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# Immunohistochemical Expression of CD163 (Macrophage Marker) in Oral Epithelial Dysplasia Induced in Experimental Animals and its Relation to Microvessel Density (An animal Study)

Mohammed Sheikh[1], Prof. Dr. Louloua Fathy[2], Ass. Prof. Dr. Nermine Raouf[3]
*1 Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University, Egypt)*
*2(Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University, Egypt) 3(Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Cairo University, Egypt)*
*Corresponding auther: Mohammed Sheikh*

***Abstract:****CD163, is significantly associated with the aggressive behavior of oral squamous cell carcinoma (OSCC). Although the phenotypes of tumour associated macrophages (TAMs) in different types of solid tumors have been extensively characterized, the phenotypes and functional properties of the TAMs that infiltrate premalignant lesions remain to be determined. The expression of the angiogenic activity has been shown to be an early and predictable characteristic of many pre- neoplastic cells, and may represent one of the earliest indications that a cell population has become committed to malignancy. Our study aims to Point out new markers that could be used for early detection of molecular changes prior to malignant transformation. A total of 48 adult male albino rats were selected. Our results showed a highly significant difference in both area percentage of CD163 immunoexpression and mean number of CD34+ blood vessels among the different experimental groups after 6 and 9 weeks of experiment.*

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## I. Introduction

Oral epithelial dysplasia (OED) is a histopathological diagnosis that is associated with an increased risk of oral cancer. The role of tobacco and alcohol, as the two major risk factors, have been well documented (Jaber, 2010). Moreover, diet as well as tobacco and alcohol have been implicated in the large increase in oral leukoplakia which is the lesion classically associated with dysplastic changes. Of these, tobacco use and alcohol are identified as major risk factors, but interaction and/or summation of all factors may play a role (Bánóczy et al., 2001). Numerous grading systems for OED exist with varying sets of assessing criteria and are largely considered subjective (Krishnan et al., 2016). Kujan et al. (2006) assessed the reproducibility of a novel binary grading system (high/low risk) of OED and compared it with the World health organization (WHO) classification 2005. TAMs are a major cellular component in the tumor microenvironment of many solid tumors. The classically activated M1 macrophages (CAMs) exhibit antitumor functions, whereas the alternatively activated M2 macrophages (AAMs) exhibit protumor functions that contribute to tumor development and progression. Although TAMs have been detected in oral squamous cell carcinoma (OSCC), little is known about their phenotype (Mori et al., 2011).

Mori et al. (2015) demonstrated that although the phenotypes of TAMs in different types of solid tumors have been extensively characterized, the phenotypes and functional properties of the TAMs that infiltrate premalignant lesions remain to be determined. CD163 is a member of scavenger receptor super family class B of the first subgroup. It is selectively expressed on cells of monocytes and macrophages lineage exclusively (Onofre et al., 2009). Angiogenesis is the process of forming new blood vessels from existing vascular networks. It is a critical event which is essential for the growth and persistence of solid tumors and their metastasis (Jiménez and Volpert, 2001). Angiogenic activity has been shown to be an early and predictable characteristic of many pre-neoplastic cells, and may represent one of the earliest indications that a cell population has become committed to malignancy. Studies have shown that vascularity increases from normal mucosa to moderate dysplasia to carcinoma (Carlile et al., 2001; Iamaroon et al., 2003 and Shieh et al., 2004). Nagatsuka et al. (2005) showed that specific antibodies against vascular endothelial cells are used for histopathological identification of microvessels in tumors. Anti-CD34 antibody is an antibody targeting the transmembranous CD34 that is detected in precursors of undifferentiated endothelial cells to differentiated endothelial cells.

## II. Material And Methods

A total of 48 adult male albino rats, maintained in the animal house as an inbred colony (obtained from the Faculty of Medicine, Cairo University, Egypt) were used in this study. Rats with an age range of 3 to 4 months and those with a weight range of 130 to200 gm were selected for carrying out this experiment

**Study Design:** An experimental study

**Study Location**: Animal house of the Faculty of Medicine, Cairo University, Egypt

**Sample size:** 48 adult male albino rats

**Sample size calculation:** A total sample size of 32 rats (8 in each of the 4 groups) will be sufficient to detect a 0.52 effect size with a power of 80% and 5% significant level. The number is increased to a total of 36 to allow for the use of non-parametric test. The sample is further increased to 48 (12 in each of the 4 groups) to allow for about 25% losses. The sample size was calculated using G*power program (University of Dusseldorf, Dusseldorf, Germany).

