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# Exploring the Inflammatory Pathogenesis of Colorectal Cancer by Assessing the Levels of the Markers Aflatoxin-B1, CARD-9, and Dectin-1

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**Abstract**

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide, with a growing incidence in developing countries such as Iraq. Chronic inflammation and exposure to environmental toxins like aflatoxin-B1 may influence disease development and progression. This study investigates the role of aflatoxin-B1, caspase recruitment domain-containing protein 9 (CARD-9), and Dectin-1 in CRC among Iraqi male patients, including newly diagnosed cases and those under chemotherapy. A total of 88 participants (60 CRC patients aged 25-70 and 28 healthy controls) were enrolled. Peripheral blood samples were withdrawn from each participant from four hospitals in Baghdad between October 2023 and January 2024. The participants were divided into three groups: 30 newly diagnosed CRC patients, 30 under treatment with chemotherapy (Folfox, Xeloda, Oxaliplatin), and 28 healthy individuals served as a control group. Serum levels of aflatoxin-B1, CARD-9, and Dectin-1 were determined by using an ELISA assay. The results showed no significant differences in aflatoxin-B1 levels across groups ($p=0.24$). However, CARD-9 levels were significantly higher in patients ($p = 0.001$), and Dectin-1 also showed significant differences ($p = 0.002$). Comparing the two patient groups, CARD-9 levels differed significantly ($p < 0.001$), while aflatoxin-B1 and Dectin-1 did not ($p = 0.1, 0.06$). Based on disease grades, CARD-9 showed significant differences in newly diagnosed patients ($p = 0.044$), while Dectin-1 showed significant differences in treated patients ($p < 0.001$). These findings highlight the potential role of CARD-9 and Dectin-1 as biomarkers in CRC progression and treatment monitoring.

**Keywords:** Colorectal cancer (CRC), aflatoxin-B1, CARD-9, Dectin-1, ELISA.

# استكشاف الامراضية الالتهابية لسرطان القولون والمستقيم من خلال تقييم مستوى المؤشرات Aflatoxin-B1 و CARD-9 و Dectin-1

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**الخلاصة**

يعد سرطان القولون والمستقيم (CRC) من الأسباب الرئيسية للمراضة والوفيات المرتبطة بالسرطان في جميع أنحاء العالم، مع تزايد حدوثه في البلدان النامية مثل العراق. قد يؤثر الالتهاب المزمن والتعرض للسموم البيئية مثل الأفلاتوكسينات على تطور المرض وتقدمه. تهدف هذه الدراسة إلى التحقيق في دور الأفلاتوكسين

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ب-1، CARD-9، و Dectin-1 في سرطان القولون والمستقيم (CRC) لدى المرضى العراقيين من الذكور، بما في ذلك الحالات الجديدة والتحت العلاج الكيميائي. تم تسجيل 88 مشاركًا (60 مريضًا بسرطان القولون والمستقيم تتراوح أعمارهم بين 25-70 عامًا و28 شخصًا سليمًا)، مع جمع عينات دم من أربعة مستشفيات في بغداد بين أكتوبر 2023 ويناير 2024. تم تقسيم المشاركين إلى ثلاث مجموعات: 30 مريضًا جديدًا تم تشخيصهم بسرطان القولون والمستقيم، 30 مريضًا تحت العلاج الكيميائي (Folfox، Xeloda، Oxaliplatin)، و28 شخصًا سليمًا. تم تحليل مستويات الأفلاتوكسين و CARD-9 و Dectin-1 في المصل باستخدام اختبار ELISA. أظهرت النتائج عدم وجود فروق ذات دلالة إحصائية في مستويات الأفلاتوكسين بين المجموعات ($p = 0.24$). ومع ذلك، كانت مستويات CARD-9 أعلى بشكل كبير في المرضى ($p = 0.001$)، بينما أظهرت مستويات Dectin-1 أيضًا فروقًا ذات دلالة إحصائية ($p = 0.002$). عند مقارنة مجموعتي المرضى، اختلفت مستويات CARD-9 بشكل كبير ($p < 0.001$)، بينما لم تختلف مستويات الأفلاتوكسين و Dectin-1 بشكل كبير ($p = 0.1$، 0.06). استنادًا إلى درجات المرض، أظهرت مستويات CARD-9 فروقًا ذات دلالة إحصائية في المرضى الجدد ($p = 0.044$)، بينما أظهرت مستويات Dectin-1 فروقًا ذات دلالة إحصائية في المرضى الذين يتلقون العلاج ($p < 0.001$). تسلط هذه النتائج الضوء على الدور المحتمل لـ CARD-9 و Dectin-1 كمؤشرات حيوية في تقدم سرطان القولون والمستقيم ومراقبة العلاج.

## 1. Introduction

Colorectal cancer (CRC) is the most common malignancy in the gastrointestinal tract-associated death in the world [1]. It arises when a group of abnormal cells proliferates uncontrollably [2]. The majority of CRC cases occur in the elderly [3].

