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Serum Levels of Interleukin 10, Interleukin 17A, and Calcitriol in Different Groups of Colorectal Cancer Patients

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Abstract

Colorectal cancer (CRC) is considered as the fourth prevalent reason of cancer deaths. Interleukin 10 (IL10) and interleukin 17A (IL-17A) act as anti-inflammatory and pro-inflammatory cytokines, respectively, both being able to exert antitumor or pro-tumor roles in cancer. Serum levels for IL-10, IL-17A, and calcitriol were measured by ELISA. The CRC patient sample (n =90) consisted of newly diagnosed, before surgery, and after surgery groups, the results of which were compared with those of healthy subjects (n=30). IL-10 levels demonstrated significantly lower (P<0.001), significantly higher (P<0.001), and insignificant differences in the three groups, respectively, as compared to the control. Serum IL-17A levels were significantly higher (P<0.001) in all patient groups compared with healthy subjects, whereas calcitriol levels were significantly lower in most patients. In conclusion,low levels of IL-10 found in CRC patients at diagnosis could be used as a diagnostic marker for the disease. However, chemo-radiotherapy caused an increase in the levels of this cytokine, which were restored to normal following surgical intervention. In addition, a contribution of serum calcitriol in changing the levels of IL-10 in CRC patients was observed. High levels of IL-17A, regardless of patient group, indicated a strong association with CRC.

Keywords: Colorectal cancer, Interleukin 10, Interleukin 17A, Calcitriol, Serum.

1. Introduction

CRC is considered as a malignant neoplasm resulting from the transformation of the epithelial cells lining the large intestine into malignant cells, where adenocarcinoma grows from the adenomatous polyps (Ahuja and Nettles, 2014).CRC accounts for around 608,000 deaths worldwide, being the fourth prevalent reason of cancer deaths, with an overall contribution of 8%. Despite remarkable development in clinical protocols, about 50% and 95%of CRC patients have still been reported to die in stages III and IV, respectively (Heitman *et al*., 2009).A number of factors are possibly associated with higher susceptibility to CRC development, including exposure to chemical and physical carcinogens (Ahuja and Nettles 2014) as well as beingpositive for rhesus factor (Alqudah *et al*., 2018).The treatment of CRC patients depends on several factors, including the stage of the disease; during stage I, approximately 95% of the patients are curable by surgical intervention, whereas this proportion declines to 65-80% in Stage II. In stages III and IV, more than one type of treatment is applied before surgical removal, such aschemo-radiotherapy (Ahuja and Nettles, 2014).

Some proteins secreted by immune cells are known to serve as biomarkers in cancer (Youssry et al., 2019). IL-10 is defined as an immunosuppressive protein that is produced in response to inflammatory adaptive and innate immune responses.IL-10 acts as a feedback regulator by preventing the production of cytokines by T cell/macrophages and inhibiting their antigen presenting capacity (Mittal and Roche, 2015).This cytokine is produced by T helper 1 (Th1), T helper 2 (Th2), and T regulatory (T-reg) cells, but also by macrophages and monocytes (Taylor*et al*., 2006). In addition, T helper 17 (Th17) cells can control immune responses negatively by secreting some immunosuppressive factors,e.g.IL-10, and hence called non-pathogenic Th17cells (Wu et al., 2018). IL-10 can suppress the response of both pro-inflammatory Th17 cytokines and macrophages by inhibiting certain inflammatory cytokines, such as IL-6 and IL-12. IL-10 deficiency leads to the stimulation of inflammatory responses, including the possibility of spontaneous tumor development in humans (Oft, 2014).

Several lines of evidence suggested the potential use ofIL-10 as a prognostic factor in CRC. High levels of IL-10 are associated with poor prognosis in CRC patients (Li

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,2019). An earlier study also reported a decreased serum IL-10 level in these patients (Abtahi, 2017).

IL-17A isanotherprotein that isproducedby Th17 cells. Previously reported evidence suggested a dual role for IL-17 in cancer. It can act as an antitumor cytokine by promoting cytotoxic T cell responses, leading to regression of the tumor. In contrast, it can encourage angiogenesis and migration of cancer cells (Murugaiyan and Saha, 2009). The anti-tumor activity of IL-17 is mediated by decreasing IL-10 and IL-13 levels, along withincreasing those of other interleukins, such as IL-6 and IL-12 (Razi *et al*., 2019).

