Original Research Article

The Egyptian Journal of Immunology
Volume 29 (4), 2022: 33–45.
www.Ejimmunology.org

Flow cytometry assessment of monocyte subsets alteration in hepatocellular carcinoma post hepatitis C virus infection

Fatma Ali[1], Reham Hammad[1], Fatma M. Kotb[2], Reda B. Aglan[3] and Mona H. Alrayes[1]

[1]Department of Clinical Pathology, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt.
[2]Department of Internal medicine, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt.
[3]Department of Hepatology & Gastroenterology, National Liver Institute Menoufia University, Menoufia, Egypt.

Corresponding author: Reham Hammad, Department of Clinical Pathology, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt.
Email: reham.hammad@azhar.edu.eg.

Abstract

Hepatocellular carcinoma (HCC) is assumed to be an immunogenic malignancy since 90% of cases develop in environments with ongoing inflammation. Monocyte subsets contribute to tumoral immunity. Most HCC patients are discovered at late stages, which lowers their survival chances. We aimed to determine whether altered frequency of monocyte subsets contribute to post hepatitis C virus infection-liver cirrhosis (HCV-LC) development to HCC. This cross-sectional study enrolled 105 patients classified as post HCV-HCC (n=72) and post HCV-LC (n=33) patients. The monocyte subsets frequency was assessed by flow-cytometry. There was a significant increase in intermediate monocytes and decrease in non-classical monocytes in HCC group when compared to the LC group (P = 0.001 and 0.006, respectively)**.** Intermediate monocyte frequency was positively correlated with cholesterol and triglycerides (r = 0.296, $P < 0.002$ and r = 0.247, $P < 0.011$, respectively). The receiver operating characteristic (ROC) curve revealed that intermediate monocytes percentage at a cutoff ≥ 0.625% and non-classical monocytes percentage at a cutoff ≤ 0.61% differentiated between patients with HCV-LC and those with HCV-HCC with a sensitivity of 76.4% and 69.4%, respectively, while both revealed low specificity of 51.5%. According to logistic regression analysis, only the triglyceride level was found to be an independent risk factor for HCC development [OR =1.014 (11.001–1.026), P = 0.031]. Finally, we concluded that post-HCV-HCC is characterized by an upregulation of intermediate monocytes and a downregulation of non-classical monocytes when compared to Post-HCV-LC. Intermediate and non-classical monocytes frequency can aid to screening biomarkers for HCC development. Intermediate monocyte frequency may be linked to hyperlipemia. The level of triglycerides is proposed as an independent risk factor for HCC emergence.

Keywords: Monocyte subsets, hepatocellular carcinoma, hepatitis C virus infection, liver cirrhosis, hyperlipemia.

Date received: 23 July2022; **accepted:** 11 Augustl 2022

Introduction

Hepatocellular carcinoma (HCC) is the fourth most prevalent cause of cancer-related deaths globally and the sixth most frequently diagnosed cancer.[1] Palliative care is also necessary because most HCC patients are already in an intermediate-advanced stage when their diagnosis is made.[1] Background chronic liver disease, which eventually causes liver cirrhosis, is a risk factor for HCC development.[2]

HCC has been screened for and monitored using imaging technology such as computed tomography and alpha-fetoprotein, a biomarker connected to the disease. Due to the existing methods' low sensitivity, HCC is still difficult to be effectively identified in its early stages.[3] With a 1–8% annual risk in individuals with HCV–induced cirrhosis, chronic hepatitis C virus (HCV) is responsible for one-third of all HCCs worldwide.[4] So urgent need for noninvasive biomarkers is justified to facilitate the screening and early diagnosis of HCC post HCV.

The HCC is regarded as an immunogenic malignancy since 90% of cases arise in environments with ongoing inflammation. This inflammation contributes to tumor growth and is linked to a higher tumor immunogenicity.[2] Therefore, immunotherapeutic techniques would be the most appropriate therapeutic options to use in these sorts of carcinomas.[5]

As innate immune cells, monocytes phagocytose microbes and produce oxygen reactive species. They also play a role in cellular processes such as tissue repair and regeneration during heart diseases. Monocytes are heterogeneous, multifunctional immune cells that exhibit great plasticity.[6] Numerous receptors expressed by monocytes keep track of environmental changes. They are essential for the development of inflammation and for host defense.[7]

According to the surface expression of the two IgG low affinity receptors, CD14 and CD16, circulating monocytes are divided into three subsets: classical ($CD14^{high}$ CD16-), intermediate ($CD14^{high}$ CD16+), and non-classical monocytes ($CD14^{dim}$ CD16 high).[6] Of the circulating monocytes, about 85% are classical monocytes, 5-10% intermediate monocytes, and 5-10% non-classical monocytes.[7]

Peripheral blood monocytes play crucial roles in the pathophysiology of inflammatory diseases by continuously replenishing hepatic macrophages and dendritic cells. Previous research studies have already revealed functional abnormalities in both cell subsets as well as decreased absolute numbers and frequencies of circulating FcRI+ monocytes and myeloid dendritic cells in HCC patients.[8]

It has been widely accepted that various monocyte subsets can regulate a variety of inflammatory and infectious disorders, such as Sjögren's disease, type 1 diabetes, cardiovascular disease, chronic kidney diseases, and many others, in different ways.[9]

Further understanding of the variations in immune cell populations may assist to identify the possible immunotherapies due to its immunogenic origin. Consequently, the current work focused on assessing whether altered frequency of monocyte subsets would contribute to post hepatitis C virus infection-liver cirrhosis (HCV-LC) development to HCC.