According to the Iraqi Cancer Registry (ICR, 2023), CRC ranks fourth among cancer types. In Iraq (2021), the colorectal cancer number and percentage were 2493(10.54%), and the crude incidence rate of CRC/100000 population was 6.05. The numbers and percentage of CRC in males were 1326 (12.51%), while females recorded a number and percentage of 1167(7.88%). In Baghdad, CRC recorded a number and percentage of 617(10.44%). Males recorded a number and percentage of 334(14.03%), while females recorded 283(7.08%) [4].
Inflammation can result in immunosuppression, providing a preferred background for tumor development. Inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [5,6]. Inflammation greatly affects the etiology, development, and progression of invasive colorectal tumors [7,8].
Mycotoxins like aflatoxin-B1 has a damaging effect on the gastrointestinal tract due to ingestion of contaminated food, commonly manifested as inflammation, necrotic changes, damage to intestinal barrier function, impairment of secretory activity, and alteration in enterocyte metabolism [9,10]. Aflatoxin-B1 can induce immunosuppressive and immune stimulatory effects [11]. Relevant to the activation of the pro-inflammatory response are those Pattern Recognition Receptors (PRRs) involved in the stimulation of pro-inflammatory antifungal immunity, including caspase recruitment domain-containing protein 9 (CARD-9) and dendritic cell-associated C-type lectin-1 (Dectin-1) [12]. *In vivo* Dectin-1 mediated CARD-9 activation after vaccination drives both expansion and activation of Ag-specific cytotoxic T-lymphocyte (CTL), resulting in a long-lasting CTL response that is sufficient to protect mice from tumor challenge. It is an adaptor protein that is abundantly expressed in myeloid cells, including Neutrophils, Macrophages, and Dendritic cells (DC). CARD-9 contains a CARD-9 domain at the N-terminus that mediates homology interaction between CARD-9-containing molecules and a coiled-coil region at the C-terminus that functions as an oligomerization domain [13]. The levels of CARD-9 expression vary in different organs; it is abundant in organs rich in cells associated with immune response, such as the bone marrow, spleen, lung, and lymph node, but it is not expressed in organs such as the kidney, liver,

intestine, colon, and brain. CARD-9 is required for the downstream signal transduction of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-lectin receptors (CLRs) such as Dectin-1, Dectin-2, and mince utilize the signaling pathway involving spleen tyrosine kinase (Syk) CARD-9 [14]. Upon pathogen recognition via the CLRs, Syk is phosphorylated and induces the activation of protein kinase Cδ, which mediates the recruitment and activation of CARD-9. CARD-9 is considered a crucial activator of the immune response against fungi [15,16], and its crucial role in cytokines /chemokines, secretion, and ROS production in different cell and organ systems is closely associated with tumor formation and metastasis [17]. Dectin-1 is the most exemplary CLR that interacts with CARD-9 to activate the inflammasome with the production of pro-inflammatory cytokines and chemokines, including Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-2 (IL-2), Interleukin-10 (IL-10), Interleukin-23 (IL-23), C-X-C Motif Chemokine Ligand 2 (CXCL2), Interferon-gamma (IFN-γ), and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) upon fungal infection [18].

CARD-9-mediated immune response is critically involved in carcinogenesis [19]. The clinic pathological analysis shows that *CARD9* expressions positively correlate with tumor invasion and metastasis [15]. It was observed that CARD-9 promotes the tumorigenesis of the large intestine, with reduced viability selectively in male mice but not in female mice. CARD-9 signaling is important for the function of innate lymphoid cells (ILCs) after intestinal epithelial injury in mice. CARD-9-mediated IL-1β secretion from myeloid cells is essential for the activation of ILCs and the release of IL-22 from ILCs, which in turn activates STAT3 signaling in intestinal epithelial cells (IECs) and subsequently regulates IEC proliferation [12] .

CARD-9 can promote the inflammation associated with carcinogenesis. However, CARD-9 is also reported to prevent the development of tumors and act as a central regulator to ensure long-lasting antitumor immunity for Dectin-1-mediated activation of $CD4^+$ T cells and $CD8^+$ cytotoxic T-cells [18].

CARD-9 and Dectin-1 are key innate immune components influencing both inflammation and tumorigenesis. CARD-9 not only mediates antifungal immunity but also regulates gut microbiota, impacting colorectal cancer risk [20]. Dectin-1 recognizes fungal β-glucans and damage-associated molecular patterns (DAMPs), modulating tumor-associated macrophage responses [21]. Aflatoxin-B1, beyond its hepatotoxicity, forms DNA adducts and induces epigenetic changes in the gastrointestinal tract, promoting inflammation and increasing cancer risk [22]. These biomarkers provide valuable insights into CRC pathogenesis and immune dysregulation.

The current study aims to understand the crucial role of both CARD-9 and Dectin-1 in the pathogenesis of CRC patients and their relationship with aflatoxin-B1 serum levels in two patient groups (newly diagnosed and under treatment) to assess the therapeutic effect of routine chemotherapy on these markers which consider an innate immunological marker that can promote inflammation.