IL-17A is produced by the epithelial cells in CRC patients, where it is inversely correlated with the expression of p53.IL-17 was also suggested as a valuable serum marker for CRC development (Radosavljevic *et al*., 2010).

Calcitriol (1α,25-dihydroxy vitamin D3) can be classified as a seco-steroid hormone. Vitamin D3 in humans is converted to calcitriol as an active final vitamin D3 metabolism product (Feldman et al., 2018). Several studies indicated that the normal range of serum calcitriol fluctuates with different stages of age, having values of 18-64, 18-78, and 25-45 pg/mL in serum samples tested from elder males, elder females, and younger adults, respectively (Reynolds*et al*., 2016; Weil *et al*., 2018). The endocrine system for calcitriol was first discovered in animals, where the decreased calcium level in serum was demonstrated to cause an increased calcitriol production(Feldman et al., 2018).Calcitriol relationship with CRC has been investigated but with controvery in the reported results.For example, an earlier study found that calcitriol level in serum decreases as the CRC stages progress (Niv et al., 1999),while another report showed no association between calcitriol and CRC development (Lee et al., 2011). Calcitriol affects certain inflammatory processes that are known to be responsible for the development of cancer, including the expression of IL1β, IL6, and IL17 cytokines, as well as the activity of cyclooxygenase 2 enzyme (Van Harten-Gerritsen *et al*., 2015). Also, calcitriol can enhance the expression of IL-10 by T cells (Heine et al., 2008).

Overall, the previously published data about the possible associations between CRC development and the levels of IL-10, IL-17A, or calcitriol are either controversial or insufficient. Therefore,conducting this study was necessaryto clarify the relationship between the changes in serum IL-10, IL-17A, and calcitriol levels and status of CRC patientsin terms of chemo-radiotherapy and surgical intervention. It is also equally essential to seek the possibility of using these parameters as diagnostic markers for CRC development and to find the potentialinterrelationship among these chemical mediators.

2. Materials and Methods

The present study included 90 patients with primary colorectal adenocarcinoma of various disease and treatment stages (44females and 46 males, age range 28-62 years), along with30 healthy subjects (14females and 16males, age range 27-63 years). Tumor lymph node metastasis-based classification was applied for the staging of the patients. Three groups of equal numbers of CRC patients (n=30) were involved in this study. First, the newly diagnosed group of patients who were not subjected to treatments and surgical intervention, involving all disease stages. Second, the before surgery group of patients who were subjected to chemo-radiotherapy, involving stages III, and IV. Third, the after surgery group of patients who were not subjected to any type of cancer treatment before and after the surgery, involving stages I and II. Patients with kidney failure, heart disease, diabetes, family history, chronic digestive problems, intestinal polyposis, smoking habit, and alcoholism were excluded from the study. Patients were diagnosed by medical consultant doctors. Permissions were obtained from the medical city hospitals in Baghdad, Iraq, and approved by the University of Technology's institutional ethical committee, Baghdad, Iraq (Ref. No. AS 1811-12-9- 2018) in accordance with the Helsinki Declaration of 1975 revised in 2000. All participants were informed about the study design and objectives and signed an informed consent before the collection of any data or samples. Table 1 lists the disease-related characteristics of the study participants.

Table 1. Disease characteristics of CRC patients involved in the present study, with number of cases based on stage, grade, and tumor location.

TNM stage	Case (n)	Tumor location	Case (n)
Stage I	23	Cecum	4
Stage II	23	Ascending colon	11
Stage III	22	Hepatic flexure	5
Stage IV	22	Transverse colon	8
		Splenic flexure	6
Grade	Case (n)	Descending colon	15
Grade1	20	Sigmoid colon	12
Grade 2	58	Recto-sigmoid junction	10
Grade3	12	Rectum	19

Blood specimens were collected from CRC patients and healthy subjects then separated by centrifugation. Serum samples were stored at -40º C until analysis. Sandwich Enzyme linked immunosorbent assay (ELISA) kits were used to measure the serum levels of calcitriol (MyBioSource-U.S.A), IL-10, and IL- 17A (Elabscience-U.S.A). The method is based on the concept of the binding of the antigen (e.g., proteins or hormones) to its specific antibody, which allows its detection in a fluid sample with very small quantities (Gan and Patel, 2013).

