinical Research Network system.

Histopathology in liver:
Liver biopsies were formalin-fixed, paraffin-embedded, and examined for study using hematoxylin-eosin staining. The histopathology was evaluated by two experienced hepato-pathologists independently. Liver biopsies scoring was examined by an expert pathologist not knowing the patients' status and genotype. The grading of fibrosis was done based on the extent of fibrosis "No fibrosis (stage F0), "Early fibrosis (centrilobular pericellular and periportal fibrosis; F1&F2), and "Late fibrosis (bridging fibrosis; F3) by utilizing histological staging system, clinical biochemistry assays, National Institutes of Health Nonalcoholic Steatohepatitis Clinical Research Network system and NAFLD clinical research network (Kleiner, 2005).

Blood Sample collection from Biopsy proven NAFLD patients:
The blood samples of biopsy-proven NAFLD patients in three groups i.e. Group 1 (no fibrosis; n=10), Group 2 (early fibrosis; n=7) and Group 3 (late fibrosis; n=6) were collected to diagnose the progressive stages of NAFLD by quantitative gene expression of inflammatory biomarkers including IL-6, TNF-α and IL-2R-α. The present study was approved by Ethical Research Committee (ERC), The University of Lahore, and Lahore, Pakistan.

RNA Isolation and cDNA synthesis:
For RNA isolation, total RNA was extracted using blood samples using Invitrogen™ PureLink™ Total RNA Blood Kit (Cat. No. K156001), according to the manufacturers' protocol. The quality and extracted RNA concentration was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The integrity of RNA was confirmed by using 2% agarose gel and cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™). The synthesized cDNA was stored at -20°C until quantitative real-time PCR.

Quantitative Realtime PCR analysis:
To evaluate the expression levels of targeted inflammatory genes including IL-6, TNF-α and IL-2R-α along with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal control for data normalization. The primer designed for three inflammatory biomarkers and GAPDH as an internal control are presented in table 3. Quantitative real-time PCR was performed using final amount of 20 µL containing 10 µL of 2X SYBR Green PCR universal master mix (Thermo Scientific™), 5 µL of reverse-transcribed cDNA, and 10 pmol of each primer using Mic qPCR Cycler (model #634002, Bio Molecular Systems Australia) under the following cycling status: 95°C for 5 s, 35 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 15 s. All qPCR reactions were performed in duplicate.

Statistical Analysis of qPCR Experiments:
To calculate relative gene expression, Ct values were obtained for early, late fibrosis NAFLD patients and healthy control subjects. ΔCt was obtained by deducting the geometric mean of the Ct values of reference gene NADPH from the Ct values of the gene of interest (i.e. IL-6, TNF-α & IL-2R-α). Fold change gene expression was calculated by using the $2^{-\Delta\Delta ct}$ method Differences in expression between fibrotic tissue and its corresponding normal control were analyzed for significance. P-value < 0.05 was considered statistically significant.

Results
a) Histology and Histopathology analysis of Liver biopsies from NAFLD patients and healthy controls
Histopathological examination served as a crucial tool for diagnosing diseased or abnormal tissue. By comparing tissue samples with those from a control group, it provided valuable information for staging and grading chronic liver conditions like non-alcoholic steatohepatitis (NASH) as well as early and late fibrosis. Additionally, histopathology allowed for the assessment of a liver's response to treatment and the function of a liver transplant.
All sample diagnoses were histologically validated by a board-certified pathologist before molecular analysis. Group 1 (2 subjects) showed no fibrosis, Group 2 (3 subjects) exhibited early fibrosis, and Group 3 (2 subjects) displayed late fibrosis. Hematoxylin and eosin (H&E) were used for histological analysis. Histopathological slides were diagnosed using criteria from a scoring system for human NAFLD established by the National Institutes of Health Nonalcoholic Steatohepatitis Clinical Research Network system and NAFLD clinical research network (Kleiner, 2005). Information on donor age and gender was also collected.
A histology report of a normal liver Group 1 showed no fibrosis, described the typical microscopic architecture of the organ without any fat accumulation and inflammation signs. It focused on the normal appearance of liver cells (hepatocytes), blood vessels, bile ducts, and connective tissue. This histology slide of normal liver biopsy sample of group 1 showed under different microscope magnification. The small section of this slide was taken. For visualization purpose, we have used Hematoxylin and eosin (H&E) dye. The blue marked region showed under magnification of microscope mirror in **figure 1A** liver hexagonal unit and portal triad with bile ductule while red marked region showed arteriole venule. While **figure 1B** showed 10 x magnification of normal liver biopsy slide. The blue marked region in **figure 1C** under 40x magnification showed normal liver parenchyma with normal hepatocyte hexagonal unit with central vein. The normal liver exhibited regular hexagonal-shaped lobules with a central vein at their core. Hepatocyte cords radiated outward from this central vein. At the corners of lobules, portal triads were found, containing branches of the hepatic artery, portal vein, and bile duct. Abnormal features such as ballooning degeneration, steatosis, or necrosis were absent.

