

An Observational Study showing Dipeptidyl Peptidase-4 (DPP-4) Activity and Gene Expression Variation in Chronic Liver Disease (CLD) Patients from a Tertiary Care Hospital of Eastern India

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Abstract

Introduction: The studies in animal models of cirrhosis suggest that dipeptidyl peptidase type 4 (DPP-4) enzymes play a crucial role in disease pathogenesis. In this clinical observational study, activity of DPP-4 and related gene expression were analysed in chronic liver disease patients. **Objectives:** To understand the DPP-4 enzyme activity variation in the common types of chronic liver disease by assessing plasma and peripheral blood mononuclear cell (PBMC) DPP-4 activity and comparing with healthy controls and to explore DPP-4 gene expression in PBMC. **Methods:** We recruited 130 study subjects in four cohorts—46 nonalcoholic fatty liver disease (NAFLD), 23 non-alcoholic cirrhosis (NAC) excluding viral aetiology, 21 alcoholic liver disease (ALC), and 40 control subjects. Blood samples were analysed for relevant biochemical parameters and plasma DPP-4 activity. PBMC fraction was used for the DPP-4 activity assay and gene expression analysis. **Results:** We found that lower plasma DPP-4 activity among patient cohorts but this was not statistically significant. The PBMC DPP-4 activity was significantly lower in NAFLD cohort. In the same cohort, DPP-4 gene expression in PBMC fraction was significantly increased ($P < 0.05$). There was significant correlation between plasma DPP-4 activity and liver injury marker alanine aminotransferase (ALT) among NAFLD ($\rho = 0.459$, $P < 0.01$), NAC ($\rho = 0.475$, $P < 0.05$), and ALC ($\rho = -0.572$, $P < 0.01$) patients. Plasma DPP-4 activity modestly predicted ALT plasma level (beta coefficient = 0.489, $P < 0.01$). **Conclusions:** The PBMC DPP-4 activity and DPP-4 gene expression gets significantly altered in NAFLD patients. Plasma DPP-4 activity also shows correlation with ALT levels in CLD patients. The role of DPP-4 in disease pathology in NAFLD and other forms of CLD needs to be explored.

Keywords: Chronic liver disease, cirrhosis, dipeptidyl peptidase-4, gene expression, non-alcoholic fatty liver disease

INTRODUCTION

The term ‘chronic liver disease (CLD)’ encompasses a large group of disease entities. It covers varying aetiologies such as infections, metabolic disorders, alcohol abuse and genetic abnormalities. The most commonly encountered chronic liver diseases are alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD) and CLD associated with hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.^[1]

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Submitted: 03-Apr-2022

Revised: 15-Apr-2022

Accepted: 22-Apr-2022

Published: 04-Aug-2022

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How to cite this article: Barkondaj B, Nargis T, Chakrabarti P, Mukhopadhyay S, Biswas K, Ganguly D, *et al.* An observational study showing dipeptidyl peptidase-4 (DPP-4) activity and gene expression variation in chronic liver disease (CLD) patients from a tertiary care hospital of Eastern India. *Indian J Endocr Metab* 2022;26:245-51.

Access this article online

Quick Response Code:



Website:
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DOI:
10.4103/ijem.ijem_139_22

Dipeptidyl peptidase-4 (DPP-4)/CD26 is a peptidase that is widely distributed in numerous tissues. It exists in membrane bound form as well as circulating soluble form in plasma.^[2] Although physiological functions of DPP-4 are not yet fully characterized, two distinct regulatory roles on metabolism are known. Firstly, the soluble form cleaves N-terminal dipeptides from a variety of substrates such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) leading to impairment of insulin secretion from pancreatic beta cell.^[3] Secondly, the membrane bound form regulates immunological cell signalling including T cell activation and adenosine deaminase activity.^[4]

Although a lot of research on CLD is being conducted worldwide, current knowledge and understanding of CLD pathology and therapeutics is still inadequate. There is no proven effective treatment available which can reverse or halt disease progression. In animal models, DPP-4 inhibition has shown improvement of insulin resistance and liver steatosis.^[5] Elevated serum DPP-4 level and liver canalicular activity of DPP-4 has been reported in experimental liver cirrhosis.^[6,7] Despite evidence of DPP-4 role in CLD, there is inadequate knowledge about DPP-4 activity variation in different subgroups of CLD patients and about mechanisms of such activity variation. Keeping this in mind, we investigated DPP-4/CD26 biology in CLD patients.