3. Statistical Analysis

Data were analyzed using the software package IBM SPSS Statistics version 24 for Windows 10. Parameterswere normally distributed and the results were shown as mean ±SD. Differences between the groups were tested by means of one-way ANOVA test and independent t-test. Correlations were analyzed by Pearson's correlation coefficient. The receiving operating characteristic (ROC) curve was used to find cutoff values and the area under the curve (AUC). Data were considered significant at $P < 0.05$.

4. Results

All CRC patients were divided into groups depending on sex (female and male) and age (≤50 and >50 years).

Serum levels of IL10, IL17A, and calcitriol showed no significant differences for sex and age when these groups were compared to each other .The results demonstrated that serum levels of IL-10, IL17A, and calcitriol were significantly higher (P <0.001) in the before surgery group as compared to the newly diagnosed group, as shown in Table 2.

Table 2: Serum levels of IL-10, IL-17A and Calcitriol in CRC patients in terms of sex and age for all patient (n=90) and in terms of exposure to therapy (Chemo-radiotherapy) for 60 patients without tumor removal.

Group	Case (n)	IL-10 (Mean±SD) pg/mL	*p value*	IL-17A (Mean±SD) pg/mL	*p value*	Calcitriol (Mean±SD) pg/mL	*p value*
Sex							
Female	44	18.02±3.35	>0.05	77.18±11.55	>0.05	56.21±7.66	>0.05
Male	46	19.62±2.22		85.09±10.34		52.79±6.09	
Age							
≤50	47	17.90±3.77	>0.05	82.07±3.12	>0.05	55.33±5.30	>0.05
>50	43	19.76±3.87		80.19±2.24		53.69±4.55	
Therapy*							
Without	30	11.02±2.98	<0.001	71.61±12.15	<0.001	48.85±5.57	<0.001
With	30	28.12±3.70		85.14±9.87		55.99±4.98	

* Only 60 CRC patients: Newly diagnosed group (n=30, no chemo-radiotherapy), before surgery group (n=30, neoadjuvant chemo-radiotherapy).

The results also showed that IL-10 levels were significantly lower in the serum of the newly diagnosed group (11.02±2.98 pg/mL, *P*<0.001), but significantly higher in the before surgery group (28.12±3.70 pg/mL, *P*<0.001) as compared to healthy subjects (17.41±3.20 pg/mL). However, no significant difference was observed between the after surgery group (17.33±3.37 pg/mL) and healthy subjects, as shown in Figure 1-A)

The results also revealed that serum IL-17A levels in the newly diagnosed,before surgery, and after surgery groups were significantly higher (71.61±12.15, 85.14±9.87, and 86.67±6.84 pg/mL, respectively) as compared to those in healthy subjects (27.77±5.35 pg/mL, *P* <0.001), as shown in Figure 1-B.

Calcitriol levels in the sera of the newly diagnosed and before surgery groups (48.85±5.57 and 55.99±4.98 pg/mL, respectively) were significantly lower compared to those in the healthy subjects (60.04 ±3.10 pg/mL; *P*<0.001 and *P* <0.05, respectively). No significant difference was found between healthy subjects and after surgery group (60.04 ±4.87 pg/mL vs 58.66 ±5.91 pg/mL, *P*>0.05), as shown in Figure 1-C.