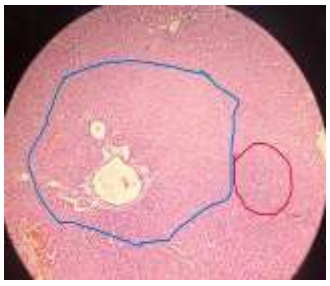

Figure 1A: Normal liver hexagonal unit marked with blue and portal triad with bile ductule, arteriole venule marked with red

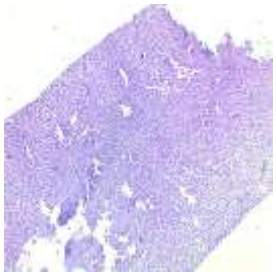

Figure 1B: Normal liver biopsy slide

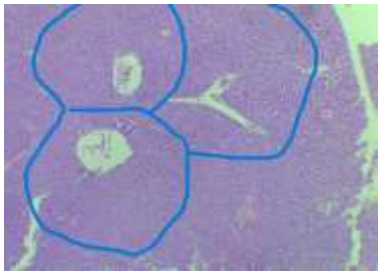

Figure 1C: Normal liver parenchyma with normal hepatocyte hexagonal unit with central vein

Figure 1: Histology images of H&E staining of normal human liver

This histopathology report of a liver with NAFLD and fibrosis revealed a spectrum of abnormalities, from simple steatosis to advanced fibrosis, depending on the severity of the disease. All sample diagnoses were histopathologically validated by a board-

certified pathologist before molecular analysis. Group 2 exhibited early fibrosis, and Group 3 displayed late fibrosis. Hematoxylin and eosin (H&E) and chromotrope aniline blue (CAB) stained sections were used for histopathological analysis. Histopathological slides were diagnosed using criteria from a scoring system for human NAFLD established by the National Institutes of Health Nonalcoholic Steatohepatitis Clinical Research Network system and NAFLD clinical research network (Kleiner, 2005).

This histopathology slide of an inflamed liver biopsy sample from group 2 was observed under a microscope. The small section of this slide was taken for visualization purpose, we have used Hematoxylin and eosin (H&E) dye. Due to fat accumulation, inflammation has been showed in **figure 2A**. The red arrow showed portal inflammation with inflammatory cells lymphocytes and plasma cells suggesting chronic inflammatory process while blue arrow-suggesting division of liver parenchyma into regenerative nodular arrangement-sign of cirrhois in the **figure 2B.** On the other hand, the red arrow showed chronic portal inflammation extending outside liver unit to meet other portal inflammation making bridge. Blue arrow showed fibrotic bands masked by inflammation extending from one unit to another like a bridge while pink amorphous acellular material with fibroblasts stained with eosin stain suggest fibrosis in the **figure 2C**.

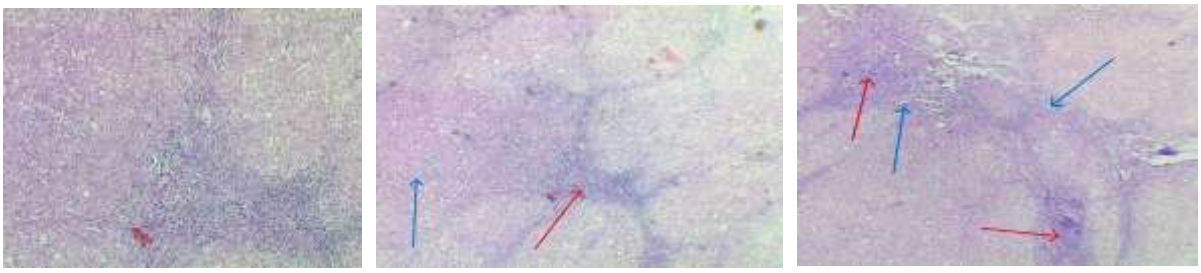

Figure 2A: Inflammation liver

(Fat accumulation)

Figure 2B:

- Red arrow- portal inflammation with inflammatory cells - lymphocytes and plasma cells suggesting chronic inflammatory process
- Blue arrow-suggesting division of liver parenchyma into regenerative nodular arrangement-sign of cirrhosis

Figure 2C:

- Red arrow-chronic portal inflammation extending outside liver unit to meet other portal inflammation making bridge
- Blue arrow-fibrotic bands masked by inflammation extending from one unit to another like a bridge
- Pink amorphous acellular material with fibroblasts stained with eosin stain suggest

Figure 2: Histopathology images of H&E staining of NAFLD human liver at early and late fibrosis stages

b) Quantitative Analysis of Inflammatory Biomarkers for fibrosis progression and diagnosis in NAFLD patients