Subjects and Methods

This comparative cross-sectional study was carried out between November 2021 and June 2022. In order to compare the frequency of monocyte subgroups, the study involved 105 patients, 72 of whom were post-HCV-HCC patients and 33 post-HCV liver cirrhosis (post-HCV-LC) patients. We included adults older than 18 years. According to recent standards, the diagnosis of HCC was histologically verified or based on specified imaging criteria [10] and only treatment naïve HCC patients were included. HCV infection was confirmed in LC patients more than six months before the study started. Patients with a history of alcoholism or autoimmune disease were excluded, as well as those with acute or chronic HBV infection (as determined by serology). HCC that not linked to HCV was also excluded.

Ethical considerations

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty

of Medicine for Girls, Al-Azhar University (No. 1072 dated November 2021). The study candidates were informed about the aim of the study and gave their informed written consent before enrolment in the study.

Sample collection

A volume of 6 ml of venous blood was drawn from each subject. The blood sample was divided into two aliquots. The first aliquot (3 ml) blood was transferred into ethylenediaminetetraacetic acid (EDTA) tube for complete blood count and flow cytometry assay. The second aliquot (3 ml) was used for separation of serum for assessment of biochemical tests.

Study methods

All patients were subjected to full history taking and general clinical exam and measuring body mass index (BMI).

-Laboratory methods: Complete blood count was performed using a full automated hematology analyzer (Sysmex, KX21N, Kobe, Japan), according to the manufacturer's instructions. Routine biochemical tests: creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, gamma GT (GGT), high-density lipoprotein (HDL), total and direct bilirubin were performed using a chemistry autoanalyzer device (Cobas Integra 400 Plus, Roche Diagnostics, Germany), according to the manufacturer's instructions.

-Serum Alpha fetoprotein (AFP) was done by the electro-chemiluminescence immunoassay (ECLIA) using (Cobas 6000, e601 module, Roche Diagnostics, Germany), according to the manufacturer's instructions.

-Abdominal computed tomography (CT) scan was performed using a scanner device (Siemens 128, German) blindly by an expert. He recorded the liver size, ascites if minimal, moderate or massive, lymph node (LN) enlargement or not, liver pattern if cirrhotic or with growth (heterogenous or focal and if single or multiple lesions), and portal vein (PV) if patent or thrombosed.

-Flow-cytometry was conducted at Al-Zahraa University Hospital using a multi-color fluorescence-activated cell sorting (FACS) flow cytometer (BD, FACSCalibur, Biosciences, San Jose, USA). Fresh EDTA blood sample was used for the assay. A volume of 50 µl of blood was incubated with 5 µl of each of CD14-PE-conjugated Ab (cat. no. A07764, lot. no.25, BD Biosciences), CD16-FITC-conjugated Ab (cat. no. P59232AA, lot no.200105, Immunotech; Beckman Coulter, Marseille, France), CD45-PerCP-conjugated anti-human (cat. no. 345809, lot no. 6039924, BD Biosciences, USA,). Then red blood cells were lysed. An adequate number of 100,000 events were acquired for analysis.

Gating strategy: Initial gate was taken on dot plot graph using forward scatter (FS) / CD45-PerCP and specified for monocytes-region (R1) then cells were examined on quadrant plot, CD14-PE and CD16-FITC for determination of monocytes subsets accordingly as classical (CD14 ^{high} CD16–), intermediate (CD14^{high} CD16+), and non-classical (CD14^{dim} CD16+) (Figure1).

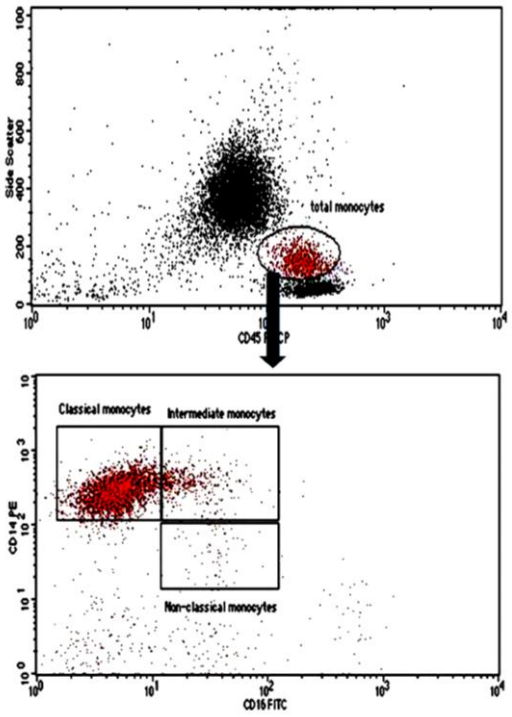

Figure 1. Gating strategy for monocyte subsets. According to CD45/SS the total monocytes-region (R1) was detected then subsets were examined on quadrant plot, CD14-PE and CD16-FITC. Classical (CD14^{high} CD16–), intermediate (CD14^{high} CD16+), and non-classical monocytes (CD14^{dim} CD16+).

Statistical methods

Recorded data were analyzed using the Statistical Package for Social Sciences, v.23.0 (SPSS Inc., Chicago, Illinois, USA). The quantitative data were presented as mean ± standard deviation (SD) and ranges when their distribution was parametric (normal). Also, qualitative variables were presented as numbers and percentages. Independent-samples *t*-test of significance was used when comparing two means, and Mann Whitney *U* test was used for two-group comparisons in non-parametric data. The comparison between groups with qualitative data was made by Chi-square test and Fisher's exact test instead of Chi-square test only when the expected count in any cell was less than 5. Multivariate logistic regression analysis: Odds ratios (OR) with 95% confidence intervals were computed to assess the overall association between each possible flow-cytometry data and the occurrence of severe BA. The confidence interval was set to 95%, and the margin of error accepted was set to 5%. Finally, a $P \leq 0.05$ was considered significant.