## 2. Materials and Methods

### *2.1. Study design*

The present study included 60 male patients with an age range of 25-75 years. Colorectal cancer, including all stages of the disease Tumor, Node, Metastasis (TNM). Patients were divided into two groups in this investigation: 30 newly diagnosed CRC patients (no treatment, no surgery) and 30 CRC patients under treatment with chemotherapy (Folfox, Xeloda, and Oxaloplatin). The recommended dosage of Xeloda is 1.250 mg/$m^2$ orally twice daily for the first 14 days of each 21-day cycle for a maximum of 8 cycles. Most often as Folfox-6:

Oxaloplatin 85 mg/m$^2$ + leucovorin 400 mg/m$^2$ intravenous (IV) then bolus of 5-fluorouracil (5-FU) 400 mg/m$^2$ and 46-hour infusion of 5-FU 2300 mg/m$^2$.
Oxaloplatin administration is via intravenous infusion (IV) over two hours. In cases of acute toxicity, the administration is extended to 6 hours. The recommended infusion rate is 1mg/m$^2$/minute or an 85 mg/m$^2$ dose over 85 minutes every two weeks [15]. All patients were diagnosed by consultant physicians (oncologists), who determined the tumor location using the ICD-O version 10. The present study also included 28 healthy subjects as a control group, without a family history of cancer. They are not subject to the effects of any medicines or dietary supplements. Healthy subjects were matched with patients in terms of age and sex.

*2.2. Exclusion criteria*

Exclusion criteria: Patients with autoimmune diseases, other types of cancer, or kidney failure.

*2.3. Samples collection*

The samples were collected from four hospitals in Medical City: Digestive and Liver Disease Teaching Hospital, Baghdad Teaching Hospital, Al-Amal National Hospital, and Al-Kadhimiya Teaching Hospital during the period from 15$^{th}$ October 2023, to 10th January 2024.
From each participant, 3 ml of venous blood was withdrawn. The blood was placed in a gel tube. Then centrifuged at 3000 rpm for 10 minutes. Serum samples were isolated and placed in Eppendorf tubes and frozen at -20 °C until use

*2.4. Principle of ELISA Kits*

The levels of aflatoxin-B1 were measured using a direct competitive ELISA method (Sun Long Biotech, China), categories as (SL0002Ot). The AFT-B1 ELISA kit employs a competitive assay using anti-AFT-B1 antibody and AFT-B1-HRP conjugate. Samples, buffer, and conjugate are incubated on a pre-coated plate for one hour. After washing, a substrate is added, producing a color change measured at 450 nm. The intensity of the yellow color is inversely proportional to the AFB-1 concentration, as sample AFT-B1 competes with the HRP conjugate for binding. A standard curve relates optical density to AFT-B1 concentration.
CARD-9 and Dectin-1 proteins in serum samples were measured using a commercial double-antibody sandwich ELISA kit (ELK biotechnology, USA) category numbers are (ELK0362, ELK7555). In this method, target antigens are sequentially bound by particular antibodies. The target antigen from the sample binds to a capture antibody immobilized on the ELISA plate. A secondary detection antibody linked to an enzyme (e.g., horseradish peroxidase) binds to the captured antigen-antibody complex. The enzyme subsequently combines with a chromogenic substrate to produce a colorimetric product whose optical density (OD) is proportional to the sample's target antigen concentration. CARD-9 and Dectin-1 levels were quantified by comparing sample OD values to a standard curve produced using known antigen concentrations from the ELISA kit. To ensure assay accuracy and consistency, the manufacturer's guidelines for sample preparation were obeyed.

*2.5. Statistical analysis*

Statistically, all data were analyzed using the SPSS program. An independent t-test and a one-way ANOVA test were used to measure the p-value using Least Significant Differences (LSD), as well as the Pearson chi-square and ROC tests. All data were presented as mean ±S.E. and p-value <0.05 was considered a significant difference Correlation was analyzed by person correlation coefficient Receiver operating characteristic (ROC) curve was used to find cut-off values and to evaluate some parameters as diagnostic markers .data were considered as significant (s) at p≤0.05, high significant (H.S) at p≤0.01 and non-significant (N.S) at P >0.05 [23].

*2.6. Ethical Approval and Study Design*

Ethical Approval and consent to participate, according to the Helsinki Declaration, Ethical permission has been obtained from the Iraqi, Ministry of Health, specifically the department in Baghdad Medical City (42768 on 9/11/2023), subject to the agreement of the patients. The oncologist referred suitable patients.

## 3. Result

*3.1. Serum level of all parameters in patients and controls*

The study results indicate differences in the serum levels of aflatoxin-B1, CARD-9, and Dectin-1 among control subjects, newly diagnosed patients, and patients under treatment, as shown in Table 1. For aflatoxin-B1, no statistically significant differences were observed among the groups; control subjects exhibited a (mean±SE) concentration of (0.149 ±0.028), newly diagnosed patients (0.133 ±0.013) ng/ml, and under-treatment patients (0.230 ±0.068) ng/ml ($p = 0.24$).