Our objectives were to investigate and compare plasma DPP-4 activity in chronic liver disease patients and healthy control subjects, CLD patients being divided into three subgroups namely, non-alcoholic fatty liver disease (NAFLD), non-alcoholic cirrhosis (NAC) excluding viral aetiology, and alcoholic cirrhosis (ALC). We also studied DPP-4 activity and gene expression in peripheral blood mononuclear cells (PBMC) of different CLD subgroups (NAFLD, NAC, ALC) and control subjects. Finally, we aimed to correlate DPP-4 enzyme activity with the established liver injury marker alanine aminotransferase (ALT).

MATERIALS AND METHODS

Study design and sample size

This study was designed as an analytical observational clinical study. We did not go for a formal sample size calculation. We used purposive sampling to recruit study subjects. On each OPD visit day, eligible study subjects were recruited in the order of their appearance after getting informed consent. Selected subjects were divided into four cohorts, namely NAFLD, NAC, ALC, and control. Institutional ethics committee approval (Inst/IEC/414 dated 11.01.2014) was obtained beforehand and the study conformed to principles enshrined in the Declaration of Helsinki. We ultimately recruited 130 study subjects (46 NAFLD, 23 NAC, 21 ALC and 40 control subjects). Subject selection criteria are given in Table 1.

Patient evaluation and procedure

Each patient underwent thorough history taking and clinical examination, including anthropometric measurements.

Ultrasonography of the whole abdomen was done. After an overnight fast, blood samples were collected for the following biochemical investigations: fasting and 2-hour postprandial plasma glucose, liver function tests, and fasting lipid profile. Viral markers for hepatitis B, C, and D were investigated in each patient to fulfil exclusion criteria.

Estimation of plasma DPP-4 activity

The DPP-4 activity was determined in plasma as the rate of 7-amino-4-methylcoumarin (AMC) cleavage per minute per millilitre from the synthetic substrate H-glycyl-prolyl-AMC. Briefly, 5 μ L of plasma was mixed with 35 μ L of assay buffer (25 mmol/L HEPES, 140 mmol/L NaCl, 80 mmol/L MgCl₂, and 1% bovine serum albumin; pH 7.8). After 5 minutes, preincubation at room temperature, the reaction was initiated by addition of 40 μ L of assay buffer containing 0.1 mmol/L of Gly-Pro-AMC substrate. After 20 min incubation, fluorescence was determined by spectrofluorometre. AMC fluorescence (excitation/emission - 380/460) was measured in a plate reader (Synergy H1 multi-mode microplate reader, Biotek).^[8] Figure 1 provides the standard calibration curve for the DPP-4 assay technique followed in the study.

Isolation of peripheral blood mononuclear cells (PBMC)

Isolation of mononuclear cells from anticoagulant treated whole blood was done with Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) technique. This method follows principle of density gradient centrifugation. Anticoagulant (EDTA) treated blood is layered on Ficoll-Paque PLUS solution and centrifuged for a period of 30 minutes at 3,000 rpm using swing bucket rotor. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated by Ficoll and sediments completely. The layer immediately above erythrocytes is the granulocyte layer. Because of their lower density, lymphocytes are found at the interface of plasma and Ficoll-Paque PLUS solution with slowly sedimenting