Inflammation being an initiator in the progression of NAFLD to advance stages such as non-alcoholic steatohepatitis (NASH) and liver fibrosis, this study investigated the relationship of quantitative differential expression of selected hepatic inflammatory genes as biomarkers for progression of NAFLD using biopsy-proven NAFLD patients in three groups i.e. Group 1 (no fibrosis; n=10), Group 2 (early fibrosis; n=7) and Group 3 (late fibrosis; n=6). The fundamental purpose of this study was to diagnose the progressive stages of NAFLD by quantitative gene expression of inflammatory biomarkers including IL-6, TNF-α and IL-2R-α on biopsy-proven NAFLD patients. The liver tissue biopsies and blood samples were collected as per defined criteria included in method section. The deranged Liver Function Tests (LFTs) for early fibrosis, late fibrosis in comparison to control subjects are shown in table 1. The mean values of the parameters of LFTs including Aspartate Aminotransferase (AST) and Alanine transaminase (ALT) were slightly higher, 50.26 U/L and 58.94 U/L respectively than normal range in the early fibrosis (F1& F2) NAFLD patients. However, Alkaline Phosphatase (ALP) and total bilirubin were in normal range 76.51 U/L and 0.57mg/dL respectively in the early fibrosis (F1& F2) NAFLD patients. Furthermore, mean values of all four LFTs parameters were deranged in the late fibrosis (F3) i.e. AST (287 U/L), ALT (406 U/L), ALP (221 U/L) and total bilirubin (1.52 mg/dL).

Table 1: Liver Function Tests (LFTs) for early fibrosis (F1& F2) and late fibrosis (F3) in NAFLD patients in comparison to control subjects; Age group (30 - 60 Yrs)

Sample IDs	**AST**	**ALT**	**ALP**	**Total Bilirubin**
	1-40 U/L	10-50 U/L	40-129 U/L	0.3-1.3 mg/dL
Normal Control Subjects				
NF1	36.7	29.9	85	0.62
NF2	82	29.9	60.4	0.27
NF3	26.9	19.4	68	0.4
NF4	38.2	37.6	65.7	0.73

NF5	38.3	38	72.3	1.25
NF6	28.1	13.1	82.3	0.3
NF7	29.9	15.4	66.5	0.73
NF8	29.7	19.3	102	0.57
NF9	28.7	18.6	63	0.23
NF10	19.8	16.2	99.9	0.58
Mean	**35.83**	**23.74**	**76.51**	**0.57**
ALT/AST Ratio		**0.66**		
Early Fibrosis (F1, F2) NAFLD Patients				
NF11	43.8	67.2	113	0.78
NF12	53.9	43.8	104	0.92
NF13	60.9	93.6	73.7	0.49
NF14	51.9	65.7	75.4	0.48
NF15	51.9	54	85.1	0.35
NF16	30.2	52.8	128	0.38
NF17	59.2	35.5	83.8	0.85
Mean	**50.26**	**58.94**	**94.71**	**0.61**
ALT/AST Ratio		**1.17**		
Late Fibrosis (F3) NAFLD Patients				
NF18	102	132	170	0.42
NF19	506	1010	493	2.13
NF20	85.3	55.2	207	1.44
NF21	686	992	142	2.85
NF22	282	172	176	1.84
NF23	60	77	135	0.41
Mean	**287**	**406**	**221**	**1.52**
ALT/AST Ratio		**1.41**		

Patients were stratified based on both liver biopsy as well as using fibroscan data. The fibroscan score considered in this study for normal subjects was between 2 and 7 kilopascals (kPA), for early fibrosis (F1& F2) between 7.5 and 10 kPA and above 10 kPA (10-14kPA) for late fibrosis (F3) of NAFLD patients as per criteria given by Memorial Sloan Kettering Cancer Center (https://www.mskcc.org/cancer-care/patient-education/understanding-your-fibroscan-results).
The representative Fibroscan profiles for normal subjects and late fibrosis (F3) in NAFLD patients are shown in figure 3 as an example.

Figure 3A: Fibroscan profile of normal subject

Figure 3B: Fibroscan profile showing early fibrosis (F2) in NAFLD patients

Figure 3C: Fibroscan profile showing late fibrosis (F3) in NAFLD patients

The fibroscan scores for normal control subjects and early as well as late fibrosis of NAFLD patients is shown in table 2. In liver biopsy stratified groups of NAFLD patients, the mean values of Fibroscan Score for early fibrosis (F1 & F2) and late fibrosis (F3) NAFLD patients were 8.64 Median kPA and 12.02 Median kPA respectively.

Table 2: The fibroscan scores for normal control subjects and early (F1, F2) as well as late fibrosis (F3) of NAFLD patients

Normal Control Subjects		**Early Fibrosis (F1, F2) NAFLD Patients**		**Late Fibrosis (F3) NAFLD Patients**	
Patient IDs	**Fibroscan Score Median kPA**	**Patient IDs**	**Fibroscan Score Median kPA**	**Patient IDs**	**Fibroscan Score Median kPA**
NF1	4.8	NF11	8.5	NF18	11.5
NF2	3.8	NF12	8.1	NF19	11.1
NF3	4.5	NF13	9.3	NF20	10.7
NF4	2.8	NF14	9.8	NF21	12.4
NF5	5.6	NF15	9.5	NF22	12.8
NF6	6.2	NF16	7.8	NF23	13.6
NF7	3.3	NF17	7.5	-	-
NF8	5	-	-	-	-
NF9	6.5	-	-	-	-
NF10	4	-	-	-	-
Mean*	**4.65**	**-**	**8.64**	**-**	**12.02**