CARD-9 serum levels were significantly elevated in newly diagnosed patients (6.77 ±0.60) ng/ml, $p < 0.001$ compared to control subjects (3.57 ±0.44) ng/ml, and the under-treatment patients (6.74 ±1.39) ng/ml. This suggests that treatment may not significantly affect CARD-9 levels, as no notable differences were found between the newly diagnosed and under-treatment groups.

Dectin-1 levels followed a similar trend, being significantly higher in newly diagnosed patients (0.939 ±0.195) ng/ml, $p = 0.002$) compared to controls (0.264 ±0.044) ng/ml. Although treatment reduced Dectin-1 levels (0.594 ±0.100) ng/ml, no statistically significant difference was recorded ($p = 0.06$, NS).

**Table 1:** Serum level distribution of studied markers among the studied groups (control, newly diagnosed, and under treatment).

| Groups | Parameter concentration (Mean±S.E.) | | |
|---|---|---|---|
| | Aflatoxin-B1 (ng/ml) | CARD-9 (ng/ml) | Dectin-1 (ng/ml) |
| Control | 0.149±0.028 | 3.57±0.44 | 0.264±0.044 |
| newly diagnosed patients | 0.133±0.013 | 6.77±0.60 | 0.939±0.195[a] |
| Under the treatment diagnosed patients | 0.230±0.068 | 6.74±1.39 | 0.594±0.100 |
| p-value | 0.24 NS | <0.001** | 0.002** |
| Between new and under treatment | - | - | - |
| p-value | 0.1 NS | <0.001** | 0.06 NS |

NS= Non-significant, a significant difference vs. control,** highly significant

Table 2 presents the association of aflatoxin-B1, CARD-9, and Dectin-1 levels across three colon cancer grades in newly diagnosed and under-treatment patients. In newly diagnosed patients, aflatoxin-B1 levels were slightly higher in Grade III (0.159±0.018 ng/ml) compared to Grades I and II (0.154±0.028 and 0.104±0.007 ng/ml), respectively, but the differences were not statistically significant ($p=0.17$). In under-treatment patients, aflatoxin-B1 levels were high in Grade II (0.278±0.119 ng/ml), though there were no significant differences across the grades ($p=0.67$). A significant difference ($p=0.01$) was observed between newly diagnosed and under-treatment patients in Grade III. CARD-9 levels were significantly higher in newly diagnosed patients with grade III (9.81±0.70 ng/ml) compared to Grade I patients (6.02±0.72 ng/ml) ($p=0.044$). In under-treatment patients, CARD-9 levels were elevated in Grade II (8.76±2.51 ng/ml) compared to Grades I and III (4.46±0.51, 4.27±0.30 ng/ml), respectively, but these differences had no statistically significant impact ($p=0.3$). A highly significant reduction was seen between newly diagnosed and under-

treatment with Grade III (p=0.001). Dectin-1 levels showed no significant differences among grades in newly diagnosed patients (0.784±0.356, 1.150±0.269, and 0.761±0.315 ng/ml), respectively (p=0.66). However, under-treatment patients in Grade III had significantly higher levels of Dectin-1 (1.707±0.033 ng/ml) compared to Grade I (0.392±0.071 ng/ml) and Grade II (0.523±0.128 ng/ml) (p<0.001). A significant difference was observed between newly diagnosed and under-treatment patients for Grades II and III p=0.03 and p=0.05, respectively.

**Table 2:** The serum level distribution of aflatoxin-B1, CARD-9, and Dectin-1 levels across the studied groups based on the grade

| Patients groups | Grade groups | Parameter concentration (Mean±S.E.) | | |
|---|---|---|---|---|
| | | Aflatoxin-B1 (ng/mL) | CARD-9 (ng/mL) | Dectin-1 (ng/mL) |
| Newly diagnosed patients | I | 0.154±0.028 | 6.02±1.07 [a] | 0.784±0.356 |
| | II | 0.104±0.007 | 6.59±0.72 | 1.150±0.269 |
| | III | 0.159±0.018 | 9.81±0.70 [a] | 0.761±0.315 |
| p-value | | 0.17 NS | 0.044* | 0.66 NS |
| Under the treatment patients | I | 0.104±0.007 | 4.46±0.51 | 0.392±0.071 [b] |
| | II | 0.278±0.119 | 8.76±2.51 | 0.523±0.128 [b] |
| | III | 0.073±0.010 | 4.27±0.30 | 1.707±0.033 [b] |
| p-value | | 0.67 NS | 0.3 NS | <0.001** |
| Between newly and under the treatment patients | I | | | |
| p-value | | 0.5 NS | 0.2 NS | 0.3 NS |
| Between newly and under the treatment patients | II | | | |
| p-value | | 0.2 NS | 0.45 NS | 0.03* |
| Between newly and under the treatment patients | III | | | |
| p-value | | 0.01* | 0.001** | 0.05* |

Similar letters mean significant differences within the same group, NS= no significance, **= highly significant.