Table 1: Subject selection criteria for the study

Inclusion criteria	Exclusion criteria
Adult patient (≥ 18 years) of either sex. Willing to give written informed consent.	Cirrhosis with chronic hepatitis virus infections (HBV, HCV). Cirrhosis from uncommon metabolic (Wilson's disease, α_1 -antitrypsin deficiency) or drug and toxin exposure.
For NAFLD, non-alcoholic cirrhosis ethanol intake limit < 20 g per day (male) or < 10 g per day (female).	Decompensated liver disease (Child Pugh score ≥ 7 ; class B & C).
For abnormal liver function tests; AST, ALT 1.5 times upper normal limit, bilirubin (> 1.3 mg/dl), albumin (< 4 g/dl), PT time (> 15.4 s)	Patients with HIV/AIDS.
Ultrasonographic demonstration of steatotic or cirrhotic changes.	Loss of body-weight $> 10\%$ in last three months.

particles as platelets and monocytes. The lymphocytes are pipetted up from the interface and washed with phosphate buffered saline (PBS) to remove any platelets, plasma and Ficoll-Paque PLUS.^[9]

Estimation of protein concentration and DPP-4 activity in PBMC lysate

Protein concentration estimation is done, because it is essential to express the DPP-4 activity in PBMC lysate. Unlike plasma DPP-4 activity which is measured directly from plasma and expressed as nmol/mL/min; PBMC DPP-4 activity is measured as nmol/min/mg of protein. According to manufacturer guidelines, we estimated by BCA protein assay method (Pierce™ BCA Protein Assay Kit, Thermo Scientific). Figure 1 provides the standard calibration curve for plasma DPP-4 estimation while Figure 2 provides the same for BCPA protein estimation.^[10] DPP-4 activity measurement in PBMC lysate is similar to that in plasma.

Making cDNA by reverse transcription polymerase chain reaction (RT-PCR)

For converting whole cell mRNA into cDNA, we need to provide a fixed quantity of mRNA, which is estimated by NanoDrop 2000 (Thermo Scientific) and purity checked by 260/280 absorbance ratio (1.8 to 2 indicates pure RNA). We added 1 µg (1000 ng) of RNA for conversion into cDNA. We used RT-PCR reagents from Applied Biosystem, and followed their protocol. For 20 µl reaction mix, 10 µl RNA sample is added to 10 µl of RT Master Mix (10x RT Buffer, 25x dNTP Mix, 10x RT Random primers, MultiScribe Reverse Transcriptase, RNAase Inhibitor, Nuclease free water).

Gene expression analysis

Total cellular RNA was isolated from PBMC using TRIzol reagent (Invitrogen). cDNA was synthesized from 1,000 ng total RNA using cDNA synthesis kit (Roche). DPP-4 gene expression was analysed by quantitative PCR (LightCycler 96 real time PCR, Roche) using SYBR Green master mix (FastStart Universal SYBR Green Master, Roche) using following primers - forward 5'AAGTGGCGTGTTC AAGTGTG3' and reverse 5'GGCTTTGGAGATCTGAGCTG3'. Relative gene

expression was analysed by $\Delta\Delta C_t$ method and normalized by 18S RNA.

Quantification of gene expression

We used $\Delta\Delta C_t$ method to analyse gene expression data. This is a commonly used and validated method and is also known as $2^{-\Delta\Delta C_t}$ method. In this method each sample gene expression fold changes compared with control subjects and each value is internally validated with housekeeping gene expression. We used 18S ribosomal RNA gene as internal control. Threshold PCR cycle (C_t) values were determined for gene of interest and 18S gene for every patient sample (LightCycler 96 real time PCR, Roche). Amplicon amplification efficiency of target gene versus internal control gene (18S) meets standard efficiency.^[11]

Statistical methods

Descriptive summary of the data has been provided as median and interquartile range (IQR) or mean and standard error of mean (SEM), as appropriate. Shapiro–Wilk goodness of fit test was used to assess normality. The 95% confidence interval (CI) values have been presented were relevant. Numerical variables were compared between groups by Kruskal–Wallis test, with Dunn's test for pairwise post hoc comparison where the results of Kruskal–Wallis test were statistically significant. The P value less than 0.05 was considered statistically significant. Pearson's correlation coefficient (r) or Spearman's rank correlation coefficient rho (ρ) have been calculated to explore association between numerical variables. Simple linear regression was used to predict alanine aminotransferase (ALT) plasma level from possible predictor plasma DPP-4 activity. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corporation, New York, USA), and GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, California, USA) software.