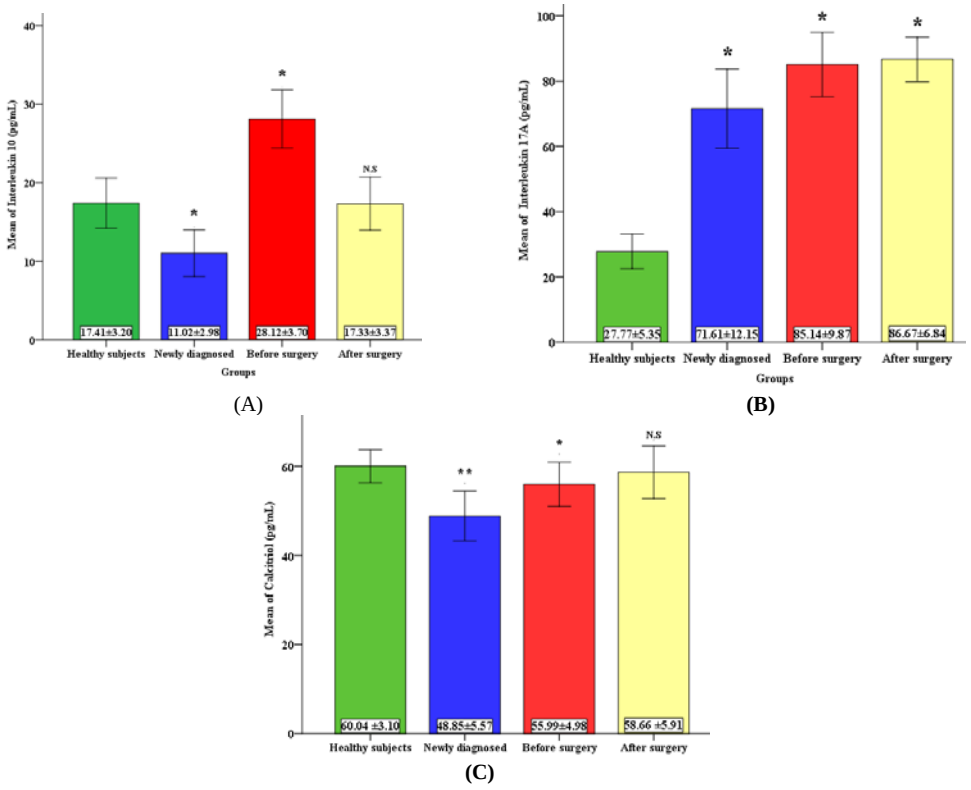
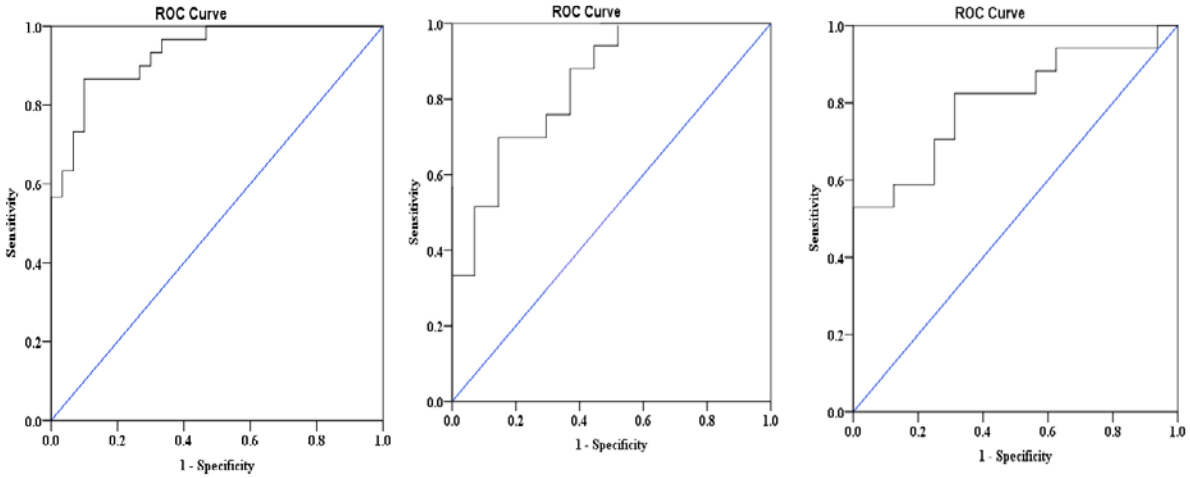


Figure 1: Levels of IL-10, IL-17A, and calcitriol in serum samples of healthy subject and CRC patient groups;**(A)**IL-10 levels in the newly diagnosed group was significantly lower while in the before surgery group was significantly higher as compared to healthy subject. No significant difference was found between the after surgery and healthy subject groups. * *P*< 0.001, N.S: Non-significant. **(B)**IL-17A levels in all CRC patient groups.The level was significantly higher as compared to healthy subjects. * *P*< 0.001.**(C)**Calcitriol levels in the newly diagnosed group and before surgery group was significant lower as compared to healthy subject. No significant difference was found between after surgery group and healthy subjects. * *P*< 0.05, ** *P*< 0.001, N.S: Non-significant.