**Subjects & selection method**:

- Numbers from 1 to 48 were written on folded papers that were placed in opaque sealed envelopes.
- Matching of threats with the numbers was done blindly through the technician in charge at the animal house.
- Each rat was attached to its number till the end, then the numbers were opened and threats were allocated in their groups according to the program's recommendations

Rats were divided into two groups as follows: Group A: Control group.
Group B: Experimental group. Each group was further subdivided into two subgroups according to the time of sacrifice (after 6 and 9 weeks from the start of painting).
Group A (Control group):
Twenty four rats were housed under the same conditions and did not receive any treatment. Group B (Experimental group) :
Twenty four rats were anesthetized by ketamine 80-100 mg/kg intraperitonealy (IP) and xylazine 10-12.5 mg/kg (IP). The buccal mucosa was painted once with a number 3 camel hairbrush according to The INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE guidlelines (IACUC guidelines 2014). Then, the rats had their buccal mucosa painted (topically) with DMBA and formaldehyde, 0.5% DMBA in acetone 3 days/week, and after 9 days 10% formaldehyde/water was used side by side with DMBA throughout the study period (6 and 9 weeks).

**Inclusion criteria:**

1. Adult rats
2. White colored rats
3. Male rats
4. Rats with weight from 130 to200 gm
5. Healthy rats

**Exclusion criteria:**

1. Female rats
2. Rats with oral or systemic disorders

### Procedure methodology

A total of 48 adult male albino rats with an age range of 3 to 4 months and with a weight range of 130 to 200 gm were selected. The rats were randomly distributed into two groups: control and experimental groups. Each group was further subdivided into two subgroups according to the time of sacrifice (after 6 and 9 weeks from the start of painting). Twenty four rats were housed under the same conditions and did not receive any treatment. Twenty four rats were anesthetized by ketamine 80-100 mg/kg intraperitonealy (IP) and xylazine 10-12.5 mg/kg (IP). The buccal mucosa was painted once with a number 3 camel hairbrush . Then, the rats had their buccal mucosa painted (topically) with DMBA and formaldehyde, 0.5% DMBA in acetone 3 days/week, and after 9 days 10% formaldehyde/water was used side by side with DMBA throughout the study period (6 and 9 weeks). Sacrifice of rats was done at 6 weeks (group A1and B1) and 9 weeks (group A2 and B2) interval from the start of painting, 12 rats for each interval . The rats were sacrificed by an overdose of the anesthetic agents (1ml/100gm). The dissected buccal mucosa of rats were processed routinely to obtain 5µ thick sections. These sections were examined histologically by H&E stain and immunohistochemically by CD163 a mouse anti-rat monoclonal antibody as a macrophage marker and also with Cd34, a mouse anti-rat monoclonal antibody as a marker for endothelial cells to evaluate angiogenesis. Moreover, the area percentage of CD163 immunoexpression and the mean number of CD34+ blood vessels in the different intervals were measured by

software Leica Qwin 500.

### Statistical analysis

Mean values for area percent of CD163 immunoreaction and mean number of CD34 positive blood vessels were collected. Data was expressed as mean ± standard deviation. Student T–test was used to compare between each of low risk and high risk groups with the control group and between the low and high risk groups. Results were considered significant at $P<0.05$ *.

Pearson test was used to correlate between area percentage of CD163 and number of CD34 positive blood vessels.

## III. Result

**I. Clinical findings:**

A total of 48 adult male albino rats with an age range of 3 to 4 months and with a weight range of 130 to 200 gms were selected.

After 6 weeks from the start of painting with (DMBA and formaldehyde) on the buccal mucosa of the experimental rats (group B1), white homogenous plaques appeared on their buccal mucosae compared with normal buccal mucosae of the control rats (group A1) which did not receive any treatment.

After 9 weeks from the start of painting, the experimental rats (group B2) showed white,wrinkled and ulcerative plaques on their buccal mucosae with facial asymmetry compared with normal buccal mucosae of the control rats (group A2) which did not receive any treatment. No loss of appetite was noticed in the experimental rats nor in the control rats during the experimental period.

**II. Microscopic findings:**

After 6 weeks from the start of drug painting , 10 cases showed mild dysplasia, 1 case showed hyperplasia and 1 case showed no obvious changes.

After 9 weeks from the start of drug painting, 6 cases showed moderate dysplasia, 5 cases showed severe dysplasia while 1 case showed mild dysplasia. Mild dysplasia cases and hyperplasia cases were considered in the low risk group, while the moderte and severe dysplasia cases were considered in the high risk group.