*Mean of fibroscan scores of three groups i.e. normal control subjects and early (F1, F2) and late fibrosis (F3)

RNA was extracted from blood samples of biopsy-proven and fibroscan verified NAFLD patients of all the three groups and quantitative gene expression analysis was performed using real-time PCR (RT-PCR) after synthesizing cDNA of all the patients. Three inflammatory biomarkers were compared in age matched NAFLD patients and control subjects i.e. IL-6, TNF-α and IL-2R-α along with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal control for data normalization. The primer designed for three inflammatory biomarkers and GAPDH as an internal control are shown in table 3. Representative real-time PCR profiles showing Ct values of all three inflammatory biomarkers for early and late fibrosis in comparison to control subjects are shown in figure 4 as an example.

Table 3: The primer sequences used to amplify the inflammatory biomarkers including IL-6, TNF-α and IL-2R-α along with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control for data normalization in Real-time PCR

Inflammatory Biomarkers (Gene Names)	**Primer**	**Primer Sequences (5'-3')**
IL-6	Forward	5'-AGGAGACTTGCCTGGTGAAA-3'
	Reverse	5'-CAGGGGTGGTTATTGCATCT- 3'
TNF- α	Forward	5'-CAGAGGGCCTGTACCTCATC-3'
	Reverse	5'-GGAAGACCCCTCCCAGATAG-3'
IL2R- α	Forward	5'-CATGGCCTACAAGGAAGGAA-3'
	Reverse	5'-TGGACTTTGCATTTCTGTGG-3'
GAPDH	Forward	5'-GAGCGAGATCCCTCCAAAATC-3'
	Reverse	5'-GGCTGTTGTCATACTTCTCATGG-3'

