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Assessment of FOXO4 Gene Expression and Serum Keap-1, Aldolase, IL-18, and IL-22 Levels in Patients with Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is a prevalent form of cancer worldwide, yet there is a scarcity of research on the serum levels of interleukins (ILs) in patients with CRC, with only a limited number of studies having been published to date. The present work aimed to investigate the contributions of selected blood molecules, namely IL-18 and IL-22, Kelch-like ECH associated protein-1 (Keap-1), aldolase (ALD), and forkhead O4 (FOXO4). The analysis involved 60 CRC patients and 30 healthy subjects who served as control. The serum levels of IL-22, IL-18, Keap-1, and ALD were performed by enzyme-linked immunosorbent assay (ELISA), while FOXO4 expression was measured by RT-qPCR. Serum levels of IL-18, IL-22, and Keap-1 were not statistically significant among patients with varying Dukes' stages, tumor sizes, histological grades, and different situations of distant metastasis of cancer cells ($P>0.05$). While ALD show a highly significant differences between patients and control group ($P<0.05$). The expression of *FOXO4* is down-regulated and was statistically different ($P<0.05$) in patients with CRC. These findings showed that levels of interleukins in the serum cannot be used as a marker for cancer. However, our results on the ALD enzyme showed a significant elevation in CRC patients in comparison with the healthy subjects, which may have future implications for confirming the incidence of colon cancer.

Keywords: Colorectal cancer, Keap-1, Aldolase, IL-18, IL-22 and FOXO4.

تقييم التعبير الجيني لجين FOXO4 ومستويات Keap-1 و Aldolase و IL-18 و IL-22 في مصل المرضى المصابين بسرطان القولون والمستقيم

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الخلاصة

سرطان القولون والمستقيم (CRC) هو شكل شائع من أشكال السرطان في جميع أنحاء العالم، ومع ذلك هناك ندرة في الأبحاث حول مستويات الإنترلوكينات (ILs) في مصل المرضى المصابين بسرطان القولون والمستقيم، مع وجود عدد محدود من الدراسات التي تم نشرها حتى الآن. يهدف العمل الحالي إلى التحقيق في مساهمات جزيئات الدم المحددة، وهي IL−18 وIL−22، و Kelch−like ECH associated protein−1 (Keap−1)، وaldolase (ALD)، وforkhead O4 (FOXO4). شمل التحليل 60 مريضًا بسرطان القولون والمستقيم و30 شخصًا سليمًا عملوا كمجموعة تحكم. تم إجراء مستويات IL−22 وIL−18 وKeap−1 وALD في المصل بواسطة اختبار الممتز المناعي المرتبط بالإنزيم (ELISA)، بينما تم قياس التعبير عن FOXO4 بواسطة RT−qPCR. لم تكن مستويات مصل IL−18 وIL−22 وKeap−1 ذات دلالة إحصائية بين المرضى الذين يعانون من مراحل مختلفة من المرض وأحجام الأورام، والدرجات النسيجية، وحالات مختلفة من النقائل البعيدة للخلايا السرطانية ($P>0.05$). بينما أظهرت ALD اختلافات كبيرة للغاية بين المرضى ومجموعة التحكم ($P<0.05$). تم تقليل التعبير عن FOXO4 وكان مختلفًا إحصائيًا ($P<0.05$) في المرضى المصابين بسرطان القولون والمستقيم. أظهرت هذه النتائج أن مستويات الإنترلوكين في المصل لا يمكن استخدامها كعلامة للسرطان. ومع ذلك، أظهرت نتائجنا على إنزيم ALD ارتفاعًا كبيرًا في مرضى سرطان القولون والمستقيم مقارنة بالأشخاص الأصحاء، مما قد يكون له آثار مستقبلية على تأكيد حدوث سرطان القولون والمستقيم.

1. Introduction

Globally, data consistently shows that colorectal cancer (CRC) has among the highest rates of new cancer diagnoses as well as the strongest links to cancer mortality in patients with various malignancy types [1]. Statistics from around the world frequently on leading causes of mortality among cancer patients introduce colon cancer to occupy the 2nd place. At the start of the 2020s, current millennium, numbers of people newly diagnosed with CRC or died from the disease exceeded 1.9 million and 930,000, respectively. With obvious differences in geographical distribution extrapolations expect an elevated burden from CRC that would reach 3.2 million newly-diagnosed cases each year by the fifth decade of the millennium [2]. The Iraqi ministry of health and environment reported in 2021 that CRC ranked third of the top ten cancers. The Iraqi population of CRC patients reached a number of 2493 persons (10.54%), with the percentage of males being 8.547 % and females being 5.75 % [3]. The recommended treatment of CRC involves antimetabolite (5-Fluorouracil: 5-FU) which, depending on the stage, might be prescribed in combination with oxaliplatin [4].

The physiological consequences of colorectal cancer (CRC) include chronic inflammation of the digestive tract tissues, which is exemplified by conditions such as Crohn's disease (CD) and ulcerative colitis [5]. NO conclusion has yet been reached in relation to the types of markers, whether tissue or blood that should be subjected to monitoring as the treatment proceeds. Reliable and potent prognostic molecules need to be identified to precisely monitor the disease as it progresses or suppressed in response to treatment protocols. One of these deeply involved molecules is aldolase A (A-2) (ALD) which actively participates in the glycolysis metabolic cycle, but also recognized as a tumor promoter during the regulation of the epithelium and mesenchyme tissue as they experience transition and the subsequent signaling pathways during the course of CRC [6, 7]. Another essential molecule in CRC

development is the Kelch-like-ECH associated protein-1(Keap-1), which associates with another molecule, namely cullin-3, to build the enzymatic complex of cullin-RING E3-ubiquitin ligase [8]. This enzyme manifests its impact on tumor regulation process through its role in the constitution of a signaling pathway that also involves Nrf2. The latter comprises an essential component in the regulation of redox homeostasis, as well as the promotion of cells to proliferate and survive during tumor development [9].

Upon immune cell activation, a notable and substantial response is elicited through the production of various low-molecular-mass glycoproteins, specifically cytokines, which play a crucial role in the immune response. These molecules are involved in the regulation of almost all types of responses by the immune cells, promoting them, for instance, to be active, migrate, proliferate, or die. In this manner, cytokines are actually involved in the main pro-inflammatory, anti-inflammatory, and homeostatic responses encompassing the innate and adaptive arms of the immune system. [10]. Among these cytokines, interleukin-18 (IL-18), previously known as IFNγ-inducing factor [11], is a 18.3 kDa protein that comprises 157 amino acids and encoded by a 1.1 kb gene, that is situated on chromosome 11 and contains 6 exogenous factors and 5 inclusions [12]. The production of this cytokine occurs by a variety of cells, including T, B, and glial cells, however, macrophages and osteoblasts, rendering it a significant pro-inflammatory protein [13]. Another important protein is IL-22, a cytokine that is physiologically involved in the promotion of processes that include wound healing and tissue repair. However, it has the unique function of acting on cells that do not belong to the hematopoietic lineage. Despite the belief of an impact of protecting the epithelial tissues, as far it is concerned with CRC, this protein was correlated with resistance to chemotherapy [14].

Transcription factors comprise another set of molecules with crucial roles in cellular signaling. Among these, the forkhead box (FOX) family comprises 19 subfamilies, the occurrence of which being extended from microorganisms up to humans. The FOX family includes the class *FOXO,* originally recognized as the Drosophila *FOXO* gene, which has the forkhead winged-helix domain of high level of conservation. Carcinogenesis is partially inhibited by *FOXO4*, in a process that involves the hindrance of the initiation of epithelial–mesenchymal transition (EMT). During the course of certain types of malignancies, e.g. gastric and lung cancers, the inhibition of EMT by FOXO is mediated by the reduction of vimentin levels while elevating those of Epithelial cadherin (E- cadherin) [15].