**A- Control group**

i. Histopathological examination of H&E stained sections in the control group

Examination of H&E stained sections showed a normal histologic appearance of the buccal mucosa of the control rats (groups A1and A2) with normal keratinized stratified squamous epithelium overlying normal connective tissue. No dysplastic changes were observed (fig. 1).

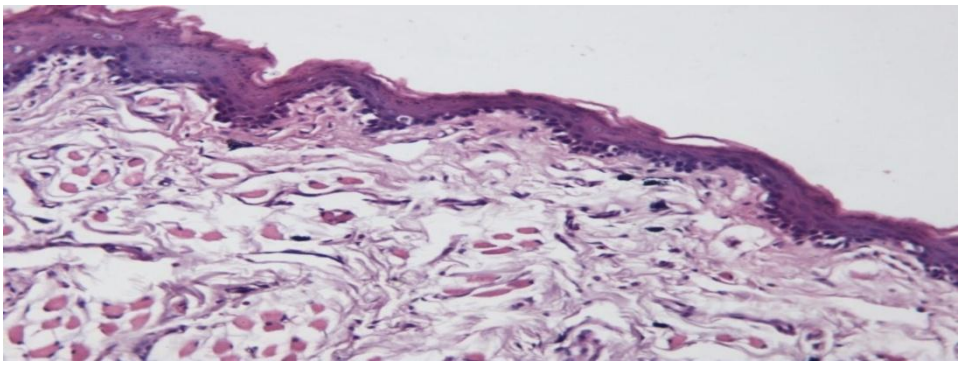

**Fig (1):** Photomicrograph of buccal mucosa of a rat in the control group revealing normal keratinized stratified squamous epithelium overlying normal connective tissue (H&E, x200).

ii. Immunohistochemical examination of CD163 stained sections in the control groups

CD163 is a specific marker for macrophages. CD163 positive macrophages appeared spindle-shaped and occasionally round-shaped cells. Immunostained sections of the buccal mucosa of the control group rats (groups A1 and A2) showed absence or few CD163 positive macrophages infiltrating the connective tissue in normal buccal mucosa of the control groups (figs. 2 and 3).

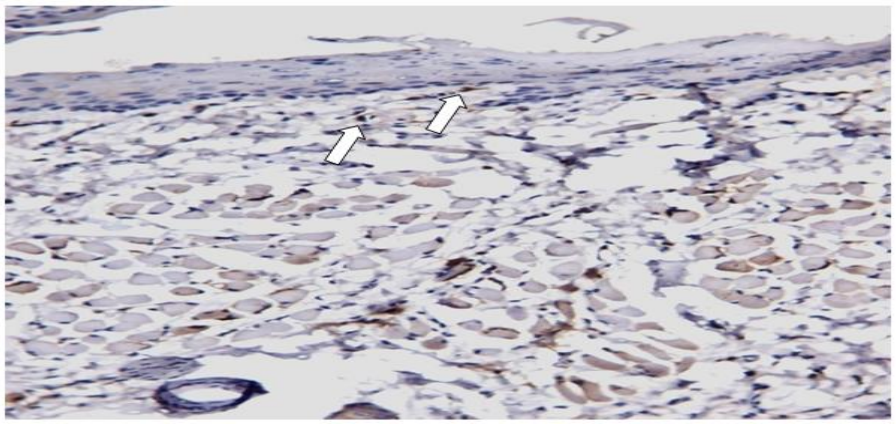

**Fig (2):** Photomicrograph showing few CD163+ macrophages (white arrows) in the connective tissue of the control group (CD163 x 200).