*3.2. Distribution of all studied parameters, serum levels according to stage*

Table 3 presents the distribution of the three studied parameters in the studied groups according to stage. aflatoxin-B1 has no significance among disease stages in newly diagnosed patients, and the results for stages I, II, III, and VI were (0.141±0.008; 0.145±0.029; 0.106±0.008; 0.162±0.024 ng/ml), respectively, p-value=0.6. CARD-9 and Dectin-1 also have no significance among disease stages between the two patient groups, with p-value = 0.3, 0.7, respectively. The results of CARD-9 and Dectin-1 for disease stages I, II, III, and IV were (6.08±2.12, 6.06±0.96, 7.04±0.86, 9.90±0.97; 0.463±0.141, 0.968±0.361, 1.171±0.332, 0.682±0.431 ng/ml), respectively. In patients under treatment, aflatoxin-B1 also has no significance among stages II, III, and IV, p-value=0.65. The results were (0.215±0.073; 0.173±0.052; 0.334±0.233 ng/ml). CARD-9 serum level has no significance among disease stages, the results were (4.39±0.70; 9.43±3.26; 5.63±1.19 ng/ml). p-value= 0.27, whereas Dectin-1 recorded a highly significance among diseases stage with p value =0.001, the results were (0.505±0.151; 0.304±0.045; 1.139±0.23 ng/ml). About the comparison between the two patient groups for each stage, we noted that aflatoxin-B1 has no significant p-value (0.34, 0.2, 0.67) for each stage. CARD-9 has no significant differences between stage II and III, p-value (0.2, 0.5), while a significance was recorded between the two patient groups, stage IV, p = 0.5. Dectin-1 has no significant differences between the two patient groups with stage I and

IV, p value (0.3, 0.34). In contrast, a significant difference was recorded between the two patient groups with stage III, P value = 0.03.

**Table 3:** Serum level distribution for three studied markers in two patient groups based on disease stage.

| Patients groups | Stage groups | Parameter concentration (Mean±S.E) | | |
|---|---|---|---|---|
| | | Aflatoxin-B1 (ng/ml) | CARD-9 (ng/ml) | Dectin-1 (ng/ml) |
| Newly diagnosed patients | I | 0.141±0.008 | 6.08±2.12 | 0.463±0.141 |
| | II | 0.145±0.029 | 6.06±0.96 | 0.968±0.361 |
| | III | 0.106±0.008 | 7.04±0.86 | 1.171±0.332 |
| | IV | 0.162±0.024 | 9.90±0.97 | 0.682±0.431 |
| p-value | | 0.6 NS | 0.3 NS | 0.7 NS |
| Under the treatment patients | II | 0.215±0.073 | 4.39±0.70 | 0.505±0.151 [b] |
| | III | 0.173±0.052 | 9.43±3.26 | 0.304±0.045 [b] |
| | IV | 0.334±0.233 | 5.63±1.19 | 1.139±0.23 [b] |
| p-value | | 0.65 NS | 0.27 NS | 0.001** |
| Between newly and under treatment patients | II | - | | |
| p-value | | 0.34 NS | 0.2 NS | 0.3 NS |
| Between newly and under treatment patients | III | - | | |
| p-value | | 0.2 NS | 0.5 NS | 0.03* |
| Between newly and under treatment patients | IV | - | | |
| p-value | | 0.6 7 NS | 0.05* | 0.34 NS |

Similar letters mean significant differences within the same group, NS= no significance, **= highly significant.

*3.3. Distribution of all studied parameters, serum levels according to type of treatment*

Table 4 illustrates the distribution of serum levels of the studied parameters in patients under treatment. Aflatoxin-B1 has no significant difference among the three types of treatment (Folfox, Oxaloplatin, Xeloda). The results were (0.184±0.058; 0.324±0.185; 0.179±0.079 ng/ml), respectively, with p-value = (p =0.63). CARD-9 also has no significance among the three types of treatment. The results were (8.81±3.05; 5.95±1.05; 4.02±0.58 ng/ml), respectively, with p value = 0.4. In comparison, Dectin-1 recorded a significance among the three types of treatment (0.433±0.114; 0.956±0.219; 0.375±0.098), p value=0.03.

**Table 4:** Serum levels distribution among the three types of treatment in patients under treatment group.

| Patients groups | Type of treatment | Parameter concentration (Mean±S.E.) | | |
|---|---|---|---|---|
| | | Aflatoxin-B1 (ng/ml) | CARD-9 (ng/ml) | Dectin-1 (ng/ml) |
| Under the treatment patients | Folfox | 0.184±0.058 | 8.81±3.05 | 0.433±0.114[a] |
| | Oxaloplatin | 0.324±0.185 | 5.95±1.05 | 0.956±0.219[a] |
| | Xeloda | 0.179±0.079 | 4.02±0.58 | 0.375±0.098[a] |
| p-value | | 0.63 NS | 0.4 NS | 0.03* |

Similar letters mean significant differences within the same group.

*3.4. The Correlation among the studied parameters*

Table 5 explores the relationship among the three studied parameters. aflatoxin-B1 does not correlate with either CARD-9 or Dectin-1. CARD-9 has a very weak negative correlation with aflatoxin-B1, the correlation was (-0.059), and there was no correlation with Dectin-1, Dectin-1 recorded a very weak negative correlation with aflatoxin-B1, r = (-0.041), and a weak positive correlation with CARD-9 r=(0.055).