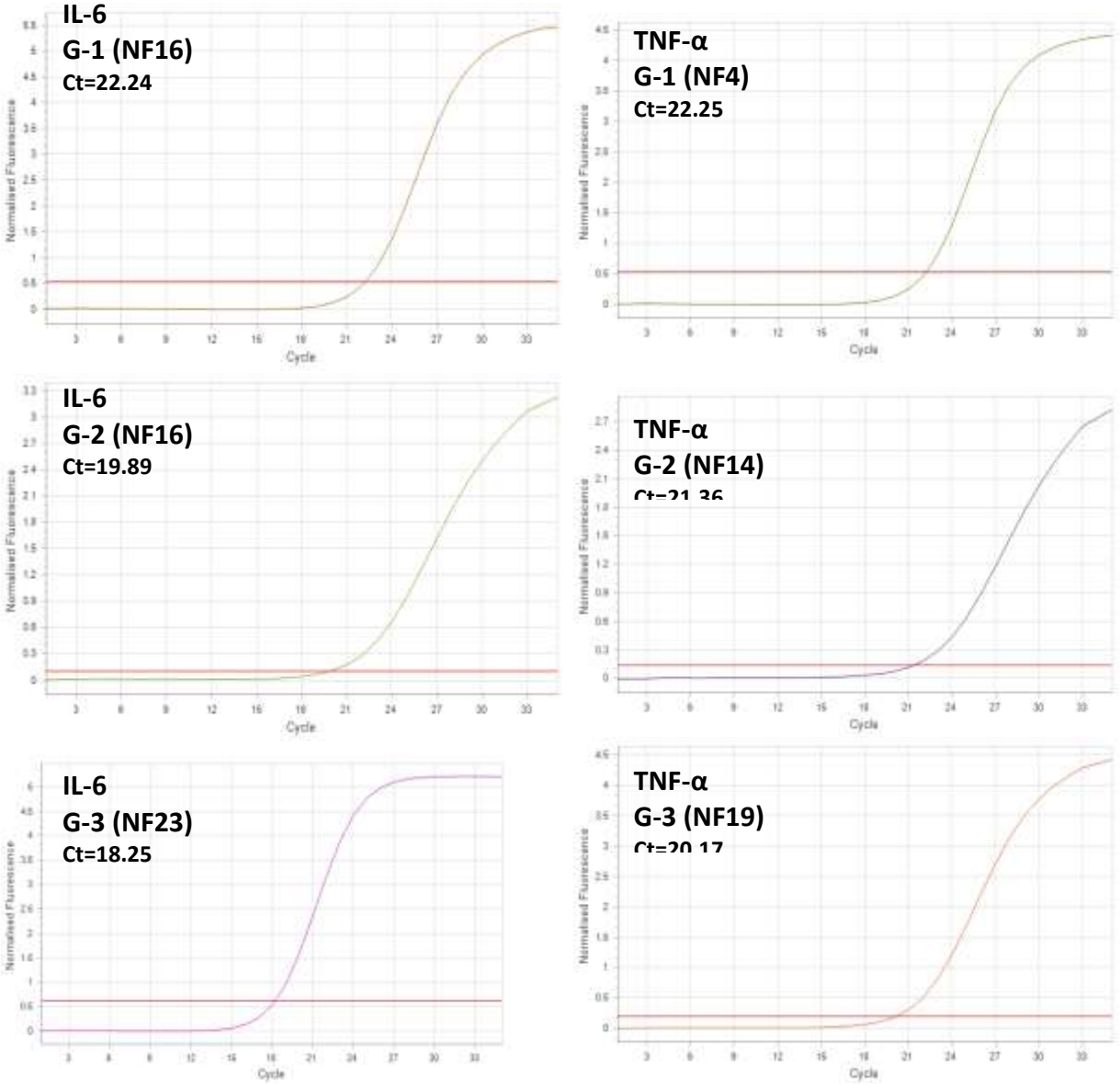

Figure 4A: Representative RT-PCR profiles of IL-6 and TNF-α in early and late fibrosis in comparison to control subjects i.e. Ct values are given for each group

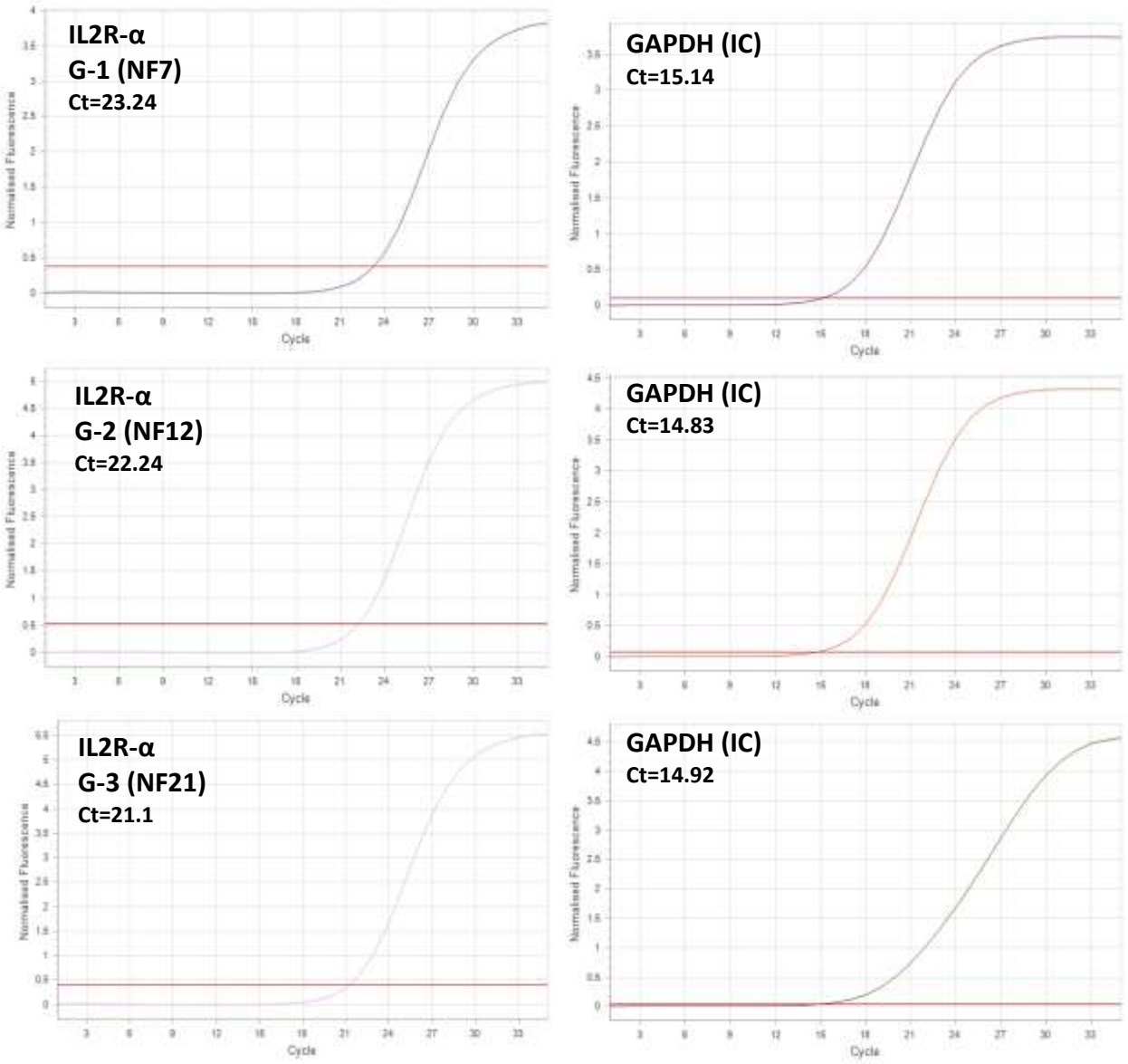

Figure 4B: Representative RT-PCR profiles of IL-2R-α and GAPDH as internal control in early and late fibrosis in comparison to control subjects i.e. Ct values are given for each group