Results

The 105 participants in this study were split into two groups: those with liver cirrhosis (LC, n = 33) and post-HCV HCC (n = 72). Table 1 displays the demographic details and clinical information of the study individuals.

Table 1. Demographic and clinical data of the 105 study participants, as divided in the two study groups.

		post-HCV-HCC group		post-HCV-LC group		*P* value
		Mean±SD		Mean±SD		
Age/year		62.31±7.63		57.94±12.51		0.030
BMI Kg/m^2		29.83±4.11		31.25±6.21		NS
		Count	%	Count	%	
Gender	male	57	79.2%	26	78.8%	NS
	female	15	20.8%	7	21.2%	
Ascites	massive	4	5.6%	4	12.1%	0.005
	moderate	8	11.1%	10	30.3%	
	mild	17	23.6%	2	6.1%	
	no	43	59.7%	17	51.5%	
Liver lesion pattern	multiple	20	27.8%	0	0.0%	-
	heterogenous mass	1	1.4%	0	0.0%	
	focal	51	70.8%	0	0.0%	
	cirrhotic	72	0.0%	33	100.0%	
Liver mass number	single	34	47.2%	0	0.0%	-
	multiple	38	52.8%	0	0.0%	
	no	0	0.0%	33	100.0%	
PV patency	thrombosed	15	20.8%	0	0.0%	-
	partially occluded	2	2.8%	0	0.0%	
	patent	55	76.4%	33	100.0%	
LN enlargement	yes	12	16.7%	0	0.0%	-
	no	60	83.3%	33	100.0%	
Splenomegaly	Yes	67	93.1%	31	93.9%	NS
	removed	2	2.8%	0	0.0%	
	no	3	4.2%	2	6.1%	
Lung	normal	64	88.9%	33	100%	NS
	metastasis	6	11.1%	0	0.0%	

BMI, Body mass index; LC, liver cirrhosis. *P*> 0.05 is not significant (NS).

Comparing the two groups showed that their sexes were similar, but the age of the HCC group was significantly higher than the LC group (*P*=0.03). As shown in Table 1, there was no difference in BMI between the two study groups (*P*=0.238). There was a significant increase in circulating intermediate monocytes and significant decrease in non-classical monocytes observed in HCC group of patients when compared to the LC group as shown in Table 2, Figure 2.

Table 2 illustrates the results of the comparison of laboratory parameters between the groups under study.

Table 2. Comparison of laboratory data between post-HCV-HCC and post-HCV-LC groups.

	Post-HCV-HCC group			Post-HCV-LC group			*P* value
	Median	1st quartile	3rd quartile	Median	1st quartile	3rd quartile	
AFP ng/ml	1.89	1.12	2.51	0.86	0.64	1.02	< 0.001
AST U/L	63.00	51.50	105.00	37.00	25.00	67.00	< 0.001
ALT U/L	52.00	36.00	65.50	25.00	18.00	35.00	< 0.001
ALP U/L	112.50	89.00	166.50	113.00	81.00	140.00	< 0.001
GGT IU/L	61.00	45.00	78.00	37.00	24.00	61.00	< 0.001
Total Bilirubin mg/ dl	1.50	0.95	3.10	1.70	0.90	5.00	NS
Direct Bilirubin mg/ dl	0.84	0.40	1.64	0.80	0.30	2.55	NS
Cholesterol mg/dl	155.50	121.50	205.50	136.00	110.00	153.00	0.002
Tri Glycerides mg/dl	133.00	95.00	197.50	75.00	63.00	113.00	< 0.001
HDL mg/dl	34.00	26.00	38.10	42.00	34.10	45.00	0.009
Creatinine mg/dl	1.01	0.80	1.36	1.16	0.80	2.00	NS
TLC × 10^3 /mm^3	6.78	4.30	11.45	7.50	4.60	11.70	NS
PLT × 10^3 /mm^3	173.00	125.00	232.50	97.00	62.00	198.00	0.001
Liver mass size/cm (largest diameter)	6.00	4.75	8.10	.	.	.	-----
Total monocytes %	5.72	4.07	8.06	6.00	4.66	7.50	NS
Classical monocytes %	4.05	2.86	5.74	4.85	3.21	6.15	NS
Intermediate monocytes %	1.03	0.66	1.61	0.60	0.40	0.96	0.001
Non-classical monocytes %	0.35	0.15	0.70	0.62	0.30	1.38	0.006

ALT, alanine aminotransferase; AST, aspartate aminotransferase, ALP, Alkaline phosphatase; AFP, alpha fetoprotein, BMI, Body mass index; GGT, gamma glutamyl transferase; LC, liver cirrhosis; HDL, high-density lipoprotein; PLT, platelet; TLC, total leukocytic count. *P* > 0.05 is not significant (NS).