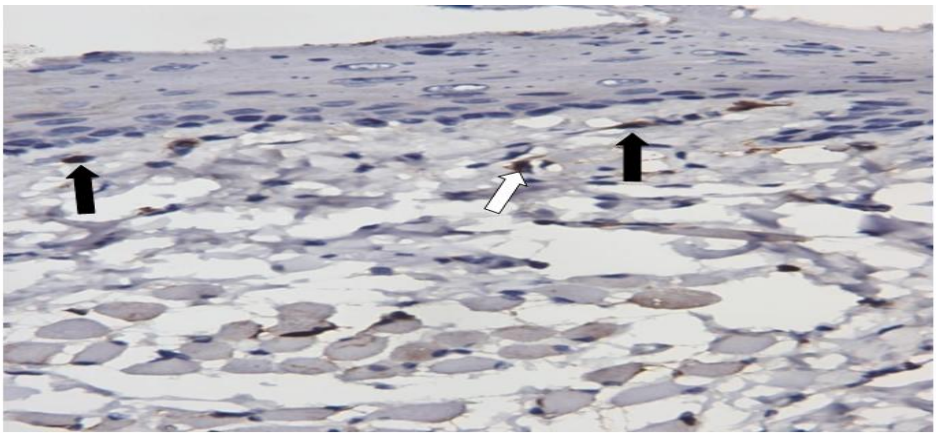

**Fig (3):** Higher magnification of the previous photomicrograph showing CD163+ macrophages in the connective tissue of the control group. Some macrophages were rounded (white arrow) and some were elongated (black arrows) (CD163 x 400).

iii. Immunohistochemical examination of CD34 stained sections in the control group

CD34 Immunostained sections of the buccal mucosa of the control group rats (groups A1 and A2) showed few newly formed blood vessels in the connective tissue. They were small in size and had a nearly rounded lumen. (figs. 4,5 and 6).

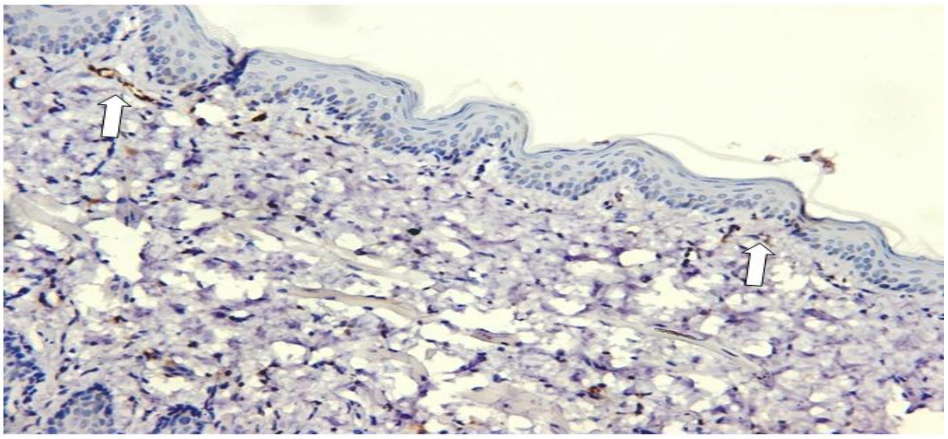

**Fig (4):** Photomicrograph showing few newly formed CD34 positive blood vessels (white arrows) in the connective tissue of normal buccal mucosa of the control group (CD34 ×200).

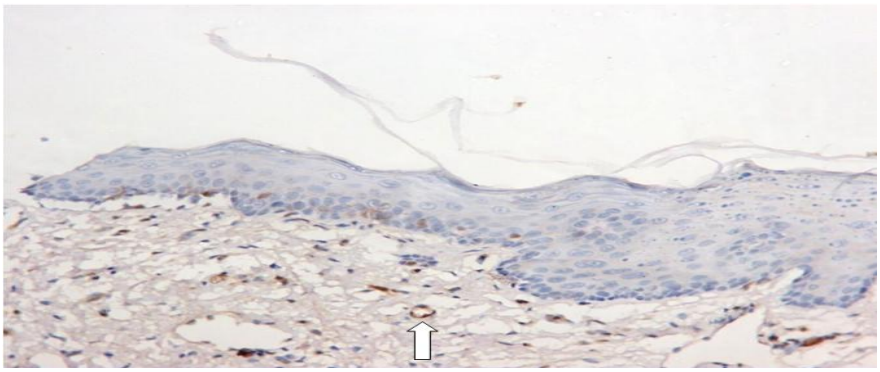

**Fig (5):** Photomicrograph showing few newly formed blood CD34 positive vessels with a rounded lumen (white arrow) in the connective tissue of normal buccal mucosa of the control group (CD34 ×200).