RESULTS

Baseline demographic and clinical characteristics

The study population consisted of 130 CLD patients and healthy volunteers. During the recruitment process we

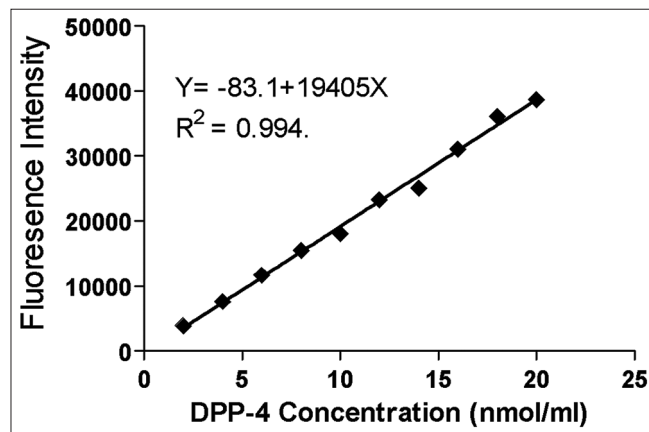


Figure 1: Standard curve: Fluorescence intensity versus DPP-4 concentration

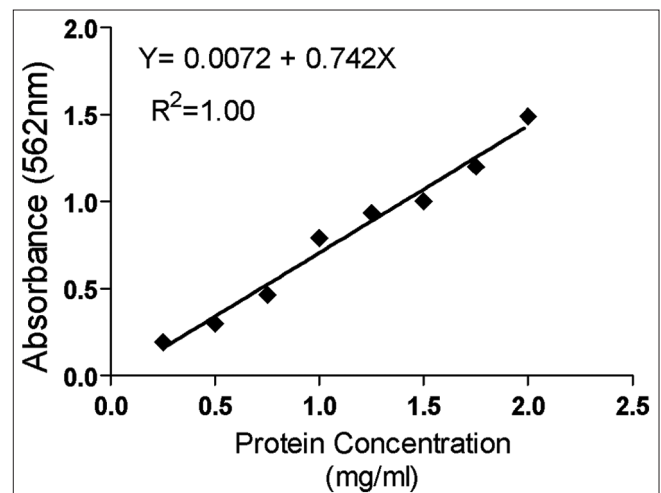


Figure 2: Standard curve for BCA protein estimation

screened 70 patients of NAFLD, from whom 46 consented to participate. Similarly, 23 subjects of non-alcoholic cirrhosis were included from among 30 screened. For alcoholic cirrhosis, we screened 35 and recruited 21 patients. Out of 80 healthy volunteers screened, 40 were recruited. Baseline characteristics of the study cohorts are presented in Table 2 and their statistical comparisons versus control in Table 3. As expected, the patient cohorts differ from control subjects with respect to body mass index (BMI) and lipid profile parameters for NAFLD patients and liver function test parameters for all patient groups.

Comparison of plasma and PBMC DPP-4 enzymatic activity

In our study, plasma DPP-4 enzyme activity was lower in NAFLD, NAC and ALC patients compared to controls [Figure 3]. The median DPP-4 enzyme activities were 17.92 (control), 14.80 (NAFLD), 14.33 (NAC), and 13.50 (ALC) in nmol/mL/min. However, the differences were not statistically significant compared to control. We also measured lysate PBMC DPP-4 activity in these four groups. The median values were 16.13, 6.30, 8.03, and 6.21 nmol/mg protein/min in control, NAFLD, NAC, ALC, respectively. PBMC DPP-4 activity was significantly lower in NAFLD patients compared to control subjects. From these figures we can say that plasma DPP-4 and PBMC lysate DPP-4 activity shows some variation across different types of CLD.