Analysis of the ROC curve was used for testing the possibility of using IL-10, IL-17A, and calcitriol levels in the serum to diagnose CRC disease in the newly diagnosed patient group. The results showed that, for IL-10, AUC value was 0.93, the 95%-confidence interval (CI) was from 0.87 to 0.99 at *P*<0.001, and Cutoff value was 14.39 pg/mL, calculated at maximum sensitivity and specificity (87% and 90%, respectively). For IL-17A, the results showed an AUC value of 0.82, CI of0.67 to 0.97 at *P* <0.001, and Cutoff value of 67.18 pg/mL, calculated at maximum sensitivity and specificity (68% and 85%, respectively). Regarding calcitriol, the result showed an AUC of 0.80, CI of 0.64 to 0.98 at *P*<0.001, and Cutoff value of 53.14 pg/mL, calculated at maximum sensitivity and specificity (82% and 69.7% respectively), as shown in Figure 2.



(A) (B) (C)

Figure 2: ROC curve for IL-10 (A) , IL17A (B), and calcitriol (C) ; low levels of IL-10 and acalcitriol , also high levels of IL-17A, can give an indication for the presence of CRC and hence they can be used as diagnostic markers.Only one significant positive correlation was found, which was between serum levels of IL-10 and calcitriol (r =0.71; *P*<0.001),as shown in Figure 3, whereas correlations between levels of IL-17A and both IL-10 and calcitriol were non-significant, for all CRC patient groups. In the healthy subjects, all correlations between these parameters were non-significant. As shown in (Table 3),levels of IL-10, calcitriol and IL-17A showed non-significant correlations with the values of body mass index (BMI)of healthy subjects (18.6-24.2 kg/m^2) and patients (18.3-24.8 kg/m^2).In addition, significant correlations between the serum levels of these chemical mediators and age were absent, as shown in Table 4.

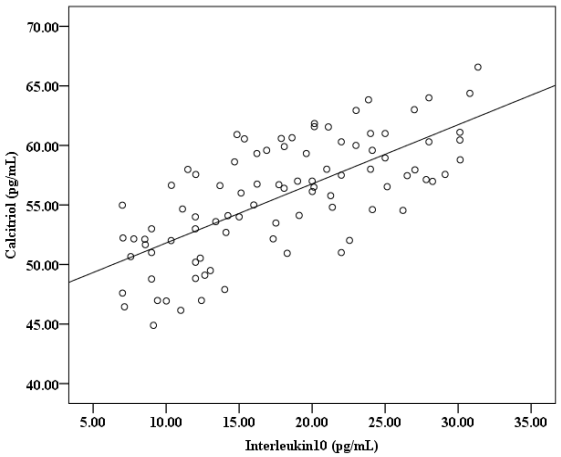


Figure 3: Significantly positive correlation between IL-10 and calcitriol in the serumof CRC patients.

Table 3. Correlations between parameters in serum for CRC patients and Healthy subjects

Correlation between	CRC Patients r (*p* value)	Healthy subjects r (*p* value)
IL-10 and Calcitriol	0.70 (<0.001)	0.45 (>0.05)
IL-10 and IL-17A	0.41 (>0.05)	0.05 (>0.05)
IL-17A and Calcitriol	0.02 (>0.05)	-0.34 (>0.05)

Table 4. Correlations between both BMI and age with the tested parameters in sera of patients and Healthy subjects

Correlation between	CRC Patients r (*p* value)	Healthy subjects r (*p* value)
IL-10 and Age	0.28 (>0.05)	0.36 (>0.05)
IL-17A and Age	-0.05 (>0.05)	-0.33 (>0.05)
Calcitriol and Age	0.05 (>0.05)	-0.02 (>0.05)
IL-10 and BMI	0.18 (>0.05)	-0.04 (>0.05)
IL-17A and BMI	-0.03 (>0.05)	-0.27 (>0.05)
Calcitriol and BMI	0.21 (>0.05)	0.06 (>0.05)

5. Discussion

Colorectal cancer patients' groups showed no significant differences in terms of age and BMI, which excludes the effects of these two factors on the studied parameters. (Table 2, 4). Also, the levels of IL-10, IL-17A, and calcitriol measured in the sera of CRC patients in the present study confirm the results obtianed by other research groups in various populations (Abtahi, 2017, Niv et al., 1999, Di Caro et al., 2016, Karabulut et al.,2016).