**Table 5:** Correlation between immunological factors in colorectal cancer.

| Parameter | | Aflatoxin-B1 | CARD-9 | Dectin-1 |
|---|---|---|---|---|
| Aflatoxin-B1 | Pearson Correlation | 1 | | |
| | Sig. (2-tailed) | | | |
| CARD-9 | Pearson Correlation | -0.059 | 1 | |
| | Sig. (2-tailed) | 0.583 | | |
| Dectin-1 | Pearson Correlation | -0.041 | 0.055 | 1 |
| | Sig. (2-tailed) | 0.704 | 0.608 | |

*3.5. The correlation among the studied parameters*

This test is used to identify a good predicted marker using Receiver Operating Characteristic (ROC) curve analysis. ROC and Area under the Curve (AUC) are measurement points used to determine how tests can distinguish between patients and healthy individuals

As shown in Table 6 and Figure 4, which illustrate the results of the ROC test for Aflatoxin-B1, no significant differences were observed for Aflatoxin-B1 and Dectin-1. However, CARD-9 showed significant differences. AUC was 0.53, and the sensitivity and specificity were 83% and 37%, respectively, while the cut-off was 0.0825. This evidence shows that aflatoxin-B1 may be a fair marker for the detection of CRC disease.

The statistical analysis using the ROC test of CARD-9 data revealed a high AUC of CARD-9, which was 0.75, whereas the sensitivity was 63% the specificity was 90%, and the cut-off was 5.99 (Table 6 and Figure 4). Thus, these results supported the theory that CARD-9 could be used as a good biomarker for detecting the disease.

The results of the current study are illustrated in Table 6, using the ROC test of Dectin-1 observed AUC (0.78) with no significant difference ($p<0.6$), and the sensitivity of 67% and specificity of 93%, while the cut-off was 1.66. This evidence supports the idea of using Dectin-1 levels as a good marker for detecting CRC.

**Table 6:** ROC curve results for all studied parameters in patients with colorectal cancer compared with controls.

| Parameters | AUC | Cut off | Sensitivity | Specificity | p-value |
|---|---|---|---|---|---|
| Aflatoxin-B1 | 0.53 | 0.0825 | 83% | 37% | 0.076 NS |
| CARD-9 | 0.76 | 5.99 | 63% | 90% | <0.001** |
| Dectin-1 | 0.78 | 0.66 | 67% | 93% | 0.06 NS |