This commonly shared structural motif of *FOXO4* gene family comprises 110 amino acids, being essentially involved in DNA binding [16, 17]. FOXO takes important parts in a wide range of activities that lead various types of cells to proliferate, differentiate, resist stress, achieve metabolic reactions, or die. It is also implicated in conditions beyond malignancies, such as diabetes mellitus. The inhibition of this protein typically takes place via the PI3K/Akt signaling pathway, preventing *FOXO* from translocating into cellular nuclei. It is also involved in the regulation of transcriptional responses that occur in an independent manner from direct DNA binding, through the joint actions of various transcription factors [18]. The present study aims to evaluate the differences in the concentrations of IL-18, IL-22, KEAP-1, and aldolase in the sera of CRC patients in comparison with those in healthy subjects. Possible involvement of *FOXO4* gene expression, tested by means of RT-qPCR, was also addressed.

2. Materials and Methods

2.1. Patients and healthy subjects

The current study was performed on 60 CRC patients (25-85 years) and 30 healthy subjects (31-82 years) who served as a control group. Exclusion criteria included diabetes,

thyroid dysfunction, and any other type of cancer, as well as children. Patients and healthy subjects were males and females who all provided a written consent. The Ethics Committee at the Division of Biotechnology (Department of Applied Sciences, University of Technology) and the Baghdad Medical City Complex / Oncology Teaching Hospital (Iraqi Ministry of Health and Environment) approved the study protocol (Ref. No. 51895, Dec 7, 2022). The disease diagnoses were made by medical specialists. Treatment history stated that fifty-four patients were treated with 5-fluorouracil (5fu; Onko, Turkey), while six cases were non-treated. Demographical and clinical characteristics, such as family history, body mass index (BMI), duration of disease, gender, tumor site, grade, and stage were obtained from a questionnaire sheet prepared previously.

2.2. Preparation of serum samples and ELISA detection

Radial vein blood (5 ml) was sampled from all participants with disposable syringes in a tube and subjected to centrifugation (3000 rpm, 10 min). Storage of the separated sera was achieved at -20 °C until being analysed. Freezing and thawing cycles were avoided. The parameters of Keap-1 (Cat. No. SL3379Hu), aldolase (Cat. No. SL0085Hu), and cytokines, namely IL-18 (Cat. No. SL0980Hu) and interleukin-22 (Cat No. SL0988Hu), were assessed by using Enzyme-Linked Immunosorbent Assay according to manufacturer instructions[19]. All kits in our current study were purchased from Sunlong, China.

2.3. Gene expression

To determine the gene expression of the *FOXO4* gene, total RNA was isolated from blood samples. For the extraction of RNA with Trizol, a *TransZol Up* Plus RNA Kit was used, and for cDNA the kit of *EasyScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix was employed. The sequences of the primers used were as follows: the forward primer was 5'-TTGAGGCCAGAGTCTGAGGT-3' and the reverse primer was 5'-CTCCGAGATAGCAGGGAATG-3'. For the housekeeping gene GAPDH (reference gene glyceraldehyde-3-phosphat dehydrogenase) the sequences were GAAATCCCATCACCATCTTCCAGG for the forward and GAGCCCCAGCCTTCTCCAT for the reverse primers.

A volume of 20 µl was adopted for the reaction, comprising 10 µl master mix, 1 µl RT mix, 1 µl random primer, 1 µl oligo dt, 3 µl RNA_{ase} free water, and 7 µl RNA. PCR cycling conditions were set; cDNA synthesis at thermal cycler steps included three stages, the first at 25 ºC for 10 min, the second at 42 ºC for 15 min, and finally, to inactivate the enzyme, at 85 ºC for 5 sec. Gene expression experiments were achieved in a reaction volume of 20 pl. Volumes of reaction components comprised 10 µl of Master mix Syper Green, 3µl of CDNA, 1µl of forward primer, 1µl reverse primer, and 5 µl of nuclease-free water. The stages and temperatures being involved are explained as follows; Stage 1, i.e. the denaturation, was performed in a temperature of 94 ºC for 60 sec. Stage 2 the denaturation was at 94 ºC for 5 sec, the annealing was at 58 ºC for 15 sec, and the extension was at 72 ºC for 20 sec. Stage 3, the dissociation was at 65-95 ºC for 1 sec. Protocols provided with the kits were followed in each of the respective tests.

2.4. Statistical Analysis

Data analysis was achieved by means of the specialized SPSS-28 software (Statistical Packages for Social Sciences- version 28), however, their presentation was performed in the form of the parameters of frequency, percentage, mean, standard deviation (SD), and range (minimum-maximum values). Significant differences (where $P<0.05$) shown by independent means were realized by means of Students-t-test [20]. Whereas those involving more than two independent means were realized by means of ANOVA test. Cutoff and area under the

curve (AUC) values were extracted by means of the receiving operating characteristic (ROC) curve.

3. Results and discussion

3.1. Baseline data and clinical factors

Clinical and disease attributes (gender, age, duration of disease, BMI, and family history) of CRC patients and their healthy counterparts are listed in Table 1. The values of the mean age and the SD for both patients and healthy participants are as follows; the mean age in the patient was (58.1± 13.1 years) and the control group was (59.7 ± 11.2 years), with non-significant differences being observed. The percentage of the group whose ages ranged from 50 to 59 years were the highest, and this is an indication that the risk of colon cancer increases with age, in addition to other factors that may sometimes cause cancer [21].

The distribution of groups according to gender revealed an approximately matched ratio between females and males in both groups. Males comprised 53.3% of the healthy control group, compared to 58.3% of the patient group. The percentage of females in the control group was 46.7%, while that in the patients was 41.7%, exerting insignificant differences, with a p-value of 0.652. Regarding disease duration, the mean value was (8.1 ± 8.3 months) in the patients, which were divided into three subgroups (<6 months, 6-11 months, =>12 months). The mean of BMI (kg/m^2) showed no significant difference between patients and control (P-value > 0.05). Both healthy subjects and CRC patients were in the range of (25.83-26.19) Kg/m^2, which is considered as overweight. The BMI is a measure of human body fat based on height and weight. A person's weight is calculated in kilograms or pounds and divided it by the square of height in meters or inch. Hence, its unit is kg/m^2 or lb/inch2 [22]. An ideal BMI lies within the range of ($18.5 \leq BMI \leq 24.9$). Also BMI provides information about nutritional status in adults [23].

In the present study, the patients were divided, based on the family history with cancer, as patients who had a family history (22, 36.7%) and those with no family history (38, 63.3%). These results reveal a higher number of patients with no family history than those who had family history. Earlier findings suggested that the majority of CRCs are sporadic and non-inherited [24], suggesting that our patients are influenced more by factors other than family history, probably involving environmental conditions. Previous findings also described an association between about 20% of colon cancer cases and familial clustering, with higher risk of developing CRC among first-degree relatives of colorectal adenomas patients or those diagnosed with invasive CRC [25].

Figure 1 shows the percentage distribution of tumor stages and grades in the patients groups. The present study revealed that the patient distribution across the four stages was as follows: stage one, 3 patients (5.0%); stage two, 11 patients (18.3%); stage three, 24 patients (40.0%); and stage four, 22 patients (36.7%). Despite clinical developments, about 50% and 95% die by this disease in stage III and stage IV of CRC, respectively [26]. We also assessed the cancerous cell grades among our patient's cohort, which was classified into 3 levels (from I to III). The most frequent was grade II (44, 73.3%), while grade 1 had 4 patients (6.7%) and grade III had 12 patients (20.0%), as shown in Figure 1. These results are similar to those previously published by another group, who found that grade II was the most frequent grade II (914, 73.8%), followed by grade III (216, 17.4%), and grade I (55, 4.4%) [27].