Comparison of DPP-4 gene expression across the cohorts

Quantitative gene expression analysis was done in comparison to 18S gene; a ribosomal constitutive gene. Here all measurements are in fold changes with respect to 18S gene. DPP-4 gene expression differed in our study cohorts. Relative fold change values were control (2.04 ± 0.44), NAFLD (35.38 ± 18.47), NAC (5.18 ± 1.52), and

ALC (22 ± 7.98). So, the PBMC DPP-4 gene expression was higher in NAFLD, NAC, and ALC patients, but only NAFLD patients had statistically significant higher value ($p < 0.05$). Contrasted to PBMC lysate DPP-4 enzyme activity, which was mostly elevated in NAC patients, DPP-4 gene expression was elevated in NAFLD patients. Up to this point, it can be said that plasma DPP-4 activity pattern was not influenced by PBMC expression pattern [Figure 4].

Plasma DPP-4 activity versus ALT (as marker for liver injury)

Alanine aminotransferase is a sensitive and specific marker for liver injury. In many previous liver injury studies, ALT has been used as a standard marker for hepatocyte damage. In our study we compared ALT plasma level with DPP-4 plasma activity. The ALT median values (U/L) in different cohorts were 12.22 (Control), 44 (NAFLD), 40 (NAC), and 95.2 (ALC). As a reflection of the disease process ALT values were significantly higher ($p < 0.001$) in all 3 CLD (NAFLD, NAC and ALC) groups, compared to controls.

Correlation analysis [Figure 5] of the plasma DPP-4 activity with plasma ALT level showed moderate correlation both in NAFLD ($\rho 0.459$, $P < 0.01$), and in NAC ($\rho 0.475$, $P < 0.05$) cohorts. However, there was no correlation ($\rho 0.003$, $P > 0.05$) in control subjects. Importantly ALC patients showed good negative correlation ($\rho -0.572$, $P < 0.01$). Thus, although plasma DPP-4 activity was not statistically different among the cohorts, it had good correlation with ALT as liver injury marker [Table 4, Figure 5]. Simple linear regression analysis, using serum ALT as dependent variable and plasma DPP-4 as predictor variable was done for NAFLD patients. As Table 5 indicates, the standardised regression coefficient was 0.401 with 95% CI ranging from 0.122 to 0.856. This reinforces a modest association between the two.