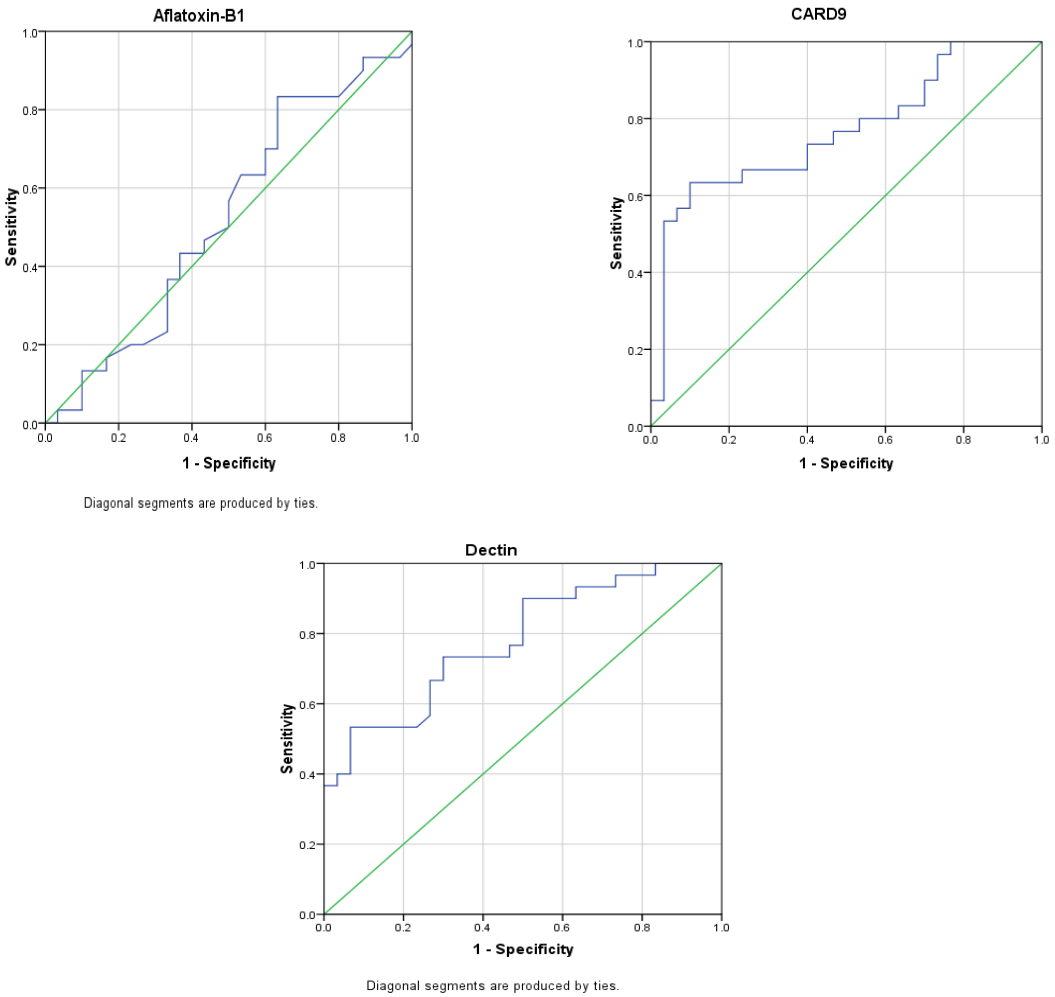


**Figure 1:** The ROC curve of all parameters in the studied groups.

## Discussion

The findings of this study demonstrate distinct patterns in serum levels of aflatoxin-B1, CARD-9, and Dectin-1 among control subjects, newly diagnosed patients, and those under treatment.

The lack of statistically significant differences in aflatoxin-B1 levels between control, newly diagnosed, and under-treatment groups suggests that aflatoxin-B1 may not be directly involved in the early progression or therapeutic response in this context. A study by Marchese *et al*., supports this finding, where no correlation between aflatoxin-B1 levels and cancer progression was found, particularly in CRC patients [24]. Aflatoxin-B1 undergoes phase metabolism in the liver, then is excreted in the bile and urine after conjugation [25]. This may be the reason for the decreased serum level and the absence of significant differences among the studied groups [26].

In the current study, CARD-9 serum levels recorded a highly significant increase in two patient groups versus the control group, p-value =0.001. This result is consistent with the study by Pagano *et al*, which reported an increased level in patients and its link with poor survival, implying its sustained engagement in inflammation [27]. A study revealed that its levels may be reduced after successful chemotherapy in patients with lymphoma; it is also possible that the levels vary depending on the cancer type and chemotherapy characteristics [28].

Dectin-1 levels were significantly higher in newly diagnosed patients compared to the control treatment, leading to a reduction; however, the difference between newly diagnosed and under-treatment patients was not statistically significant. This suggests that while treatment impacts Dectin-1 levels, its effect may be limited. The study by Peng *et al*, found similar results, where Dectin-1 played a critical role in immune responses and remained elevated in chronic inflammatory conditions [25]. On the other hand, Pagano *et al*, [23], and Li *et al*, [29] studies reported persistent Dectin-1 elevation even after treatment in some patients, highlighting the complex interplay between treatment and immune modulation.
Aflatoxin-B1 levels were slightly higher in Grade III compared to Grades I and II, although the differences were not statistically significant for grade in newly diagnosed patients. This suggests that aflatoxin-B1 levels may not strongly correlate with colon cancer grade. These findings align with research by Gong *et al*, [30], was reported that aflatoxin-B1 levels tend to vary inconsistently in cancer patients, possibly due to differing environmental exposures or genetic susceptibility. In under-treatment patients, the highest aflatoxin-B1 levels were observed in Grade II patients, though there were no significant differences between grades. Newly diagnosed patients had significantly higher aflatoxin-B1 levels than under-treatment patients in Grade III, and therefore, our results suggest that treatment might help reduce aflatoxin-B1 levels in the more advanced cancer stages.

In newly diagnosed patients, the mean CARD-9 was higher in Grade III than in Grade I. This implies that CARD-9, the protein involved in regulating innate immune response, may be more active at the progressive stages of cancer. This is further in line with a study reported that increased CARD-9 levels indicate poor cancer prognosis [31]. For under-treatment patients, CARD-9 was highest in Grade II, but there were no significant differences among the grades. Thus, considerable differences between newly diagnosed and under-treatment Grade III patients for CARD-9 allow for the discussion of the influence of treatment on immune responses, as a study mentions that CARD-9 is suppressed by successful cancer therapies [32].

There was no significant difference in Dectin-1 levels between newly diagnosed patients with different grades. The pattern recognition receptor that contributes to immune and fungal responses does not seem to be affected by cancer grade in the early stages of diagnosis. However, the under-treatment patients with Grade II had significantly higher Dectin-1 levels than patients treatment Grade I and II. This indicates that Dectin-1 may be upregulated in later cancer stages during treatment and can be attributed to cancer relevance. Similarly, for newly diagnosed and under-treatment patients, the differences for Grade II and Grade III Dectin-1 were statistically significant as well, adding more complexity to the understanding of the link between cancer growth, treatment, and Dectin-1. These observations are in line with those by Zheng *et al*, study, which noted that Dectin-1 can be upregulated in chronic inflamed cases of cancer patients [33].
For the disease stage, the results presented in Table 4 can give useful insights into the distribution of aflatoxin-B1, CARD-9, and Dectin-1 levels in newly diagnosed and under-treatment colon cancer patients at different stages of cancer. These results contribute to the identification of the stage-related behaviour of these factors and the action of the treatment process. Comparing the newly diagnosed patients by the stages, the aflatoxin-B1 levels remain comparatively lower and constant, with no marked differences. This means that the aflatoxin-B1 levels do not have a direct positive correlation with the cancer stage, as suggested by Gong *et al*, [30], and evidence that aflatoxin-B1 exposure rises with cancer progression is weak. Likewise, in the under-treatment patients, there were no differences across the stages, indicating that the treatment does not affect aflatoxin-B1 levels. The

absence of fluctuations in protein levels may mean that aflatoxin-B1 is not pivotal to stage-dependent disease development or therapeutic outcomes.