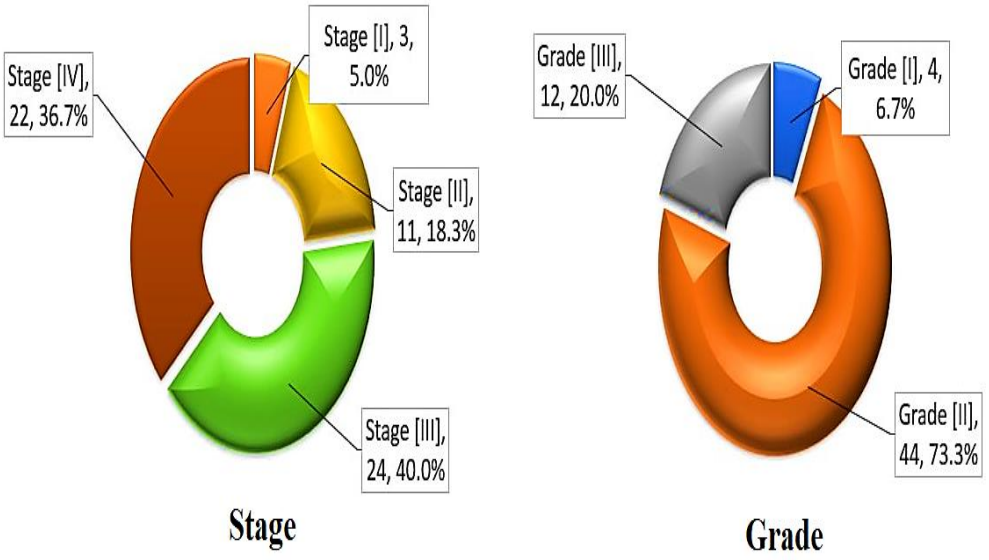

Figure 1: Characteristics of CRC patients with percentage of cases based on stage and grade

Table 1: The clinical and laboratory characteristics of colorectal cancer patients and healthy subjects

Demographic characteristics	Group		P- Value
	Patients no. (%) n=60	Control no. (%) n=30	
Age (Years)			
Mean ± SD	58.1 ± 13.1	59.7 ± 11.2	
< 50	13 (21.7)	3 (10.0)	**0.429**
50 – 59	20 (33.3)	9 (30.0)	
60 – 69	17 (28.3)	10 (33.3)	
≥70	10 (16.7)	8 (26.7)	
Gender			
Male	35 (58.3)	16 (53.3)	**0.625**
Female	25 (41.7)	14 (46.7)	
Duration of disease (Years)			
<6 months	32 (53.3)	-	-
6- 11 months	16(26.7)	-	
≥ 12 months	12 (20.0)	-	
Mean ± SD (8.1 ± 8.3)			
BMI (kg/m^2) Underweight (<18.5) Normal (18.5-24.9) Overweight (25-29.9) Obese (=>30)	25.83 ± 5.73	26.19 ± 3.37	**0.574**
Family History of Cancer			
Have family history	22 (36.7)	-	-
Without family history	38 (63.7)	-	

3.2. IL-18 and IL-22 levels in CRC patients and healthy subjects

As illustrated in Figure 2A, there were only minor variations in IL-18 levels between the patient group and the healthy control group, with values of 38.62±9.83 pg/ml and 39.57±18.97 pg/ml, respectively (p-value=0.755). This finding might have resulted from low sample size. Another cause could be that the patients involved in the present study were with different stages of tumor progression that could be differentially affected by the antitumor activity of IL-18. This led to the increased serum level of IL-18 in early stages, while in stages III and IV, the level was gradually decreased reaching equilibrium with the control group.

As shown in **Figure 2B,** disease duration was distributed as < 6 months (39.05±7.775) and >6 months (37.82±13.00 pg/ml)**.** The current results showed no significant differences (p value 0.647) between these two groups. The cases were also distributed regarding tumor stage into three groups. In Iraq specifically, the number of Stage I CRC cases is very low due to the lack of early screening programs. Therefore, stage I and stage II were combined into one group. As seen in **Figure 2C,** IL-18 levels in patients with stages I and II (42.91±14.24 pg/ml) were higher in comparison with those in stages III (38.21±8.41pg/ml) and IV (36.34±7.096 pg/ml). Higher level was also recorded in stage II patients in comparison with those in stage IV, with insignificant variations (p-value >0.05).

As shown in **Figure 2D**, IL-18 level in patients with grade1 (39.109±10.429 pg/ml) was higher than that in patients with grades II (38.775±5.845 pg/ml) and III (36.564±8.668 pg/ml), but these increments were not statically significant (p-value >0.05). In the present study, the participants were also classified into two groups based on treatment, where treated and non-treated patients revealing non-significant variations in IL-18 levels at p value > 0.05 **(Figure 2E).**

Table 2 describes insignificant variations in IL-18 level in patients with CRC group when compared according to age, gender, BMI, family history and chronic illness. An earlier study also revealed that IL-18 level was insignificantly associated with age, gender, and BMI [28]. IL-18 serum levels in patients with tumor cells located in the rectum site were higher than those in patients with other sites of colon involvement, but these increments were not statically significant. Patients with rectum and sigmoid tumors more frequently than at other sites. In rectum, IL-18 were (6%) (47.317±19.038 pg/ml), small intestine (2%) (40.600±12.304 pg/ml), cecum (3%) (40.533±4.325 pg/m), ascending colon (4%) (34.100±8.540pg/ml), transverse colon (13%) (38.331±6.650 pg/ml), descending colon (6%) (41.050±12.287 pg/ml), sigmoid (14%) (35.914±7.443 pg/ml), rectosigmoid (10%) (38.050±8.706pg/ml), anus (2%) (33.100±5.657 pg/ml). IL-18 level in non-treated patients was 43.617±7.813 pg/ml and after the first dose of chemotherapy, it was decreased to 37.333±5.238 pg/ml, while in the patient who received >6 doses of chemotherapy, it was 39.425±10.824pg/ml.

These findings agree with those of a previous study which showed that a significantly increased occurrence of rectum and sigmoid tumors (77.8%) in comparison with those located in other sites of the colon [29]. The findings proposed a possible induction of IL-18 to be produced as a reaction to direct influence of tumor tissue or indirect influences from relevant events. While another study about CRC patients in the Iranian population indicated an elevation in serum IL-18 concentration in CRC patients. IL-18 represents a highly essential cytokine due to its roles during CRC development. This molecule acts in synergy with IL-12, leading to the induction of IFN-γ to be produced and type I T cell response to be favored [30].

The similar findings related to the association of IL-18 with CRC were observed previously by Han and his coworkers, who indicated that IL-18 level is not affected by clinical histopathology grades [21]. IL-18 significant contribution to anti-tumor defense mechanisms

was documented [31]. A previous study performed on gastric cancer patients found that IL-18 level was also not significantly changed in patients versus controls [32]. Other findings regarding IL-18 expression in cancer demonstrated that IL-18 was significantly lower in colon cancer tissues than in normal tissues, with this downregulated expression being happened on the early stage of the disease. Their results suggested that IL-18 might function as a suppressing molecule to colon cancer via the inhibition of their proliferation. Such results mark the possibility to utilize IL-18 as a prognostic marker in colon cancer [33].

IL-22 Interleukin is a molecule that belongs to IL-10 family of cytokines. This protein exerts different activities during immune responses. As implied by the name, IL-22 secretion occurs predominantly by Th22 cells, a novel subtype of T helper cells [34]. The results of the present work demonstrate that the mean value of IL-22 levels in the patients was (49.42±11.44pg/ml), whereas in the control group, it was (48.09±12.27pg/ml) with a p-value of 0.612, showing no significant variations **(Figure 3A).** In terms of disease duration, the patients were distributed as groups with < 6 months (46.38± 8.641pg/ml) and with > 6 months (55.06±13.88pg/ml), with IL-22 level being significantly higher in the latter group **(Figure 3B).** The results indicated that IL-22 levels were higher in patients diagnosed during <6 months compared to those diagnosed within >6 months. This finding may be one of the factors that contributed to the non-significant IL-22 sera levels in both the patient and control groups.