Levels of IL-10 in CRC patient groups involved in this study revealed differences from those in healthy subjects (Figure 1-A). IL-10 levels were significantly lower in the newly diagnosed group. IL-10, cyclooxygenase 2 (COX-2), and Interleukin 1β (IL-1β) are known to be involved in the same pathway. IL-1β was reportedto induce COX-2

synthesis through the activation of the nuclear factor κB (NF-κB), while IL-10 was reportedto block the induction of IL-1β to activate NF-κB in epithelial cells of the intestine (Al-Ashy et al., 2006, Andersen et al., 2013). COX-2 is an enzyme that increases prostaglandin secretion, where the high expression of this enzyme is associated with CRC in humans by affecting angiogenesis (Rao et al., 2004). Hence, the low levels of IL-10 in the serum reportedly lead to an increased risk of CRC disease, by elevating COX-2 expression. IL-10 deficiency increases the number of colon tumors in CRC mouse models (Tomkovich et al., 2017). A previous study showed that IL-10 level was low in the plasma of newly diagnosed CRC patients, but with non-significant differences compared with the control (Yamaguchi et al., 2019), whereas another work demonstrated significantly lower differences (Abtahi, 2017).

The present study recorded significantly highIL-10 levels in the before surgery group, which was subjected to more than one type of treatment. IL-10 tends to perform a protective function in CRC animal models, which is mediated by regulatory T cells.IL-10 was needed to decrease the tumor burden in mice (Erdman et al., 2005).

However, no significant differences were recorded in the levels of this cytokine in the after surgery group, in which tumor is completely removed without chemo-radiotherapy or any type of treatment. In a study that described CRC patients who did not experience relapse after surgery, IL-10 levels in the serum were significantly loweras compared to their state before surgery (Di Caro et al., 2016).

IL-17 is known to have a dual role in cancer because it can act as an antitumor cytokine by promoting cytotoxic T cell responses, leading to the regression of tumor (Murugaiyan and Saha, 2009).IL-17 is considered as one of the most common cytokines that promotes angiogenesis and tumor development, where it isassociated with poor prognosis in CRC (Liu et al., 2011). It is involved in a potential mechanism of stimulating the growth of tumor due to its proangiogenic effect, through enhancing the production of vascular endothelial growth factor (VEGF) which is considered as an important proangiogenic factor in many human cancer types (Razi et al., 2019).

The results of the present study showed significantly higher levels of IL-17A in the serum for all CRC patient groups (Figure 1-B).These results are consistent with those reported by previous studies related toIL-17A levels in the plasma and serum of newly diagnosed CRC patients (Yamaguchi et al., 2019, Wang et al., 2019), as well as those desribing patients before or after surgical removal (Radosavljevic et al., 2010, Karabulut et al., 2016).In the present study, the high levels of IL-17A in the after surgery group may be due to an uncompleted treatment plan, possibly involving the prescription of adjuvant chemotherapy doses to destroy the remaining cancer cells, if found.Also,the samples were collectedfrom the patients after 21 days of surgical removal, which is a short period to restore a normal health state. In addition, there is a possibility of bacterial infection for some patients as a result of surgery (Al-Awaysheh, 2018) .

Calcitriol synthesis occurs in the intestinal cells besides its synthesis in the kidney. Both receptors of vitamin D and calcidiol 1-monooxygenase can be expressed in the normal and malignant cells of the human colon (Matusiak et al., 2005). Also, a correlation exists between calcitriol levels in the serum and those in the colonic tissues in humans (Wagner et al., 2012). Calcitriol can inhibit the proliferation of the colonic epithelial cells. Therefore, low levels of calcitriol facilitate colorectal carcinoma development and affect its biological behaviour. Levels of $25(OH)D_3$ and calcitriol were reported to have different relationships in the same set of colorectal carcinoma patients. A previous study found that $25(OH)D_3$ in the serum of patients of all disease stages were higher as compared to those of the healthy individuals, whereas the levels of calcitriol were significantly lower in patients of advance stages (Niv et al., 1999).