Table 2: Baseline demographic and laboratory parameters of the study cohorts

Variables	Control (n=40)	NAFLD (n=46)	Non-alcoholic cirrhosis (n=23)	Alcoholic cirrhosis (n=21)
Male : Female	21 : 19	16 : 30	11 : 12	21 : 0
Age (years)	42.0 (36.0-52.0)	41.5 (35.0-49.75)	44.0 (33.0-48.0)	40.0 (35.0-48.0)
Body mass index (kg/m ²)	25.19 (23.14-26.72)	25.97 (24.38-30.22)	22.96 (19.00-24.84)	22.95 (20.07-24.53)
Fasting blood glucose (mg/dl)	97.5 (91.75-104.25)	99.5 (90-112)	93.5 (84-110)	98.5 (88.75-106)
Postprandial blood glucose (mg/dl)	139.0 (119.8-153.3)	137.5 (114.8-148.0)	146.0 (145.0-154.0)	135.0 (128.8-140.0)
Diabetes (%)	0	13.04	21.73	0
Total cholesterol (mg/dl)	180.5 (160.3-211.0)	228.0 (197.0-243.0)	214.5 (130.5-226.5)	180.0 (128.0-192.5)
Triglycerides (mg/dl)	119.0 (78.0-159.0)	145.0 (125.0-240.0)	158.0 (139.3-184.5)	100.0 (97.5-125.0)
LDL (mg/dl)	105 (87.2-132.5)	160 (115-180.5)	106 (73.35-142)	124 (117.5-130.5)
HDL (mg/dl)	47.0 (39.8-56.3)	40.0 (35.0-48.5)	36.0 (27.0-45.72)	44.0 (30.0-46.0)
VLDL (mg/dl)	29.2 (18.3-39)	31.0 (25.0-48.0)	22.7 (20.1-27.3)	20.0 (18.5-25.0)
Total bilirubin (mg/dl)	4.90 (4.50-5.02)	0.70 (0.60-0.97)	1.10 (0.80-1.6)	1.77 (0.90-4.22)
Albumin (g/dl)	0.7 (0.57-1)	4.4 (4.0-4.8)	2.9 (2.67-3.6)	3.6 (2.7-4)
ALT (U/L)	12.2 (8.7-17.5)	44 (31.0-60.0)	40 (30.0-58.0)	95.2 (62.0-147.1)
AST (U/L)	14.8 (8.7-21.8)	43 (31.0-66.0)	44 (27.3-65.3)	84.6 (49.8-146.8)
Plasma DPP-4 activity (nmol/ml/min)	17.92 (8.70-28.78)	14.80 (4.10-33.09)	14.33 (3.96-23.99)	13.50 (1.45-23.83)
PBMC DPP-4 activity (nmol/min/mg pr)	16.13 (13.85-17.45)	6.30 (2.10-11.38)	8.03 (5.02-14.61)	6.21 (0.98-12.47)

DISCUSSION

The target population for this analytical observational study consisted of both CLD patients and healthy volunteers. CLD patients were categorised to three common subgroups, namely non-alcoholic fatty liver disease, non-alcoholic cirrhosis, and alcoholic cirrhosis. The disease cohorts had no difference with control subjects with respect to age, fasting and postprandial

plasma glucose, and fasting lipid profile parameters (except raised total and LDL-cholesterol in NAFLD group). However, liver function test parameters (total bilirubin, albumin, AST, and ALT) differed significantly in all three disease cohorts from control subjects. These variations are to be expected.^[12]

It is known that DPP-4 is present in two forms—a free-soluble form in plasma and membrane bound form that occurs in T-lymphocytes.^[13] Plasma DPP-4 activity represents the sole soluble form of the enzyme. It did not show any statistically significant activity variation across the disease cohorts [Table 2 and Figure 3], despite differences in the median values. However, PBMC lysate DPP-4 activity showed significantly decreased level between the NAFLD and control cohorts. NAC patients had higher PBMC DPP-4 activity but this was not statistically significant. This lack of difference in DPP-4 activity with control subjects goes against the use of DPP-4 inhibitors in the management of NAFLD and allied forms of CLD, contrary to some of the reported experience.^[14-16]

We further investigated DPP-4 gene expression in all cohorts by real time PCR after getting no difference in plasma and PBMC DPP-4 activity. In NAFLD patients, DPP-4/CD26 showed significant higher level of gene expression [Figure 4]. So, in this cohort, higher PBMC DPP-4 gene expression is not relating with unchanged plasma enzyme activity. This suggests that that DPP-4 gene expression is increased in peripheral blood inflammatory cells without corresponding rise in plasma DPP-4 activity. This difference may be explained partly by heterogeneity in soluble/circulatory and plasma membrane bound form of DPP-4 enzyme. Further, there may be differential shedding of DPP-4 enzyme from inflammatory cells in the blood from the main site of inflammation at liver.^[17]

We compared the plasma DPP-4 activity with liver injury marker ALT. This analysis showed positive and moderate correlation in NAFLD and NAC patients but moderate negative correlation in ALC patients [Table 4 and Figure 5]. Though the correlations are not strong, they are suggesting role of DPP-4 in pathogenesis of NAFLD and NAC. Simple regression analysis