Considering that the level of IL-22 increases in people diagnosed with cancer over a long period. IL-22 was shown to play a critical part in the progression of inflammatory diseases, but its biological function in carcinogenesis remains obscure [35]. Our results suggest that the increment in IL-22 level in patients who were diagnosed with more than 6 months of disease may be due to its role in tumor progression. In regard to CRC stages, IL-22 levels showed a highly significant increase in patients with stage I, II (56.11±16.60pg/ml). This level decreased gradually in stage III (48.58±8.83 pg/ml) and stage IV (46.07 ±8.32pg/ml) with a p value of (0.030) (**Figure 3C**). Regarding tumor grade, the mean level of IL-22 was (50.07±10.53 pg/ml) in grade I, (50.50±12.33pg/ml) in grade II, and (45.26±7.34 pg/ml) in grade III patients, with a p value of 0.614, also showing non-significant differences **(Figure 3D)**. In the present work, the cases were also distributed as two groups of patients who received chemotherapy and patients without chemotherapy. The results revealed that IL-22 serum levels were significant between the control group and the untreated patients (p value < 0.05), as well as between the treated and un-treated patients (p value < 0.05) **(Figure 3E)**.

IL-22 level did not show significant variations in patients with regard to age, BMI, family history, medical history, tumor site, and gender as presented in **(Table2)**. IL-22 level in the patients at the period of pre-treatment was (54.33±11.28pg/ml), which was higher than that in the patients under treatment. The level after the second dose was (49.622±10.253 pg/ml), after third dose was (53.06±14.74 pg/ml), and after the fifth dose was (48.886±10.500 pg/ml). In patients who took more than six doses of chemotherapy, the mean level of IL-22 was (48.838±11.079 pg/ml), while in those with more than 12 doses it was (44.87±10.65pg/ml).

A previous study on CRC reported similar findings regarding IL-22 levels, indicating no significant differences based on the clinical characteristics of the study subjects [36]. Current findings showed that the level of IL-22 after treatment was decreased. It was shown by an *in vitro* analysis that a dose of 5-FU chemotherapy, even if it is non-toxic, was found to cause the induction of immunogenic alterations in CRC cells [37]. Accordingly, the present work performed an evaluation of the impacts of the immunological alterations that appeared in the peripheral blood following the treatment with 5-FU of the therapy in CRC patients. Other findings related to the role of the IL-10 family in CRC were similar to those of the current study, revealing insignificant differences between the expression of IL-10 in CRC patients and the control group [38].

The current study also revealed that IL-22 level was lower in the advanced stages of the disease. Our results are in line with those reported by another group which demonstrated significantly reduced levels of IL-17A and IL-22 mRNA transcripts in tissues with advanced tumor in comparison with those in non-tumor tissues [39]. Thus, elimination of cells that produce these two cytokines might have occurred in the advanced stages of CRC An elevation in IL-22 level was reported in CRC patients following surgical resection of the tumor, i.e. in the earlier stages of the disease, which are similar results to those found in the present study [39]. However, other reports proposed the promotion of cell survival and proliferation by the action of IL-22 and through the stimulation of STAT3, supporting the notion that this cytokine is involved in the pathogenesis of different types of cancer. Like the other members of the IL-20 subfamily, IL-22 is not independently involved in carcinogenesis. It has a role in the promotion of tumor in nascent tumors of epithelial origin, including lung, liver, pancreatic, colon and gastric cancers. All these types of malignancies have cells that produce IL-22, as well as IL-22R1^{+} cancer cells that proliferate in response to IL-22. This cytokine does not only promote cells to survive and proliferate, but is also involved in the induction of cell mobility, angiogenesis, and tissue dysplasia [40]. Previous findings suggested that the instability recorded in IL-22 levels may be associated with tumorigenesis and tumor progression in CRC and liver cancer, with notable effects of chemotherapy on these levels in CRC patients [41, 42]. However, the levels of IL-22 were not statistically significant, possibly due to low sample size. IL-22 is also produced by Th17 cells [43], and studies have revealed that the 5fu chemotherapy can suppress Th17 activity [44]. Since most of the patients in this study were treated with 5fu, the lack of a statistical difference in IL-22 levels between patients and healthy controls could be explained by the effects of this treatment.

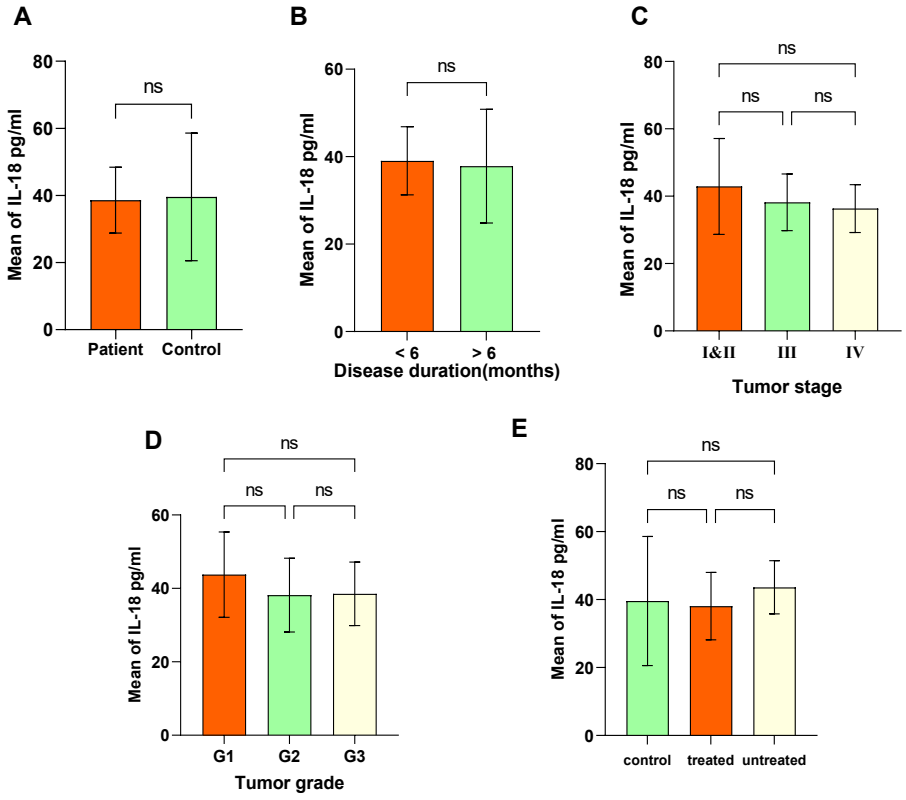

Figure 2: (A) Comparison between patient and control groups in IL-18 level. **(B)** IL-18 level with disease duration. **(C)** IL-18 level with tumor stage. **(D)** IL-18 level with tumor grade. (**E**) IL-18 levels with treated, untreated, and control groups.