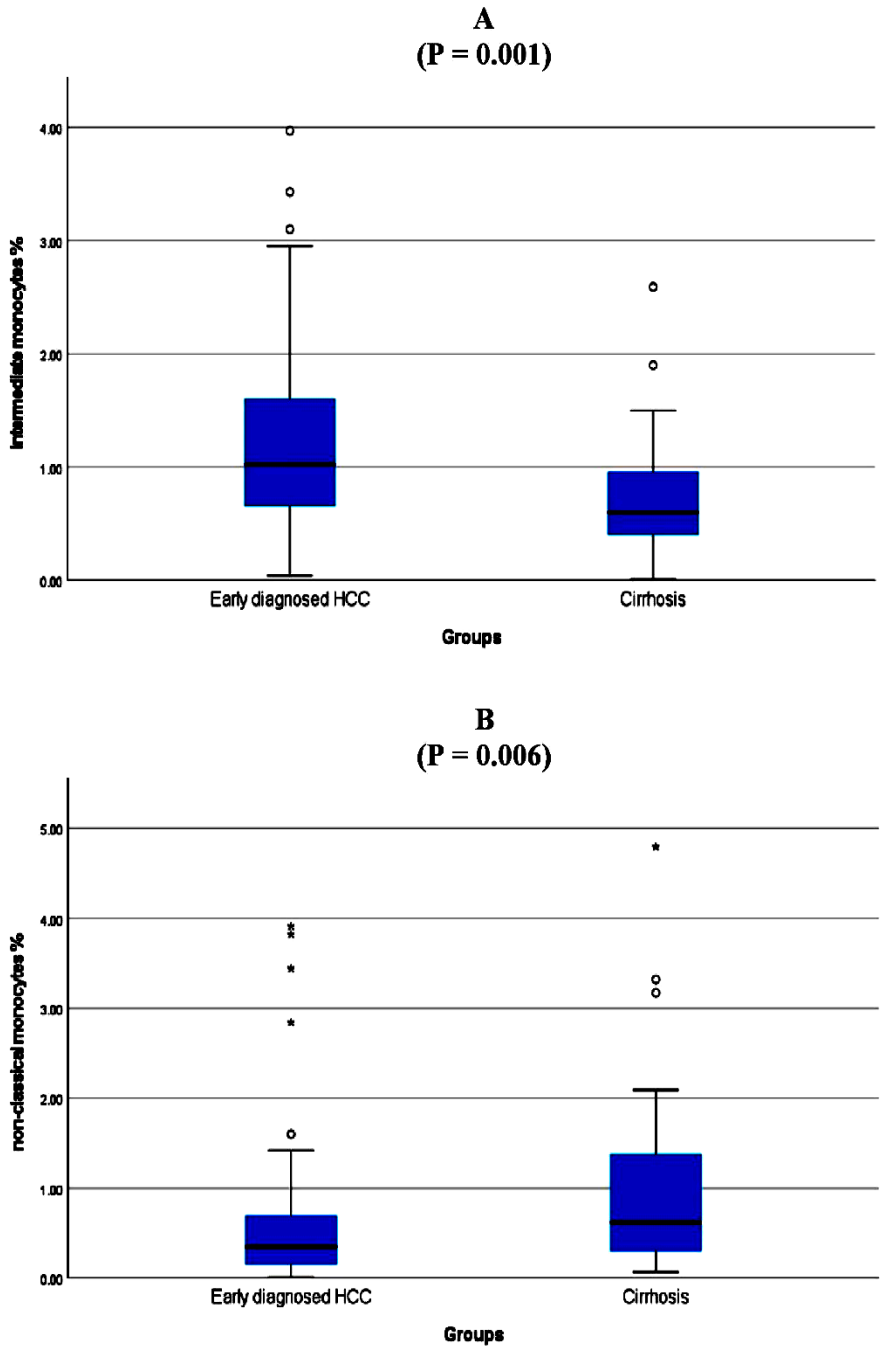

Figure 2. Comparison of intermediate and non-classical monocytes in the study groups. [A] There was a significant increase in intermediate monocytes in the HCC group when compared to liver cirrhosis group (*P*=0.001). [B] There was a significant decrease in non-classical monocytes in the HCC group when compared to liver cirrhosis group (*P*=0.006).

Regarding correlation studies in all 105 study participants, there was a significant positive correlation between the frequency of intermediate monocytes with BMI (r = 0.239, *P* < 0.014). There was a significant positive correlation between the frequency of intermediate monocytes with AST, cholesterol, and triglycerides (r = 0.242, *p* < 0.013; r = 0.296, *P < 0.002*; r = 0.247, *P* < 0.011, respectively). There were significant negative correlations between the frequency of non-classical monocytes and ALT and GGT (r = -0.316, *P* < 0.001; r = -0.226, *P < 0.02*1, respectively) as shown in Table 3.

Table 3. Correlation of total monocytes %, classical monocytes %, intermediate monocytes % and non-classical monocytes % with all study markers in all 105 study participants.

		Total monocytes %	Classical monocytes %	Intermediate monocytes %	non-classical monocytes %
BMI Kg/m2	Correlation Coefficient	-0.017	-0.055	0.239	0.158
	P value	NS	NS	0.014	NS
Albumin mg/dl	Correlation Coefficient	-0.034	0.025	0.061	0.128
	P value	NS	NS	NS	NS
AFP ng/ml	Correlation Coefficient	-0.012	-0.070	0.098	-0.186
	P value	NS	NS	NS	NS
AST U/L	Correlation Coefficient	0.152	0.063	0.242	-0.177
	P value	NS	NS	0.013	NS
ALT U/L	Correlation Coefficient	-0.062	-0.045	0.046	-0.316
	P value	NS	NS	NS	0.001
GGT IU/L	Correlation Coefficient	0.082	0.089	0.019	-0.226
	P value	NS	NS	NS	0.021
Cholesterol mg/dl	Correlation Coefficient	0.185	0.068	0.296	0.050
	P value	NS	NS	0.002	NS
Triglycerides mg/dl	Correlation Coefficient	0.089	0.028	0.247	-0.029
	P value	NS	NS	0.011	NS

ALT, alanine aminotransferase; ALP, Alkaline phosphatase; AFP, alpha fetoprotein, BMI, Body mass index; GGT, gamma glutamyl transferase; LC, liver cirrhosis. *P* > 0.05 is not significant (NS).