Mean CARD-9 levels were significantly higher in stage IV under-treatment patients than in other stages, but with no significant differences. Higher levels of CARD-9 in stage IV might also be a result of higher immune response in more advanced cancer, as Zhong *et al*, study, discovered that CARD-9 is a biomarker of poor prognosis in cancer [34]. In the under-treatment group, CARD-9 was downregulated in all stages, but most prominently in stage IV. This suggests that in advanced stages of cancer, treatment suppresses immune activation kindling and thus may provide a favorable prognosis by attenuating anti-tumour CARD-9 inflammation, and it was reported that CARD-9 acts as a central regulator to ensure long-lasting antitumor immunity [31].

Dectin-1 levels were highest in stage III newly diagnosed patients compared to earlier stages, though these differences were not statistically significant. Elevated Dectin-1 in more advanced stages may indicate increased immune challenges or fungal infections, as described by Zhang *et al* [31]. In under-treatment patients, significant reductions in Dectin-1 levels were observed in stage III ($p=0.03$), suggesting that treatment effectively modulates Dectin-1 levels, reducing immune disturbance. However, stage IV patients exhibited persistently high Dectin-1 levels of under-treatment, indicating that Dectin-1 may be more resistant to the treatment in advanced cancer stages.

For the type of treatment group, the aflatoxin-B1 level seemed not to vary much with the type of treatments given, which suggests that chemotherapy has little impact on aflatoxin-B1 metabolism and excretion. This supports previous research works asserting that the metabolism of aflatoxin-B1 differs with environment and genetics rather than with the treatment type [32]. This is in agreement with other research, which established that aflatoxin-B1 and its consequences have more relationships to the environment and DNA than to drug use, Cao *et al*, also discovered differences in aflatoxin-B1 concentrations can be more likely consistent with the level of tolerance in individuals than the type of treatment [26].

The mean level of CARD-9 protein expression was found to be highest in the patients receiving Folfox, but the comparison for the same across different treatments was insignificant; the CARD-9 protein levels may not change significantly. However, it could be seen that lower levels of CARD-9 in the patients treated with Xeloda might mean that there is more immune modulation that occurs with this treatment, similar to the work done by Zheng *et al*, indicating that some treatments modulate immunity more comprehensively [33]. The increased levels of CARD-9 in Folfox-treated patients, relative to the controls, indicate that immune functions or the pathways associated with innate immunity are more pronounced with this regimen, as elucidated by Zhong *et al* [34]. If true, this may suggest lower levels in Xeloda-treated patients are more representative of an immune suppressive effect that lessens CARD-9-mediated inflammation.

Based on the type of treatment, Dectin-1 serum level shows a consistency, the lowest level was in Xeloda followed by Folfox, then Oxaloplatin, and this is combined with a high significance. This marked difference indicates that Dectin-1, a molecule associated with immune response to fungal pathogens, may be more sensitive to some chemotherapeutic agents, and that Oxaloplatin may cause increased Dectin-1 expression. Such observations are in concordance with earlier findings by Zhang *et al*, who pointed out that the inverse of Dectin-1 levels differ after certain treatments and are indicators of immune activation and inflammation state [31] . The existence of such a result indicates that Oxaloplatin may more significantly affect the downregulation of Dectin-1, which may explain the activation of the immune response or fungal recognition pathways. In the Zhang *et al*, study, it was pointed out that there are treatments that can upregulate Dectin-1, and this is normally said in conditions that involve activation of the immune organs [33]. Aflatoxin-B1 undergoes phase metabolism

in the liver and is then excreted in bile and urine after conjugation, so this may explain the decreased serum level and absence of a significant difference among the three studied groups [24].

## 4. Conclusion

The results presented in this study can give useful insights into the distribution of aflatoxin-B1, Dectin-1, and CARD-9 levels in newly diagnosed and under-treatment colon cancer patients at different grades and stages of CRC. These results contribute to the identification of the stage-related behavior of these biomarkers and the action of the treatment process. CARD-9 is associated with the development of colon cancer and functions as a regulator of antitumor immunity, suggesting that the involvement of Dectin-1 in cancer needs to be evaluated based on the type and stage of the malignancy. This indicates that some treatments modulate immunity more comprehensively. Inverse Dectin-1 levels differ after certain treatments and are indicators of immune activation and inflammation state. We can conclude that Dectin-1 and CARD-9 may be considered good biomarkers to evaluate the efficiency of treatment in patients with colorectal cancer, but aflatoxin-B1 may not be directly involved in the early progression or therapeutic response.

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## Conflict of interest

There are no conflicts of interest.

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