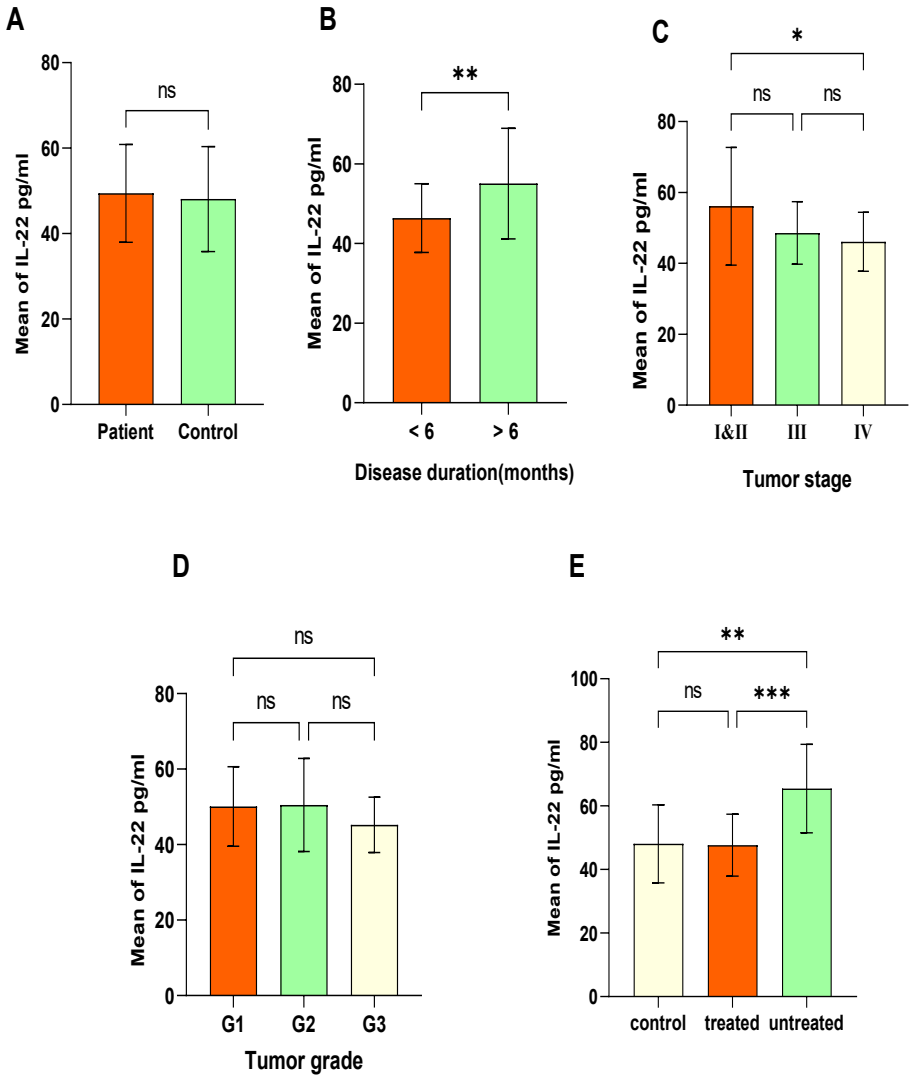

Figure 3: (A) Comparison between patient and control groups in IL-22 level. (B) IL-22 level with disease duration. (C) IL-22 level with tumor stages. (D) IL-22 with tumor grade. (E) IL-22 with treated un treated and control

Table2: Variations between patient subgroups in IL-18 and IL-22 levels.

Colorectal carcinoma			IL-18 (pg/mL)	P value	IL-22 (pg/mL)	P value
		No	Mean±SD		Mean±SD	
Age (years)	<50years	13	41.085±14.845	0.745	49.692±15.863	0.742
	50---59	20	38.485±10.446		48.170±11.002	
	60---69	17	38.053±6.976		48.600±10.612	
	=>70years	10	36.650±2.932		52.960±6.887	
Gender	Male	35	38.711±11.216	0.935	50.489±12.379	0.399
	Female	25	38.492±7.690		47.924±10.023	
BMI (Kg/m^2)	Underweight (<18.5)	5	36.580±6.156	0.690	47.340±6.529	0.588
	Normal (18.5-24.9)	22	40.173±11.886		50.577±13.543	
	Overweight (25-29.9)	21	38.862±8.992		50.829±11.196	
	Obese (=>30)	12	36.200±8.616		45.700±9.190	
Smoking	Current smoker	8	38.538±11.263	0.766	48.300±12.557	0.813
	Ex-smoker	8	41.012±15.110		51.762±18.377	
	Not	44	38.200±8.569		49.198±9.874	
Past medical history	Yes	23	39.452±8.799	0.612	52.091±12.031	0.155
	No	37	38.103±10.499		47.759±10.886	
Family history of cancer	Yes	22	40.068±10.620	0.391	51.364±12.244	0.321
	No	38	37.782±9.380		48.295±10.951	
Colon site	Appendix/Small intestine	2	40.600±12.304	0.679	53.150±7.425	0.882
	Cecum	3	40.533±4.325		43.567±4.717	
	Ascending colon	4	34.100±8.540		50.175±8.484	
	Transverse colon	13	38.331±6.650		49.585±9.091	
	Descending colon	6	41.050±12.287		50.000±14.880	
	Sigmoid	14	35.914±7.443		51.700±10.645	
Rectal site	Recto-sigmoid	10	38.050±8.706	0.294	45.530±10.984	0.581
	Rectum	6	47.317±19.038		52.900±21.064	
	Anus	2	33.100±5.657		43.200±3.960	
Number of doses	No	6	43.617±7.813	0.804	54.333±11.282	0.553
	1---2	9	37.333±5.238		49.622±10.253	
	3---4	10	38.800±15.307		53.060±14.740	
	5---6	7	36.900±8.120		48.886±10.500	
	>6	16	39.425±10.824		48.838±11.079	
	Long	12	36.867±7.848		44.867±10.648	

3.3 Assessment of Keap-1 and aldolase levels in patients and control groups

Figure 4A shows that keap-1 levels were higher in the CRC patients (132.9 ± 39.87 pg/ml) than the healthy control group (119.7± 55.83 pg/ml)**,** but this variation was not statistically significant (p value = 0.099). The same results were recorded by Chang *et al.* who also found that Keap-1 levels were higher in the patients than the control group, with the differences being also statically non-significant [45]. Keap-1 expression was significantly elevated in CRC patients according to another prior study [46]. Regarding disease duration, patients were distributed into the < 6 months subgroup, in which Keap-1 level was

131.1±44.00 pg/ml) and the > 6 months group (136.2±31.52 pg/ml), with a p value= 0.662), as shown in **Figure 4B.**

Regarding CRC stage, keap-1 level in the combined Stages I and II was 139.9±12.96 pg/ml, while in stage III it was 140.3±38.68 pg/ml and in stage IV it was 120.3±37.76pg/ml (p-vale =0.181) **(Figure 4C).** As related to the grades of cancer cell, the level of keap-1 in grade I patients was (136.9±64.88 pg/ml), while in grade II patients it was (136.2±39.59 pg/ml) and in grade III patients it was (119.3±31.62 pg/ml), with a p-value = 0.425 **(Figure 4D).** Regarding the treatment, the patients were classified into the pre-treatment and post-treatment subgroups, where keap-1 level showed no statistically significant differences when compared with each other and with the control group **(Figure 4E).**

As shown in Table 3, the results demonstrate that keap-1 has no relation with the clinical characteristic of patients, including the age, BMI, gender, family history, smoking status, medical history, and number of doses. According to available reports, there is a significant involvement for oxidative stress in the course of CRC [47]. An analysis of different kinds of malignancies revealed an extensive variability in the rate of *Keap-1* somatic mutations occurring within the Nrf2 interacting domain. The most frequent mutations were recorded in patients with gallbladder cancer (30.7%), followed by ovarian clear cell carcinoma (29%), and NSCLC (19%). Keap-1 is a crucial regulatory protein of Nrf2, with mutations in this gene being reported in a variety of malignancies, including breast cancer. Reduced mutation rates were reported in cases of endometrioid endometrial tumors (8.5%), non-clear cell ovarian cancer (8%), hepatocellular carcinoma (8.9%), colorectal cancer (7.8%), biliary duct carcinomas (5%), breast cancer (2%), prostate cancer (1.3%) and gastric cancer (1%) [48]. In these patients, the typical activities of the Keap-1 protein were lost, e.g. Nrf2 was not inhibited and its translocation to the nuclei was not blocked. As a consequence, these molecule accumulated in the nuclei and caused tumor-promoting impacts [49].

A previous study found that when Keap-1 is upregulated, tumor's ability to develop, progress, and invade other tissues is inhibited. Similar reports demonstrated a significantly reduced level of Keap-1 in osteosarcoma as compared to normal bone tissue. Other reports found no correlation between Keap-1 level and certain clinical and pathological characteristics, including gender and age [50].