According to the ROC curve analysis, intermediate monocytes frequency, at a cutoff of ≥ 0.625 and an AUC of 0.698, can distinguish development of post-HCV HCC from post-HCV LC, with 76.4% sensitivity and 51.5% specificity (*P*< 0.001), while non-classical monocytes frequency had 69.4% sensitivity and 51.5 % specificity at a cutoff of < 0.61 and an AUC of 0.667. (*P*= 0.003) (Table 4, Figure 3).

Multivariate analysis was performed in the 105 study participants to test study markers' flow-cytometry data as predictors of HCC development as estimated in Table 5.

Table 4. Roc curve analysis for percentage of intermediate monocytes and non-classical monocytes as discriminators of post HCV-HCC development from post HCV-LC.

	Area Under the Curve	*P* value	95% Confidence Interval		Cut off	Sensitivity %	Specificity %
			Lower Bound	Upper Bound			
Intermediate monocytes frequency	0.698	< 0.001	0.594	0.803	0.625	76.4	51.5
Non-classical monocytes frequency	0.667	0.003	0.556	0.778	< 0.61	69.4	51.5

P > 0.05 is not significant (NS).

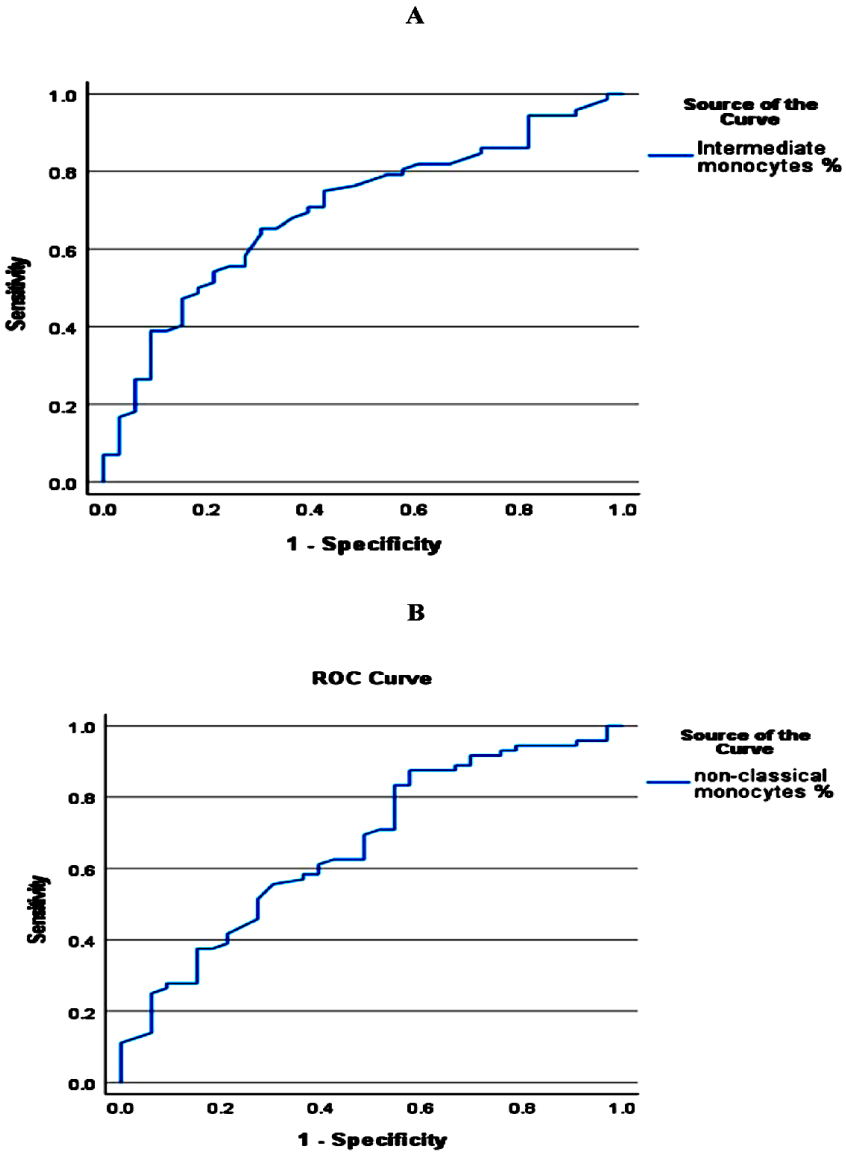

Figure 3. ROC curve analysis. [A] for discriminative power of intermediate monocytes between patients with post-HCV liver cirrhosis and HCC. [B] for discriminative power of non-classical monocytes between patients with post-HCV liver cirrhosis and HCC.

Table 5. Logistic regression analysis of percentage of total monocytes, classical monocytes intermediate monocytes, non-classical monocytes as predictors of post-HCV-HCC development after adjustment for confounders.

		P value	OR	95% C.I.	
				Lower	Upper
HCC	Intermediate monocytes %	NS	2.019	0.754	5.408
	Non-classical monocytes %	NS	0.655	0.360	1.191
	Age/ year	NS	1.050	0.996	1.107
	BMI Kg/m^2	NS	0.920	0.820	1.033
	Cholesterol mg/dl	NS	1.015	0.997	1.034
	Triglycerides mg/dl	0.031	1.014	1.001	1.026

BMI. Body mass index; CI, confidence intervals; HCC, hepatocellular carcinoma; OR, Odds ratios.
P > 0.05 is not significant (NS).