The findings of the present work showed that calcitriol levels in the serum were significantly lower in thenewly diagnosed and before surgery groups, while the after surgery group showed no significant differences, when compared with the healthy subjects (Figure 1-C). For the newly diagnosed group, these findings confirm those published in an earlier study of CRC patients, where calcitriol level was lower in the serum (Niv et al., 1999). Various reasons can account for lower calcitriol levels in the serum. The before surgery group in this study received chemo-radiotherapy, whereas patients who received chemotherapy were less likely to be involved in outdoor activities and therefore have less exposure to sunlight. Other factors might involve modified diet regimes as well as the reported post-chemotherapy reduction of serum vitamin D levels in CRC patients (Fakih et al., 2009). The after surgery group, which included only Stage I and stage II patients, showed no significant differences in calcitriol levelsin comparison with healthy subjects.This may be due to the complete removal of the tumor before exposure toto chemo-radiotherapy regimes. A previous study showed that calcitriol levels in the serum are correlated negatively with CRC disease stages (Niv et al., 1999).The results of the current study suggest that the observed low levels of IL-10 and calcitriol in the serum of the newly diagnosed group can give an indication for the presence of the disease, hence these chemical mediator shave a good potential to be utilized as diagnostic markers(Figure 2-A,B).However, the observed low levels of IL-10 and calcitriol, which were lower than the cutoff values (14.39 pg/mL and53.14 pg/mL, respectively), may contribute in the pro-tumorconditions andassociate with an increased risk of developing CRC disease. Previous studies could not agree on a definite range of normal calcitriol value in the serum of humans. Also, several recent studies indicated an increase in the upper limits of the normal range, which reached 78 pg/ml (Feldman et al., 2018, Reynolds et al., 2016, Weil et al., 2018). Hence,this cutoff value can be regarded as acceptable to associate with the increased risk of CRC disease.

The present study showed that the high levels of IL-17A in the serum could be used to diagnose CRC disease (Figure 2- C).The recorded cutoff value (67.18 pg/mL) can aid in predictingthe magnitude of the risk to develop CRC. Previous studies also suggested that IL-17A level in the serum is an important diagnostic and prognostic marker for CRC disease (Radosavljevic et al., 2010, Wang et al., 2019).

This study also demonstrated a positive correlation between IL-10 and calcitriol (Figure 3, Table 3). A previous study showed that calcitriol could increase IL-10

expression by B cells in humans. B lymphocytes can produce the bioactive form of vitamin D (calcitriol) which enhances their expression of IL-10 Heine et al., 2008). In a study that examined the effects of calcitriol treatment on Th2 cell development in mice using an *in vitro* culture, it was found that IL-10 production was increased (Boonstra et al., 2001).

In the present study, IL-10, IL-17A, and calcitriol levels in the serum ofCRC patients who were subjected to treatment (Before surgery group) were significantly higheras compared to untreated patients(Newly diagnosed group) (Table 2, Figure1). In breast cancer patients under neoadjuvant therapy, serum levels of IL-10 were significantly increased as compared to prior use of IL-10 (Jabeen et al., 2018). IL-10 showed some resistance to chemotherapy via autocrine IL-10 production from the tumor cells in breast cancer (Yang et al., 2015). In a previous study, IL-17A was used as a biomarker of the response to bevacizumab in CRC metastases cases, where the higher levels in serum were reported to lead to shorter progression-free survival (Lereclus et al., 2017). Resistance to cisplatin drug in CRC patients was also reported (Sui et al., 2019). The high calcitriol levelsin the before surgery group may be due to vitamin D supplements that are given to CRC patient as a part of the treatment plan.

6. Conclusion

Low levels of IL- 10 in the serum can be used as a diagnostic biomarker for CRC, whereas high levels of IL-10 result from the effects of chemo-radiotherapy. The association of calcitriol with IL-10 indicates that calcitriol contributes in changing the levels of IL-10 in CRC patients. High levels of IL-17A in the serum, regardless of CRC group type, indicates a strong association with the disease. Early surgical treatment in the early stages of CRC restores the normal levels of IL-10, and calcitriol. The cutoff values found by this study can be used to predict health conditions in which the individuals are at risk or already having CRC.

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