The Nrf2/ Keap-1 pathway is typically involved in the protection from oxidative stress and inflammation. This pathway also plays a fundamental role in regulating the responses of cells aiming at avoiding stress causes, whether endogenous or exogenous, brought by ROS and electrophiles [51]. Oxidative stress drives Nrf2 and Keap-1 molecules to uncouple, leading the free Nrf2 protein to translocate into the nuclei. Following this, the molecule binds to the macrophage activating factor (Maf), resulting in the creation of heterodimers. These dimers also experience binding to antioxidant response elements (AREs), causing the initiation of processes during which the targeted genes are transcribed and expressed. These genes can include intracellular oxidoreductase genes, phase 2 detoxification genes, and transporter proteins encoding genes [50]. Our findings align with certain studies that have similarly documented non-significant correlations, yet diverge from those that have identified Keap-1 as a significant variable. This inconsistency highlights the intricate nature of oncology and emphasizes the necessity for additional investigations to elucidate the role of Keap-1.

The results also indicated that ALD level was highly significantly higher among those with CRC (200.3±87.4 pg/ml) than healthy control group (153.41±123.8 pg/ml). This increment was statistically significant (p value <0.05**) (Figure 5A).** Aldolase level in patients diagnosed within < 6 months was higher (201.3± 62.87 pg/ml) than that in patients diagnosed within > 6 months (198.5± 121.8 pg/ml) **(Figure 5B).** The variation in ALD level was not statically significant, which might be due to small number of patients in the subgroups. Such findings

confirm that the patients could be predicted for poorer prognosis when the expression of ALD is high [52, 53]. Recorded ALD levels in patients in stage I and II was (236.7±138.0 pg/ml), stage III was (190.0±64.40 pg/ml), and stage IV was (188.5±61.70pg/ml) **(Figure 5C).** Aldolase level also showed decrement with increasing grade of colorectal tumor. The level in grade I patients was 229.0±68.80 pg/ml, grade II was 203.7±94.42 pg/ml, and grade III was 178.6±60.26 pg/ml **(Figure 5D).** Regarding the treatment parameter, the ALD level in the treated patients was (189.5±63.41 pg/ml), which was lower than that in the untreated patients (297.8±184.9 pg/ml) and the control group (153.4± 123.8 pg/ml) **(Figure 5E).**

Furthermore, an association was described between elevated ALD level and poor prognosis of CRC patient. Such findings are also shared by the present study. ALD has the capability of promoting tumor growth via the regulation of the glycolytic pathway [46]. ALD, functioning as a crucial catalytic enzyme within the glycolytic pathway, plays a pivotal role in cancer progression via various mechanisms within the tumor microenvironment. Studies have indicated that ALD promotes cancer cell growth and spread by hastening the process of glycolysis [54]**.**

Table 3 shows that when patients were divided into subgroups, ALD shows no significant differences. As related to the baseline characteristics of our study groups, ALD showed a non-significant association with age, gender, smoking, BMI, family history, medical history and tumor site. Our aforementioned results suggest that the decreased ALD levels might be due to their active contribution in regulating cell shape and mobility. ALD has essential contributions in various physiological functions, including assisting striated muscle to contract, actin filament to be organized. Furthermore, it is among the glycolytic enzymes with the highest abundance in malignant cells [52]. The level of this enzyme could be elevated in various conditions, ranging from infectious diseases to cancer. However, the link between ALD and metabolism in tumorigenesis pathways is still unknown [55]. Upregulation of ALD was described in a multiplicity of tumor types, suggesting a contribution to the re-programming of metabolism described in tumor tissues [56-60].

The decrease in ALD level post-treatment is due to the 5fu mechanism of action because ALD plays an important role in catalyzing the metabolic processes of cells [61]. 5fu was reported to work as an anti-metabolic agent [62]. These results suggest that the ALD level is affected by 5fu chemotherapy. Such findings are similar to what we found in the current study. Previous studies demonstrated the high expression of ALD malignancies of bladder, breast, kidney, and liver, as well as in lymphoma, myeloma, and pancreatic cancer. However, lower expression was described in malignancies of the central nervous system and esophagus, as well as leukemia [63].

An association was reported between the alterations in morphology caused by ALD and the inhibition of the expression of various adherents and tight junction proteins, such as E-cadherin, β-catenin, and JAM-A. Such association was also described throughout the EMT process. When ALD is over-expressed, the expression of HIF-1α is induced. The latter molecule is recognized for its role in regulating EMT-associated transcription factors, e.g. Twist, Snail, Slug, Smad interacting protein 1 (Sip1), and zinc finger E-box-binding homeo-box 1 [64].

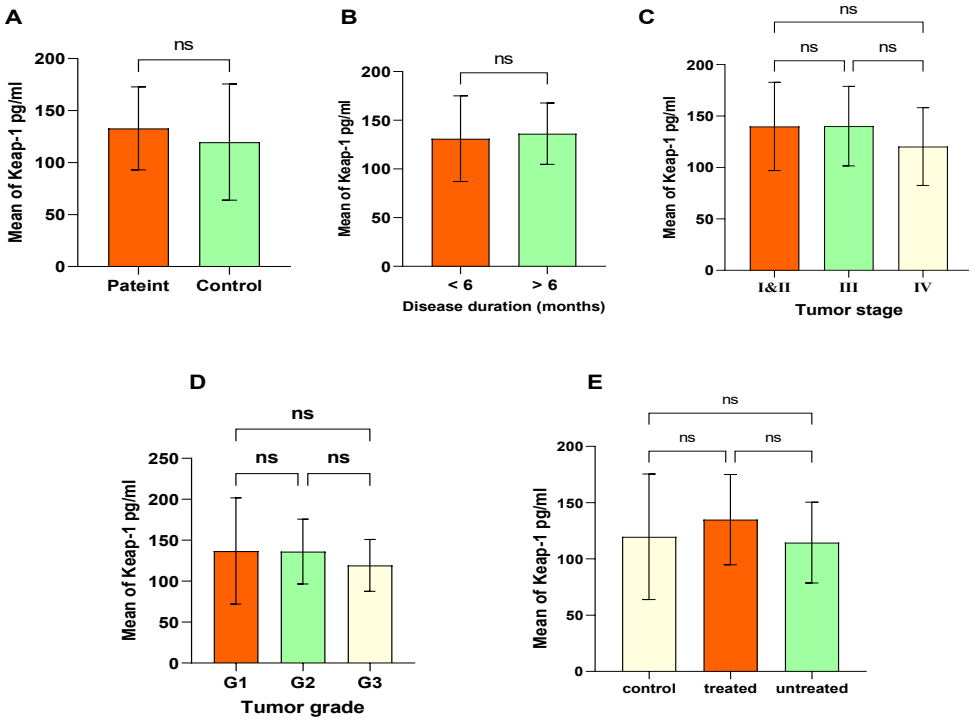

Figure 4: (A) Keap-1 level in the patient and control groups (B) Keap-1 level with disease duration (C) Keap-1 level with tumor stages (E) Keap-1 level with treatment.