Table 3: Results of statistical comparisons between patient and control groups

Variables	P (Control vs. NAFLD)	P (Control vs. NAC)	P (Control vs. ALC)
Age (years)	NS	NS	NS
Body mass index (kg/m ²)	<0.01	<0.05	NS
Fasting blood glucose (mg/dl)	NS	NS	NS
Postprandial blood glucose (mg/dl)	NS	NS	NS
Total cholesterol (mg/dl)	<0.05	NS	NS
Triglycerides (mg/dl)	<0.05	NS	NS
LDL (mg/dl)	<0.05	NS	NS
HDL (mg/dl)	NS	NS	NS
VLDL (mg/dl)	NS	NS	NS
Total bilirubin (mg/dl)	NS	<0.05	<0.001
Albumin (g/dl)	<0.05	<0.001	<0.001
ALT (U/L)	<0.001	<0.001	<0.001
AST (U/L)	<0.05	<0.05	<0.001
Plasma DPP-4 activity (nmol/ml/min)	NS	NS	NS

Abbreviations are standard and have been expanded in the text.
NS=Non-significant P value

Table 4: Correlation analysis between plasma DPP-4 activity and plasma ALT level in respective study cohorts

Control		Non-alcoholic fatty liver disease		Non-alcoholic cirrhosis		Alcoholic cirrhosis	
Rho value	P	Rho value	P	Rho value	P	Rho value	P
0.003	NS	0.459	<0.01	0.475	<0.05	-0.572	<0.01

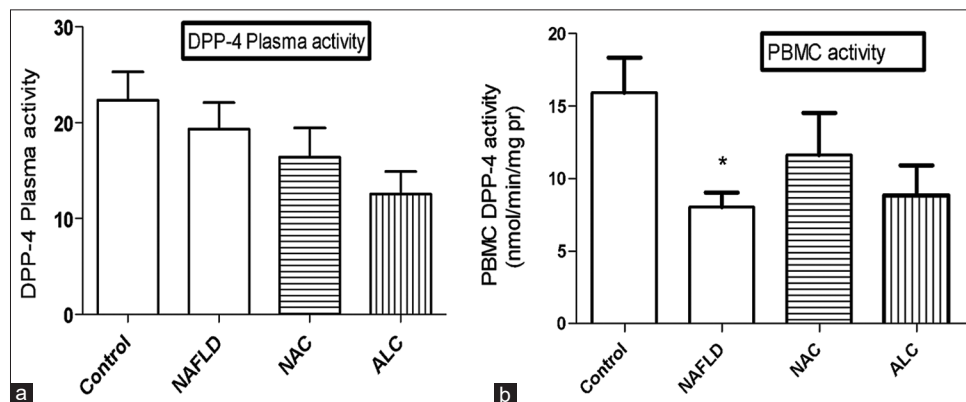


Figure 3: Bar chart showing plasma DPP-4 activity (a), and PBMC DPP-4 activity (b) in different study cohorts. Data presented as mean \pm standard error of mean; * denotes $P < 0.05$. Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAC, non-alcoholic cirrhosis; ALC, alcoholic cirrhosis

Table 5: Simple linear regression analysis of relation between ALT and DPP-4 in NAFLD patients

	Unstandardized coefficients		Standardized coefficients	T	P	95% confidence interval for regression coefficient beta	
	Beta	Std. error	Beta			Lower bound	Upper bound
(Constant)	38.293	4.874		7.856	0.000	28.426	48.161
DPP-4	0.489	0.181	0.401	2.699	0.010	0.122	0.856