Table 4A: Ct values for IL-6, TNF-α and IL-2R-α and GAPDH in control subjects

Patient IDs	Group I (n=10) Average Ct (3 Replicates)					
	IL-6	GAPDH	TNF-α	GAPDH	IL2R-α	GAPDH
NF1	22.35	15.34	22.45	15.61	23.45	15.21
NF2	22.24	15.17	23.40	15.44	23.68	15.14
NF3	22.35	14.88	22.50	14.78	23.72	14.98
NF4	22.15	14.83	22.25	14.92	23.55	14.83
NF5	22.25	15.29	22.38	14.87	22.62	15.10
NF6	21.97	14.62	23.18	15.62	23.15	14.92
NF7	22.10	15.17	22.29	14.86	23.24	15.17
NF8	22.37	14.75	23.00	14.95	23.67	14.85
NF9	21.89	14.98	23.60	15.38	23.44	14.80
NF10	22.27	15.32	22.66	14.92	23.19	15.06
Average Ct	**22.19**	**15.04**	**22.77**	**15.14**	**23.37**	**15.01**

Table 4B: Ct values for IL-6, TNF-α and IL-2R-α and GAPDH in early fibrosis (F1, F2) NAFLD patients

	Group 2 (n=7) Average Ct (3 Replicates)					
Patient IDs	**IL-6**	**GAPDH**	**TNF-α**	**GAPDH**	**IL2R-α**	**GAPDH**
NF11	20.18	14.68	20.89	15.01	21.84	14.78
NF12	19.91	15.31	20.91	14.88	22.24	14.91
NF13	20.27	15.45	21.15	14.78	21.96	15.15
NF14	20.25	15.21	21.36	15.41	22.13	15.11
NF15	20.05	15.33	21.34	14.93	22.21	14.83
NF16	19.89	15.22	21.41	15.38	21.97	14.97
NF17	20.29	15.41	21.65	15.34	22.02	14.97
Average Ct	**20.12**	**15.23**	**21.24**	**15.10**	**22.05**	14.97

Table 4C: Ct values for IL-6, TNF-α and IL-2R-α and GAPDH in late fibrosis (F3) NAFLD patients

	Group 3 (n=6) Average Ct (3 Replicates)					
Patient IDs	**IL-6**	**GAPDH**	**TNF-α**	**GAPDH**	**IL2R-α**	**GAPDH**
NF18	18.28	14.95	20.10	15.22	20.76	15.11
NF19	18.40	14.87	20.17	14.89	21.04	14.92
NF20	18.01	15.47	19.75	14.82	20.89	14.81
NF21	17.95	14.87	20.20	15.38	21.10	14.89
NF22	18.26	14.99	20.05	15.22	20.71	14.96
NF23	18.25	15.35	19.66	15.32	20.72	15.05
Average Ct	**18.19**	**15.08**	**19.99**	**15.14**	**20.87**	**14.96**

Table 5: Differential gene expression analysis of inflammatory biomarkers i.e. IL-6, TNF-α, IL-2R-α and GAPDH as internal control in early and late fibrosis in comparison to control subjects

	Inflammatory Marker (IM)	**IM Ct**	**GAPDH Ct**	**ΔCt**	**ΔΔCt**	**2-ΔΔCt/ Fold Change**	***p*-value**
Control Subjects (No fibrosis)	IL-6	22.19	15.04	7.15	-	-	-
	TNF-α	22.77	15.14	7.63	-	-	-
	IL2R-α	23.73	15.01	8.72	-	-	-
Early Fibrosis	IL-6	20.12	15.23	4.89	-2.26	**4.79**	7.59^{-08}
	TNF-α	21.24	15.1	6.14	-1.49	**2.81**	1.03^{-03}
	IL2R-α	22.05	14.97	7.08	-1.64	**3.12**	2.36^{-04}
Late Fibrosis	IL-6	18.19	15.08	3.11	-4.04	**16.45**	1.25^{-07}
	TNF-α	19.99	15.14	4.85	-2.78	**6.87**	8.43^{-05}
	IL2R-α	20.87	14.96	5.91	-2.81	**7.01**	7.58^{-06}

The quantitate changes in gene expression of inflammatory biomarkers i.e. IL-6, TNF-α, IL-2R-α and GAPDH as internal control in early and late fibrosis in comparison to control subjects is shown in table 5. All three inflammatory biomarkers (IL-6, TNF-α, IL-2R-α) showed significant upregulation in both early and late fibrosis in comparison to GAPDH housekeeping gene (Table 5). The highest quantitative changes in gene expression such as 4.79-fold (*p*-value=7.59^{-08}) and 16.45-fold (*p*-value=1.25^{-07}) were observed in the case of IL-6 in early and late fibrosis NAFLD patients respectively in comparison to other two inflammatory biomarkers (TNF-α, IL-2R-α) (Table 5). The inflammatory biomarker TNF-α and IL-2R-α also showed significant quantitative gene expression in early fibrosis as well as in late fibrosis NAFLD patients in comparison to control subjects. As shown in table 5, TNF-α was significantly over expressed 2.81-fold (*p*-value=1.03^{-03}) and 6.87-fold (*p*-value=8.43^{-05}) in early and late fibrosis NAFLD patients respectively. Similarly, IL-2R-α was also significantly over expressed 3.12-fold (*p*-value=2.36^{-04}) and 7.01-fold (*p*-value=7.58^{-06}) in early and late fibrosis NAFLD patients respectively in comparison to control subjects. The differential gene expression levels of all three inflammatory biomarkers (IL-6, TNF-α, IL-2R-α) is depicted in figure 5.