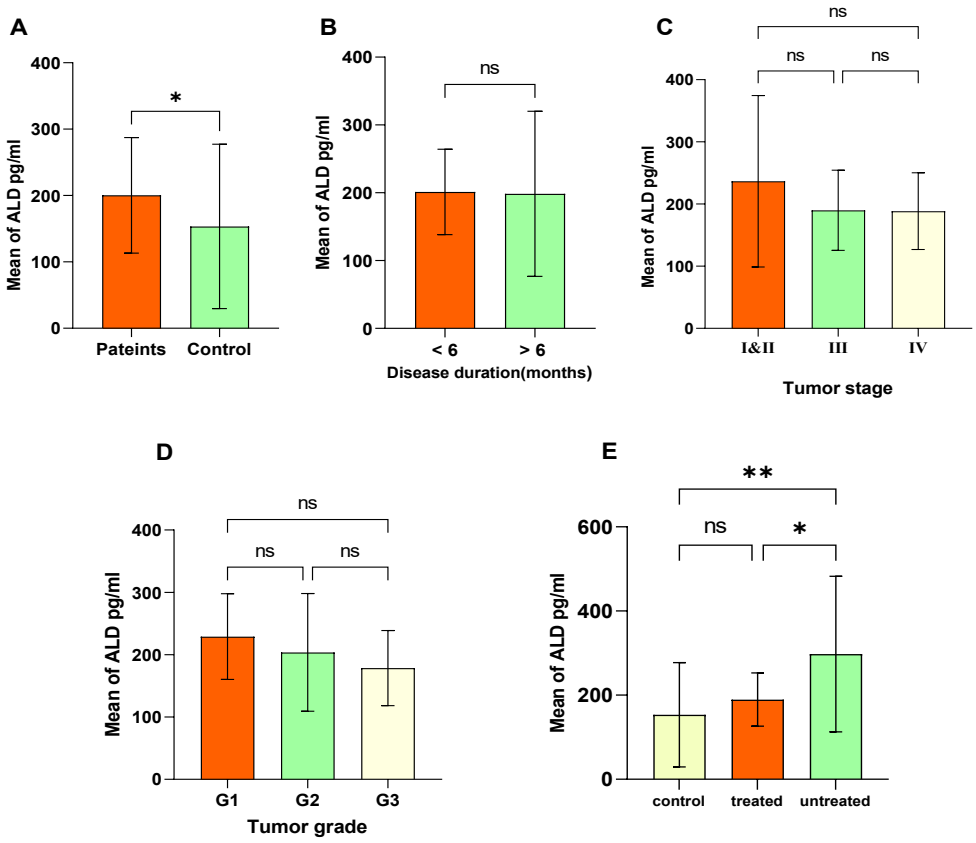

Figure 5: (A) Aldolase level in the patients and control (B) Aldolase level with disease duration (C) Aldolase level with tumor stage (D) Aldolase level with tumor grade (E) Aldolase level with treatment.

Table 3: Variations among patients' subgroups in the elvels of keap-1 and aldolase.

Colo-rectal carcinoma			Human Kelch-like ECH Ass. Protein 1 (Keap-1) (pg/mL)	P value	Aldolase (pg/mL)	P value
		No	Mean±SD		Mean±SD	
Age (years)	<50years	13	141.446±47.434	0.815	201.777±85.529	0.919
	50---59	20	130.145±44.098		189.620±72.092	
	60---69	17	128.112±35.279		193.100±45.991	
	=>70years	10	135.300±30.573		202.210±58.716	
Gender	Male	35	133.860±45.198	0.823	206.666±72.730	0.110
	Female	25	131.500±31.810		179.480±51.042	
BMI (Kg/m2)	Underweight (<18.5)	5	133.480±30.432	0.875	202.480±29.708	0.730
	Normal (18.5-24.9)	22	137.686±41.107		204.214±71.781	
	Overweight (25-29.9)	21	131.757±36.645		193.824±67.133	
	Obese (=>30)	12	125.767±49.205		178.742±64.627	
Smoking	Current smoker	8	120.500±49.428	0.649	206.900±88.752	0.818
	Ex-smoker	8	134.913±51.401		198.475±80.881	
	Not	44	134.757±36.349		192.666±59.212	
Past medical history	Yes	23	133.713±33.163	0.899	197.491±59.549	0.895
	No	37	132.357±43.970		194.000±69.715	
Family history of cancer	Yes	22	124.650±38.897	0.227	191.727±61.337	0.712
	No	38	137.639±40.168		197.429±68.516	
Colon site	Appendix/Small intestine	2	135.150±58.619	0.555	225.15±134.138	0.364
	Cecum	3	125.933±53.455		242.533±78.109	
	Ascending colon	4	119.000±31.292		185.350±52.242	
	Transverse colon	13	116.469±29.827		167.015±52.943	
	Descending colon	6	138.017±22.718		198.167±20.846	
	Sigmoid	14	141.550±40.564		201.136±62.436	
Rectal site	Recto-sigmoid	10	143.100±44.655	0.338	208.330±69.524	0.459
	Rectum	6	151.517±58.402		213.033±103.26	
	Anus	2	92.250±4.455		131.700±0.990	
Number of doses	No	6	148.283±46.883	0.757	248.950±66.993	0.078
	1---2	9	131.100±24.242		169.978±36.070	
	3---4	10	135.830±47.374		199.630±74.885	
	5---6	7	113.300±42.125		150.243±59.041	
	>6	16	132.469±35.193		210.337±56.499	
	Long	12	136.008±47.063		190.283±73.656	

It was also utilized the ROC curve analysis to test whether it is possible to employ the serum levels of IL-18, IL-12, Keap-1, and aldolase levels for the diagnosis of CRC. As related to IL-18, the results showed that the AUC value was 0.51, while the 95%-confidence interval (CI) value ranged from 0.39 to 0.64 at P-value of 0.831 and Cutoff value of 12.24 pg/mL, the calculation of which being conducted at maximum levels of sensitivity and specificity (96%

and 13%, respectively) (Figure 6A). Moreover, IL-22 demonstrated an AUC value of 0.54 and CI of 0.42 to 0.66 at P-value of 0.543 and cutoff value of 27.08 pg/mL, the calculation of which being conducted at maximum levels of sensitivity and specificity (90% and 30%, respectively) (Figure 6B). In relation to keap-1, the findings revealed an AUC of 0.631 and CI of 0.50 to 0.76 at P-value of 0.04 and cutoff value of 75.7 pg/mL, the calculation of which being conducted at maximum sensitivity and specificity (66% and 63% respectively) (Figure 6C). Finally, aldolase demonstrated an AUC of 0.76 and CI of 0.66 to 0.88 at P-value of 0.0001 and cutoff value of 79.99 pg/mL, the calculation of which being conducted at maximum sensitivity and specificity (75% and 73%, respectively), as illustrated in Figure 6 D.

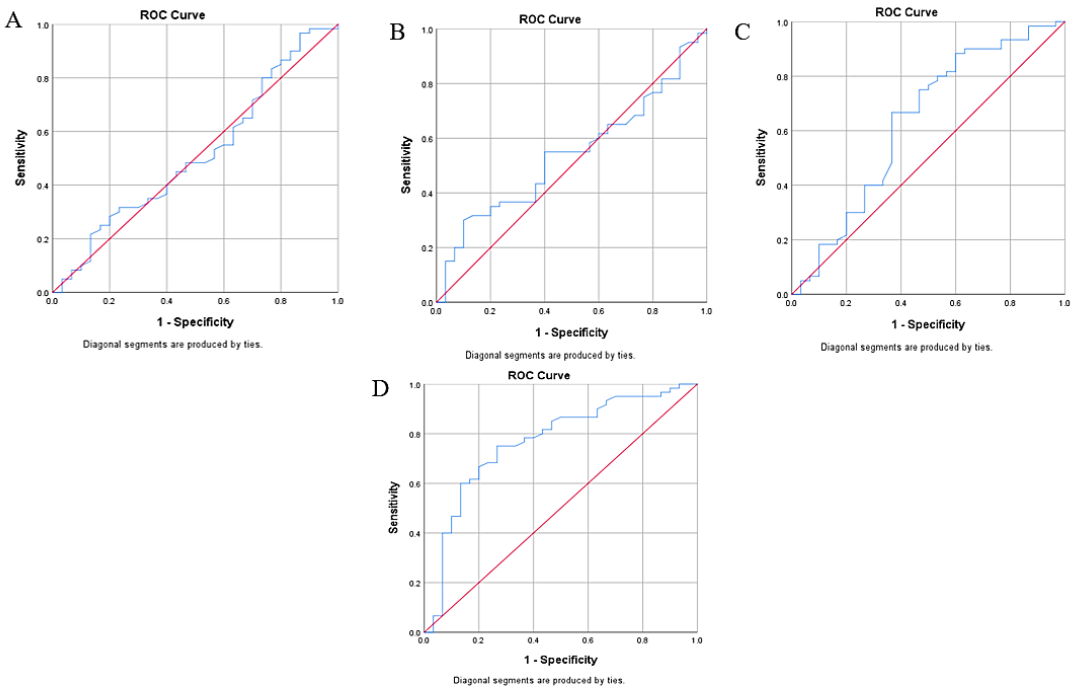

Figure 6: ROC curve analysis for IL-18 (A), IL-22 (B), Keap-1 (C), and aldolase (D).