Discussion

Monocytes play a crucial role in controlling the onset and the spread of cancer. The extracellular matrix is remodeled, angiogenesis is encouraged, tumor-associated macrophages (TAMs) and dendritic cells (DC) are differentiated from monocyte subsets, and these actions all contribute to both pro- and antitumoral immunity in cancer.[11]

In the present study we focused on assessing whether altered frequency of monocyte subsets would contribute to post HCV-LC development to HCC. There was no difference found between the frequency of total monocytes and classical monocytes between HCC and LC group**.** Still, when compared to the LC group, the HCC group of patients had a significant increase in circulating intermediate monocytes, according to the current study. Similar findings were reported by Matsubara et al., 2013, who documented the upregulated frequency of circulating intermediate monocytes in HCC and added that this subset is characteristically expressing angiopoietin receptor Tie2.[12] Additionally, Atanasov et al., 2019 observed that these Tie2-expressing monocytes (TEMs) promote tumor growth, and that their infiltration into HCC has been linked to a more aggressive behavior of the tumor in patients with HCC.[13]

Other types of tumors have shown the same pattern of elevated intermediate monocytes. For instance, a study by Prat et al., 2020 showed that ovarian cancer patients had an increase in circulating intermediate monocytes that was linked to the presence of soluble immunosuppressive mediators.[14]

Angiopoietin receptor Tie2 transmits pro-angiogenic signals and identifies a subpopulation of monocytes with pro-angiogenic activity. These TEMs promote neoangiogenesis and oncogenesis are being specifically attracted to malignancies. It was reported that TEMs are a part of the CD16+monocyte subsets that trigger protumorigenic activity in solid tumors.[13]

The (CD14+CD16+) monocytes were shown to be active in the blood and liver of individuals with liver cirrhosis, according to a research study by Tacke et al., 2012 By producing particular cytokines, these cells encourage pro-inflammatory activity, which aids in the pathogenesis of liver tumors.[15]

It was shown that monocyte secreted interferon gamma (IFNγ), encourages an increase in the expression of the chemokine (C-X3-C motif) ligand 1 (CX3CL1) on blood vessel lumen, which imposes continuous crawling of intermediate monocytes and renders them insensitive to chemokines required for their recruitment, facilitates the recruitment of circulating intermediate monocytes inside solid tumors. Chemokine receptors (CX3CR1), CXCR4, chemokine (C-C motif) Receptor 1 (CCR1), CCR2, CCR5, and matrix metalloprotease 9 (MMP9) are expressed by proangiogenic monocytes.[16]

In the present study when compared to the LC group, the HCC group had a significantly lower level of circulating nonclassical monocytes (CD14-/dim CD16+). Non-classical monocytes have antitumoral features such as tumor cytotoxicity, metastasis prevention, engulfment of tumor material, recruitment of/correlation with NK cells, and suppression of regulatory T cells. Due to the loss of their antitumoral functional impact, this considerable drop in HCC can lead to the development of HCC.[17]

Due to nonclassical monocyte's ability to preserve vascular homeostasis, they were frequently thought to be anti-inflammatory. In the identification and removal of pathogens, nonclassical monocytes serve as the initial line of defense.[18]

Nonclassical patrolling monocytes (CX3CR1high CD14^{dim} CD16+) can actively patrol the vascular endothelium under homeostatic and inflammatory settings. Patrolling monocyte subsets have been linked to wound healing and the reduction of inflammation in injured tissues.[11] They work in a variety of disease conditions to remove debris and damaged cells from the vasculature.[19] Endothelial cells (ECs) express CX3CL1, which is necessary for the CX3CL1 receptor (CX3CR1)-dependent recruitment of immune cells such as CX3CR1 non-classical monocytes.[18]

Regarding correlation studies using data of all participants (n=105), there was a significant positive correlation between frequency of intermediate monocytes with BMI (r = 0.239, *P* < 0.014). This is consistent with Devêvre et al., 2015 observation that obesity was linked to intermediate and non-classical monocytes. And they noted that obese donors' circulating monocytes expressed more CX3CR1, indicating a higher chemotactic potential for adipocytes that secrete CX3CL1.[20] This may demonstrate how obesity could be considered as a chronic systemic inflammatory disease.[21]

There was a significant positive correlation between the frequency of intermediate monocytes with AST (r = 0.242, *P* < 0.013) which highlighted the inflammatory nature of this population mentioned before in many studies.[22-23]

Additionally, we observed a positive connection between the frequency of intermediate monocytes and cholesterol and triglycerides (r = 0.296, *P* = 0.002 and r = 0.247, *P* = 0.011, respectively). A study by Zhao et al., 2019 reported that hyperlipidemia is one of atherosclerosis' risk factors, which is the pathological cause of coronary artery disease (CAD).[24] Lipid deposition, plaque formation, and inflammation in vascular intima are the main symptoms of atherosclerosis. Lipid retention and oxidation in the sub-endothelial region are crucial for the development of atherosclerotic plaques.[25] This was consistent with Zawada et al., 2016 hypothesis that atherosclerosis risk factors, like dyslipidemia, were linked to changes in monocyte subsets with a shift toward the intermediate monocytes.[26] A study by Xiang et al., 2020 observed that low serum HDL may induce upregulation of CD16 on monocytes, which may then result in an increase of intermediate monocytes in atherosclerosis patients. They also found that patients with atherosclerosis had a large increase in intermediate monocytes.[27]