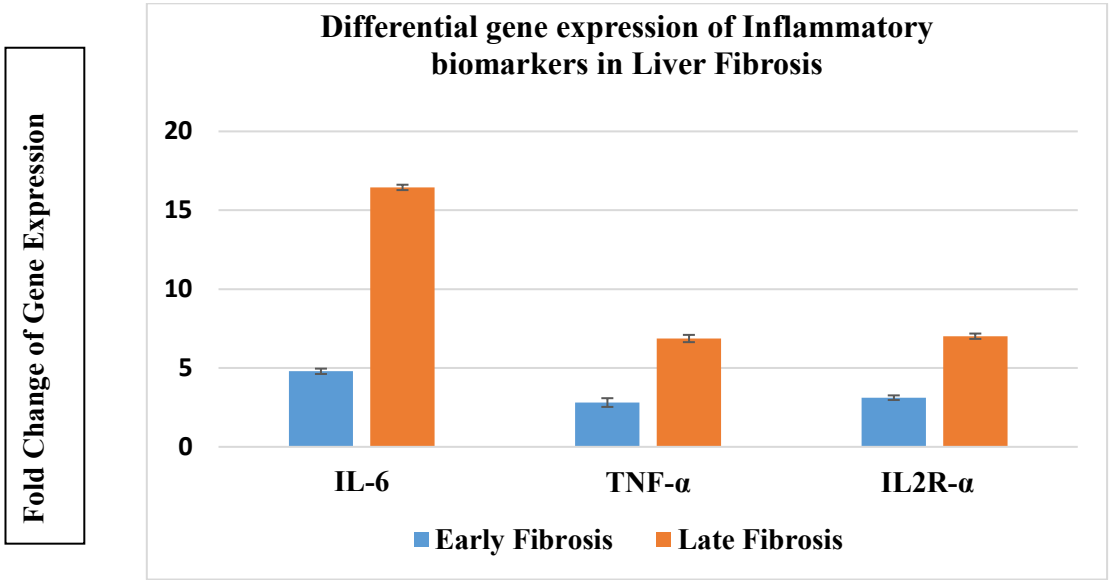

Figure 5: Quantitative gene expression of inflammatory biomarkers IL-6, TNF-α and IL2R-α in early and late Liver Fibrosis of biopsy-proven NAFLD patients

Notes: The expression levels of Inflammatory biomarkers IL-6, TNF-α and IL-2R-α were analyzed by real-time quantitative PCR using blood samples of early fibrosis (n=7), late fibrosis (n=6) and normal Control subjects (n=10) of biopsy-proven NAFLD patients. The mRNA levels of inflammatory biomarkers were normalized to the mRNA levels of GAPDH housekeeping gene as an internal control. Error bars are standard deviation between replicates.

Discussions

Inflammation in liver is an important determinant for the progression of NAFLD to advance stages such as non-alcoholic steatohepatitis (NASH) and liver fibrosis. This study explored the relationship of quantitative differential expression of selected hepatic inflammatory genes as biomarkers including IL-6, TNF-α and IL-2R-α for progression of NAFLD in to early stages of fibrosis using biopsy-proven NAFLD patients in three groups i.e. Group 1 (no fibrosis), Group 2 (early fibrosis) and Group 3 (late fibrosis). The purpose of this study was to diagnose the early progressive stages of NAFLD in comparison to stages of biopsy-proven NAFLD patients. The NAFLD patients were stratified based on the criteria of the deranged Liver Function Tests (LFTs), liver histopathology and Fibroscan data for early fibrosis and late fibrosis in comparison to control subjects.

The mean values of the parameters of LFTs including Aspartate Aminotransferase (AST) and Alanine transaminase (ALT) were slightly higher, 50.26 U/L and 58.94 U/L respectively than normal range in the early fibrosis (F1& F2) NAFLD patients. However, Alkaline Phosphatase (ALP) and total bilirubin were in normal range 76.51 U/L and 0.57mg/dL respectively in the early fibrosis (F1& F2) NAFLD patients. Furthermore, mean values of all four LFTs parameters were deranged in the late fibrosis (F3) i.e. AST (287 U/L), ALT (406 U/L), ALP (221 U/L) and total bilirubin (1.52 mg/dL). The ALT/AST ratio was increased subsequently in both early (1.17) and late fibrosis (1.41) in the NAFLD patients of this study. It has been reported previously in American cohorts that a higher ALT/AST ratio independently associate with a significantly higher risk of NAFLD and liver fibrosis (Xuan et al., 2024). Several studies have reported that ALT/AST ratio shows association with NAFLD in the general population, and hence, it may be used as diagnostic indicator of NAFLD **(**Sookoian et al., 2015**).** A cross-sectional case-control study of 6,926 participants reported that ALT/AST ratio is an independent risk factor for NAFLD **(**Fuyan et al., 2013**).** Another retrospective study of 3959 healthy women reported that the higher ALT/AST is an independent risk factors for the development and severity of NAFLD **(**Park et al., 2023**).**

In liver biopsy stratified groups of NAFLD patients, the mean values of Fibroscan Score for early fibrosis (F1 & F2) and late fibrosis (F3) NAFLD patients were 8.64 Median kPA and 12.02 Median kPA respectively which is as per criteria given by Memorial Sloan Kettering Cancer Center (https://www.mskcc.org/cancer-care/patient-education/understanding-your-fibroscan-results).