3.4. Gene expression of *FOXO4* gene

The 2-ΔΔCt of foxo4 mRNA showed significant downregulation in CRC patients in comparison with that in the healthy subjects. Nevertheless, the distribution of the patients depending on clinical and disease features revealed insignificant differences among the mean values o 2-ΔΔCt, proposing the downregulation of the *FOXO4* gene in CRC patients [46].
Analysis of the mean values of 2-ΔΔCt indicated a relative expression value of less than 1. Associations with clinical and laboratory features in the patient group indicated insignificant differences among 2-ΔΔCt values in subgroups (p-value>0.05) **(Table 4)**. A clear impact of age was noticed on gene expressions following the division of the patients into four age groups (<50, 50-59, 60-69, ≥70). *FOXO4* was significantly lower expressed in the 50-59 years old age group, with an elevation being recorded in the <50,≥70 age group.

The FOXOs, comprising a forkhead transcription factor family consisting of four members (FOXO1, 3, 4, and 6), are significantly involved in functioning as tumor suppressors by conferring resistance to oxidative stress and eliciting cell cycle G1/S arrest and apoptosis in the process of tumorigenesis. Numerous studies have presented mounting proof indicating that dysregulation [65, 66]. Numerous studies have provided mounting evidence indicating that the mis regulation of *FOXO4* expression may expedite the

advancement of various types of tumors, such as cervical, colorectal, pancreatic, and lung cancers [67].

PI3K/Akt was found to be the main pathway by which the regulation of FOXO4 activity takes place [68]. FOXO3 and *FOXO4* possibly have essential contributions in cell apoptosis, cell cycle, and fetal membrane rupture [69]. *FOXO4* is a protein that has a clear role in suppressing tumors and has a close association with the metastasis of cholangiocarcinoma [70]. Various types of cancer demonstrated irregular activation of the elements of the PI3K/Akt pathway. The overexpression of Akt was described in a variety of malignancies, such as those occurring in the colon, pancreas, and ovaries [71, 72].

Phosphatidylinositol 3-kinase (PI3K) is reportedly overactivated in patients with CRC. The signaling of this enzyme has multiple activities in the signal transduction pathways that affect different cellular processes, such as growth, transformation, and tumorigenesis. When p85α, which is the PI3K regulatory subunit, is depleted, induction of G1 phase cell cycle arrest was observed, along with the sensitization of CRC cells to 5-FU-induced apoptosis. The absence of p85α in these setups resulted in a marked reduction in AKT (and phosphorylated AKT), causing a significant reduction in *FOXO* proteins in the cytoplasmic compartment [73]. Previous research proposed the control of *FOXO* transcription factors via the PI3K/Akt signaling, with the involvement in events like cell cycle progression and cell death [74]. A marked decrease in the level of *FOXO4* protein was reported in the patients with high-grade of cancer, whereas *FOXO3* and *FOXO4* were low expressed in the bladder cancer tissues in comparison with the adjacent normal tissues which were positively correlated with each other [75].

Table 4: Gene fold of *FOXO4* gene with multiple variants.

Colorectal carcinoma		PCR (gen fold)		P-value
		No	Mean±SD	
Age (years)	<50years	13	0.84±0.83	0.045*
	50---59	20	0.29±0.36	
	60---69	17	0.34±0.36	
	=>70years	10	0.79±1.35	
Gender	Male	35	0.49±0.89	0.282
	Female	25	0.53±0.50	
BMI (Kg/m^2)	Underweight (<18.5)	5	0.88±0.70	0.069
	Normal (18.5-24.9)	22	0.33±0.60	
	Overweight (25-29.9)	21	0.56±0.50	
	Obese (=>30)	12	0.571±1.25	
Smoking	Current smoker	8	0.307±0.277	
	Ex-smoker	8	0.870±1.015	
	Not	44	0.475±0.745	
Chronic illness	Yes	23	0.594±0.96	0.653
	No	37	0.450±0.58	
Family history of cancer	Yes	22	0.482±0.46	0.696
	No	38	0.519±0.88	
Colon site	Appendix/Small intestine	2	0.348±0.08	
	Cecum	3	0.153±0.13	
	Ascending colon	4	0.184±0.18	

	Transverse colon	13	0.467±0.51	0.472
	Descending colon	6	0.724±0.45	
	Sigmoid	14	0.460±0.53	
Rectal site	Recto-sigmoid	10	0.738±1.37	
	Rectum	6	0.599±1.11	
	Anus	2	0.304±0.32	
Duration of disease (years)	<6months	32	0.528±0.64	0.575
	6---11months	16	0.605±1.09	
	=>12months	12	0.312±0.43	
Grade	Grade [I]	4	0.362±0.62	0.783
	Grade [II]	44	0.555±0.83	
	Grade [III]	12	0.371±0.43	
stages	Stage [I&II]	14	0.539±0.75	0.530
	Stage [III]	24	0.359±0.43	
	Stage [IV]	22	0.644±0.99	
Number of doses	No	6	0.16±0.08	0.703
	1---2	9	0.51±0.53	
	3---4	10	0.59±0.90	
	5---6	7	0.31±0.25	
	>6	16	0.69±1.11	
	Long	12	0.47±0.53	

3.5. Correlations among parameters in the serum of CRC patients

The serum levels of IL-18 and IL-22A demonstrated a highly significant positive correlation in the present study (r= 0.728, P-value 0.001). A significant moderate positive correlation was also observed between IL-18 and keap-1 (r = 0.639, p = 0.0001 as well as IL-18 and aldolase (r = 0.638, p = 0.0001) (Table 5). IL-22 exerted a significantly weak positive correlation with keap-1 (r = 0.548, p = 0.0001) and a moderate positive correlation with aldolase (r = 0.600, p = 0.0001). The following scoring criteria for the degrees of correlation were adopted in the present study: r = 0-0.19 is very weak correlation, r = 0.2-0.39 is weak, r = 0.40-0.69 is moderate, r = 0.7-0.9 is strong, and r = 0.9-1 is very strong.

Table 5: Correlation analyses among various factors tested in the present study.

Colorectal carcinoma		**IL-18 (pg/mL)**	**IL-22 (pg/mL)**	**Keap-1 (pg/mL)**	**Aldolase (pg/mL)**
IL-18 (pg/mL)	r	-	0.728**	0.639**	0.638**
	P	-	0.0001	0.0001	0.0001
IL-22 (pg/mL)	r	0.728**	-	0.548**	0.600**
	P	0.0001	-	0.0001	0.0001
Keap-1 (pg/mL)	r	0.639**	0.548**	-	0.735**
	P	0.0001	0.0001	-	0.0001
Aldolase (pg/mL)	r	0.638**	0.600**	0.735**	-
	P	0.0001	0.0001	0.0001	-
*Correlation is significant at the 0.05 level. **Correlation is highly significant at the 0.01 level.					

r: correlation coefficients, P: p. value

Conclusions

Serum ALD levels were elevated in patients with CRC, while IL-22, IL-18 and keap-1 levels showed non-significant variations. Aldolase enzyme has been identified as a promising biomarker for tracking the progression of CRC, offering a valuable tool for clinicians to monitor disease development and response to treatment. These findings confirm that it is difficult to forecast cancer by assessing the blood's genetic expression of immune response-related genes, which are exclusively expressed in cancer cells and tissues. As a result, this weak marker cannot be used specifically to identify cancer. The study proposes that altered serum ALD levels and *FOXO4* gene expression may be associated with the pathogenesis of CRC.

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Data availability

Data supplied in this manuscript shall be supplied upon request.

Conflict of interest

The authors declare no conflict of interest.

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