Another research study has connected the relationship between the monocyte subsets and blood lipid levels to the expression of the CD36 molecule on the surface of the cells rather than the changing frequency of the subsets in circulation. [9] The endothelial cells (EC), smooth muscle cells, and monocytes/macrophages readily take up oxidized low-density lipoprotein (ox-LDL) and other modified LDL particles through the scavenger receptor CD36, a class B scavenger receptor, and membrane glycoprotein. [28]

Non-classical monocyte frequency was significantly inversely correlated with both ALT and GGT (r = -0.316, *P*= 0.001; r = -0.226, *P*= 0.021, respectively), as demonstrated in (Table 3). Such observation may support the earlier suggestion of a study by Narasimhan et al., 2019 for the protective and anti-inflammatory activity of non-classical monocytes.[18]

According to the ROC curves, intermediate monocytes frequency, at a cutoff of ≥ 0.625 and an AUC of 0.698, was an accepted biomarker for distinguishing post-HCV HCC from LC, with 76.4% sensitivity and 51.5% specificity (*P* < 0.001). While non-classical monocytes frequency had 69.4% sensitivity and 51.5 % specificity at a cutoff of < 0.61 and an AUC of 0.667 (*P* = 0.003). According to their reasonable sensitivity and low specificity, both intermediate and non-classical monocytes percentage can be used as non-invasive screening biomarkers for post HCV-HCC development rather than being diagnostic markers. This could be of great help in low AFP secreting HCC.

In order to evaluate altered frequency of intermediate and non-classical monocytes as independent risk factors for HCC progression from post-HCV-LC, a logistic regression analysis was conducted with adjustment of cofounders, as shown in Table 5. The results showed that, neither could be considered as independent risk factor, which could be understood as tumor development is a multifactorial process that is not only dependent on a specific cellular element.

Nerveless, triglyceride level was shown to be an independent risk factor for HCC development [OR =1.014 (11.001–1.026), *P* = 0.031]. This was consistent with Ma et al., 2021 findings which showed the abnormal lipid metabolism in HCC and that lipid buildup worsened tumor growth by promoting the proliferative proliferation of cancer cells. Lipid gives tumor cells both energy and the phospholipid building blocks needed to create cell membranes. It was discovered that

HCC had significantly lower levels of medium-chain acyl-CoA dehydrogenase (ACADM), an enzyme that catalyzes the initial stage of mitochondrial fatty acid oxidation. Functionally, ACADM suppression increased triglyceride, and cellular lipid droplet levels while promoting HCC cell motility.[29]

The present study has certain limitations. The study did not assess the expression of angiogenic markers on the surface of monocytes. Also, it did not investigate the frequency of monocyte subsets in the liver tissue to evaluate the modulation of monocyte-subset recruitment into the liver and their subsequent differentiation to TAM which may represent promising approaches for therapeutic interventions in the future.

In conclusion, our study data indicated that post-HCV-HCC was characterized by an upregulation of intermediate monocytes and a downregulation of non-classical monocytes when compared to LC. Both intermediate and non-classical monocytes percentage can be used as non-invasive screening biomarkers for post HCV-HCC development rather than being diagnostic markers. The frequency of intermediate monocyte was linked to hyperlipemia. The level of triglycerides was an independent risk factor for the development of HCC.

Author Contributions

FA, processing of samples and aiding to the writing process. RH, conceptualization of the paper idea, performance of flow cytometry procedure and analysis, writing the paper and publishing correspondence. FMK, recruitment of liver cirrhosis patients and collection of their samples. RBA, recruitment of HCC patients and collection of HCC samples. MHA, conceptualization of the idea and supervising the whole process and revising the data.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) denies receipt of any financial support for the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine (Girls), Al-Azhar University, Cairo, Egypt (approval number: No. 1072 dated November 2021). In addition.

Informed consent

An informed consent form was obtained from all participants before enrollment in the study.

ORCID iD

Reham Hammad iD https://orcid.org/0000-0002-8967-9106.

References

1. Shen J, Shen H, Ke L, et al. (2022). Knowledge Mapping of Immunotherapy for Hepatocellular Carcinoma: A Bibliometric Study. *Front Immunol.;* 13:815575. doi: 10.3389/fimmu.2022.815575.
2. Yousef MH, El-Fawal HA., Abdelnaser A (2020). Hepigenetics: A Review of Epigenetic Modulators and Potential Therapies in Hepatocellular Carcinoma. *Biomed Res Int.* 2020: 9593254
3. Song T, Li L, Wu S, et al. (2021). Peripheral Blood Genetic Biomarkers for the Early Diagnosis of Hepatocellular Carcinoma. *Front Oncol.* 11: 583714.
4. Ali MAM, Harmsen WS, Morsy KH, et al. (2022). Prognostic utility of systemic inflammatory markers and chronic hepatitis C virus infection status in hepatocellular carcinoma patients treated with local ablation. *BMC Cancer.* 22(1):221. doi: 10.1186/s12885-021-09121-8.
5. Martín-Sierra C, Martins R, Coucelo M, et al. (2020). Elevated soluble TNF alpha levels and upregulated TNFalpha mRNA expression in purified peripheral blood monocyte subsets associated with high-grade hepatocellular carcinoma. *J Inflamm*; 17:14.
6. Gómez-Olarte S, Bolaños NI, Echeverry M, et al. (2019). Intermediate Monocytes and Cytokine Production Associated with Severe Forms of Chagas Disease. *Front Immunol*. 10:1671.