The differential gene expression of three inflammatory biomarkers were compared in age matched NAFLD patients and control subjects i.e. IL-6, TNF-α and IL-2R-α along with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal control for data normalization. All three inflammatory biomarkers (IL-6, TNF-α, IL-2R-α) showed significant upregulation in both early and late fibrosis in comparison to GAPDH housekeeping gene in NAFLD patients of this study. The highest quantitative changes in gene expression such as 4.79-fold (p-value=7.59^{-08}) and 16.45-fold (p-value=1.25^{-07}) were observed in the case of IL-6 in early and late fibrosis NAFLD patients respectively in comparison to other two inflammatory biomarkers (TNF-α, IL-2R-α) (Table 5). The upregulation of pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 has been reported during the sustained inflammation in liver (Moschen et al., 2010). A meta-analysis study examining a total of 19 pro-inflammatory cytokines has reported significant association for IL-6 and TNF-α with NAFLD and suggested that such inflammatory mediators may serve as potential biomarkers for early diagnosis and intervention NAFLD (Duan et al., 2022). IL-6 and its circulating coreceptor sgp130 has been reported to be higher plasma concentration in people with NAFLD in

comparison to normal individuals. The data of this study strongly supported that circulating components of the IL-6 trans-signaling system correlate with NAFLD/NASH pathogenesis and suggested that liver may be a source of these mediators in metabolic disease **(**Gunes et al., 2023**).**

The inflammatory biomarker TNF-α and IL-2R-α also showed significant differential gene expression in early fibrosis as well as in late fibrosis NAFLD patients in comparison to control subjects. The results of this study has shown that TNF-α was significantly over expressed 2.81-fold (p-value=1.03^{-03}) and 6.87-fold (p-value=8.43^{-05}) in early and late fibrosis NAFLD patients respectively. Similarly, IL-2R-α was also significantly over expressed 3.12-fold (p-value=2.36^{-04}) and 7.01-fold (p-value=7.58^{-06}) in early and late fibrosis NAFLD patients respectively in comparison to control subjects in this study.

Similar to our study, a recent study in the adult NASH-Clinical Research Network (NASH-CRN) cohort has reported the higher plasma levels of TNF-α in no/mild fibrosis and subsequently higher in significant fibrosis in NAFLD patients **(**Ajmera et al., 2017**).** Furthermore, in a multivariable analysis of significant fibrosis as compared to no/mild fibrosis has reported the increased plasma levels soluble IL-2 receptor alpha (sIL2Rα) consistent with the results of this study **(**Ajmera et al., 2017). Another study also reported that soluble IL-2 receptor alpha increased with fibrosis severity and portal inflammation. Particularly, sIL-2Rα were reported to be significantly higher in stage 3-4 fibrosis compared to stage 0-2 in NAFLD patients consistent with results of this study **(**Perito et al., 2017**).** Similar to our study, it has suggested that IL2R-α would be a useful single biomarker for early diagnosis of NASH and liver fibrosis by providing evidence of significant higher IHC of IL2R-α in NASH group than the non-NASH group in Taiwanese (Kao et al, 2021).

Conclusions: ALT/AST ratio was increased in early (1.17) and late fibrosis (1.41) in the NAFLD patients and the mean fibroscan scores observed for early fibrosis (F1 & F2) and late fibrosis (F3) of NAFLD patients were 8.64 Median kPA and 12.02 Median kPA respectively for liver biopsy stratified groups of NAFLD patients included in this study.

The quantitative differential expression of hepatic inflammatory genes as biomarkers including IL-6, TNF-α and IL-2R-α on biopsy-proven NAFLD patients showed significant upregulation in both early and late fibrosis in comparison to GAPDH housekeeping gene. The highest significant quantitative changes in gene expression such as 4.79-fold and 16.45-fold were observed in the case of IL-6 in early and late fibrosis NAFLD patients respectively in comparison to other two inflammatory biomarkers (TNF-α, IL-2R-α). Moreover, the inflammatory biomarker TNF-α and IL-2R-α also showed significant quantitative upregulation in both early fibrosis as well as in late fibrosis of NAFLD patients in comparison to control subjects. Therefore, the data of this study suggest that the quantitate expression analysis of these three inflammatory genes may be used as biomarkers for noninvasive tests for early dignosis and progression of fibrosis in NAFLD patients.

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