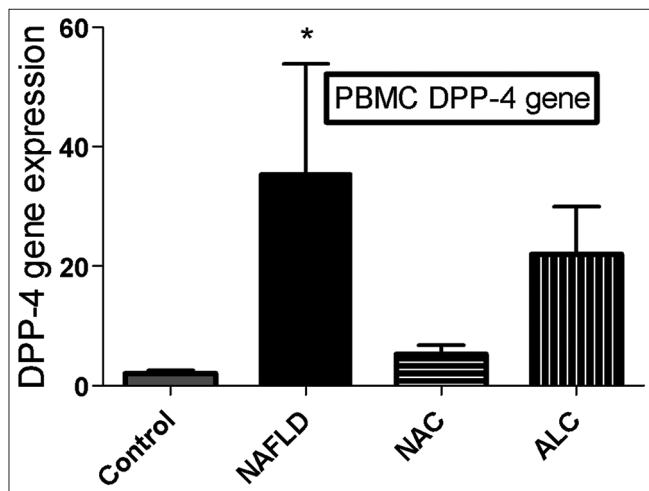


Figure 4: Bar chart showing DPP-4 gene expression in PBMC lysate in different study cohorts. Data presented as mean \pm standard error of mean; * denotes $P < 0.05$. Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAC, non-alcoholic cirrhosis; ALC, alcoholic cirrhosis

with ALT as dependent variable and DPP-4 activity as predictor showed modest model fit in NAFLD patients [Table 5].

In conclusion, it can be said that we have documented the expected variations in plasma and PBMC lysate DPP-4 activity in three common categories of CLD patients, namely NAFLD, alcoholic, and non-alcoholic cirrhosis. With NAFLD patients, compared to healthy controls, there is a significant reduction in the PBMC DPP-4 activity but no significant decline in plasma DPP-4 activity. Further, in this cohort, the DPP-4 gene expression is increased in peripheral blood inflammatory cells without rise in plasma DPP-4 activity. The plasma DPP-4 activity also shows moderate to good correlation with the established liver injury marker ALT in these disease cohorts but no correlation in healthy controls. This begets the question of whether DPP-4 is involved in the pathogenesis of chronic liver disease, which should be a fertile area for future research.

Acknowledgements

We thank all recruited subjects for their wholehearted participation in the study. We are grateful to all members of the Metabolic Disease Laboratory (laboratory 250) under Dr. Partha Chakrabarti, and Dendritic Cell Laboratory (laboratory 138) under Dr. Dipyaman Ganguly, senior scientists at CSIR-Indian Institute of Chemical Biology, Kolkata, for assistance in laboratory work. In particular we must mention Titli Nargis, Mainak Ghosh, Moumita Adak, Dipsikha Biswas, Amrit Raj

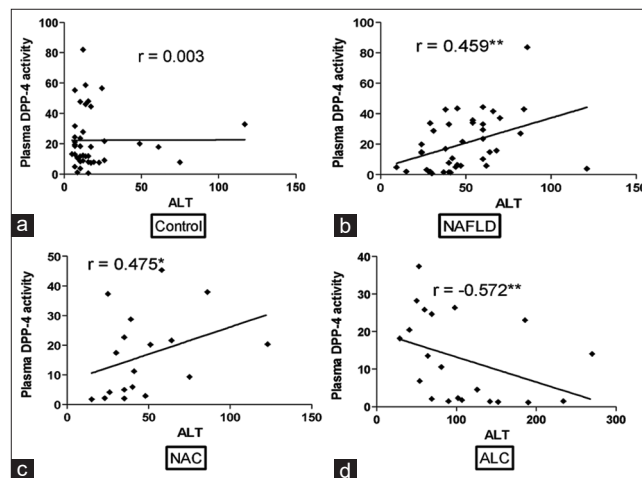


Figure 5: Scatter plots depicting correlation between plasma DPP-4 activity and ALT level in respective cohorts, namely control (a), NAFLD (b), NAC (c) and ALC (d). * denotes $P < 0.05$, ** $P < 0.01$. Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAC, non-alcoholic cirrhosis; ALC, alcoholic cirrhosis

Ghosh, Roopkatha Bhattacharya, Oindrila Rahaman, and Deblina Raychowdhury.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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