7. Coillard A, Segura E (2019). In vivo Differentiation of Human Monocytes. *Front Immunol;* 10:1907.
8. Martín-Sierra C, Martins R, Laranjeira P, et al. (2019). Functional Impairment of Circulating FcεRI⁺ Monocytes and Myeloid Dendritic Cells in Hepatocellular Carcinoma and Cholangiocarcinoma Patients. *Cytometry B Clin Cytom.* 96(6):490-495.
9. Mansour N, Abdel-Salam M, Hammad R, et al. (2021). Role of CD36 expression on circulating monocytes subsets in children on regular hemodialysis. *Egypt J Immunol*. 8(4):224-232.
10. The European Association for the Study of the Liver EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. (2018). *J. Hepatol*.; 69:182–236.
11. Olingy CE, Dinh HQ, Hedrick CC. Monocyte heterogeneity and functions in cancer. J Leukoc Biol. 2019;106 (2):309–322.
12. Matsubara T, Kanto T, Kuroda S, et al. (2013). TIE2-expressing monocytes as a diagnostic marker for hepatocellular carcinoma correlates with angiogenesis. *Hepatology.* 57(4):1416-25. doi: 10.1002/hep.25965.
13. Atanasov G, Dino K, Schierle K, et al. (2019). Angiogenic inflammation and formation of necrosis in the tumor microenvironment influence patient survival after radical surgery for de novo hepatocellular carcinoma in non-cirrhosis. *World J Surg Oncol.* 2019; 17(1):217. doi: 10.1186/s12957-019-1756-8.
14. Prat M, Le Naour A, Coulson K, et al. (2020). Circulating CD14high CD16low intermediate blood monocytes as a biomarker of ascites immune status and ovarian cancer progression. *J Immunother Cancer.* 8(1):e000472. doi: 10.1136/jitc-2019-000472.
15. Tacke F. (2012). Functional role of intrahepatic monocyte subsets for the progression of liver inflammation and liver fibrosis in vivo. Fibrogenesis *Tissue Repair.* 5S27. doi: 10.1186/1755-1536-5-S1-S27.
16. Sidibe A, Ropraz P, Jemelin S, et al. (2018). Angiogenic factor-driven inflammation promotes extravasation of human proangiogenic monocytes to tumours. *Nat Commun.* Jan 24;9(1):355. doi: 10.1038/s41467-017-02610-0.
17. Mattoscio D, Isopi E, Lamolinara A, et al. (2021). Resolvin D1 reduces cancer growth stimulating a protective neutrophil-dependent recruitment of anti-tumor monocytes. *J Exp Clin Cancer Res.* 40(1):129. doi: 10.1186/s13046-021-01937-3.
18. Narasimhan PB, Marcovecchio P, Hamers AAJ, et al. (2019). Nonclassical Monocytes in Health and Disease. *Annu Rev Immunol*. 37:439-456. doi: 10.1146/annurev-immunol-042617-053119.
19. Thomas G, Tacke R, Hedrick CC, et al. (2015). Nonclassical patrolling monocyte function in the vasculature. *Arterioscler Thromb Vasc Biol.* Jun; 35(6):1306-16. doi: 10.1161/ATVBAHA.114.304 650.
20. Devêvre EF, Renovato-Martins M, Clément K, et al. (2015). Profiling of the three circulating monocyte subpopulations in human obesity. *J Immunol.* 194:3917–23. 10.4049/jimmunol.1402 655
21. Rohm TV, Meier DT, Olefsky JM, et al. (2022). Inflammation in obesity, diabetes, and related disorders. Immunity. 55(1):31-55. doi: 10.1016/j.immuni.2021.12.013.
22. Zimmermann HW, Seidler S, Nattermann J, et al. (2010). Functional contribution of elevated circulating and hepatic non-classical CD14CD16 monocytes to inflammation and human liver fibrosis. *PLoS One.* Jun 10;5(6):e11049. doi: 10.1371/journal.pone.0011049.
23. Kapellos TS, Bonaguro L, Gemünd I, et al. (2019). Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. *Front Immunol.* Aug 30; 10:2035. doi: 10.3389/fimmu.2019.02035.
24. Zhao D, Liu J, Wang M, et al. (2019). Epidemiology of cardiovascular disease in China: current features and implications. *Nat Rev Cardiol.* 16(4):203–212.
25. Dasagrandhi D, Muthuswamy A, Swaminathan JK. (2022). Atherosclerosis: nexus of vascular dynamics and cellular cross talks. *Mol Cell Biochem.* Feb; 477(2):571-584. doi: 10.1007/ s11010-021-04307-x.
26. Zawada AM, Schneider JS, Michel AI, et al. (2016). DNA methylation profiling reveals differences in the 3 human monocyte subsets and identifies uremia to induce DNA methylation changes during differentiation. *Epigenetics*. 11(4):259–272
27. Xiang Y, Liang B, Zhang X, et al. (2020). Lower HDL-C levels are associated with higher expressions of CD16 on monocyte subsets in coronary atherosclerosis. *Int J Med Sci.* Aug 1;17(14):2171-2179. doi: 10.7150/ijms.47998.
28. Tian K, Xu Y, Sahebkar A, et al. (2020). CD36 in Atherosclerosis: Pathophysiological Mechanisms and Therapeutic Implications. *Curr Atheroscler Rep.* Aug 9;22(10):59. doi: 10.1007/s11883-020-00870-8.

29. Ma APY, Yeung CLS, Tey SK, et al. (2021). Suppression of ACADM-Mediated Fatty Acid Oxidation Promotes Hepatocellular Carcinoma via Aberrant CAV1/SREBP1 Signaling. *Cancer Res.* Jul 1;81(13):3679-3692. doi: 10.1158/0008-5472.CAN-20-3944.