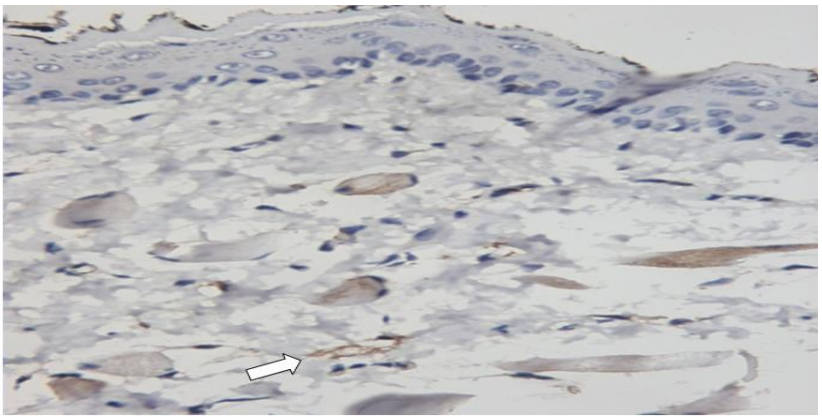

**Fig (6):** Photomicrograph showing cytoplasmic CD34 expression in endothelial cells lining the newly formed blood vessels (white arrow) in the connective tissue of normal buccal mucosa of the control group (CD34 ×400).

**B- Low risk OED group**

i. Histopathological examination of H&E stained sections in low risk OED group (group B1)

Examination of H&E stained sections of low risk OED group (group B1) revealed some dysplastic changes (cellular and architectural) occurring in the basal and parabasal layers of the epithelium. Cellular changes were characterized by abnormal variation in nuclear shape, increased N/C ratio and hyperchromatism. Architectural changes were characterized by bulbous or drop shaped rete ridges and loss of polarity of basal cells. The connective tissue showed few inflammatory cells infiltration (figs. 7, 8, 9,10,11,12,13 and 14).

**Fig (7):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing acanthosis, clubbing of the rete ridges and loss of polarity of basal cell layer ( black arrows) (H&E, x200).

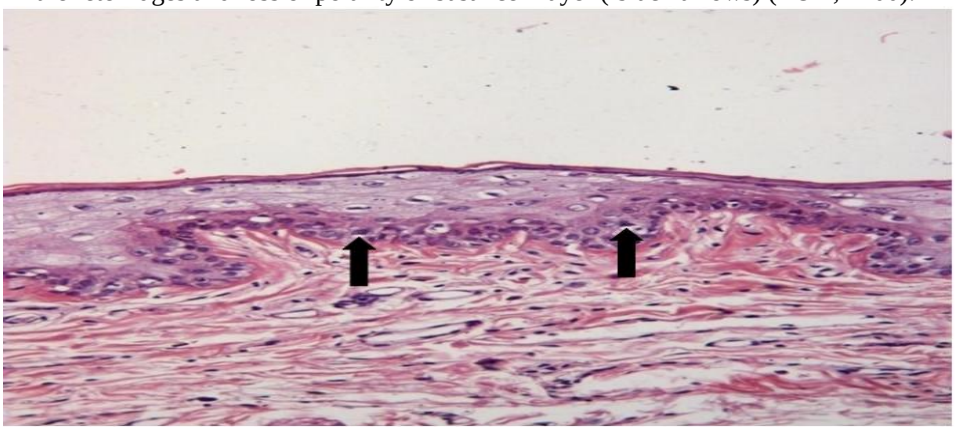

**Fig (8):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing nuclear pleomorphism and hyperchromatism (black arrows) in the lower third of the epithelial thickness. There is slight surface keratosis (H&E, x200).

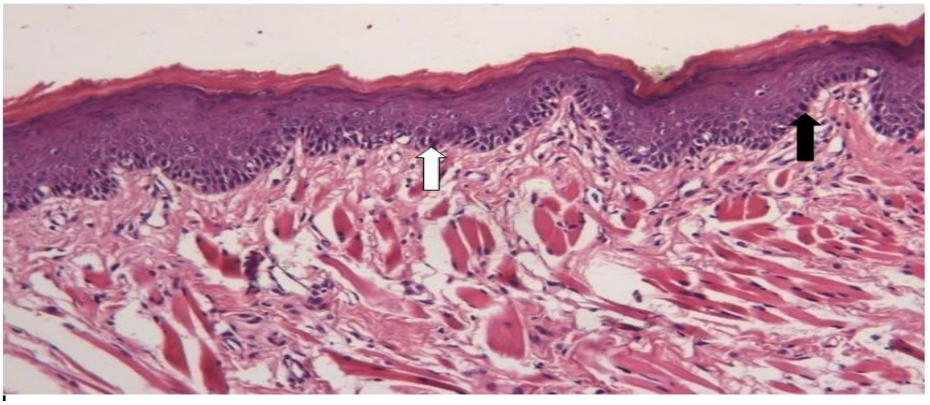

**Fig (9):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing excessive surface hyperkeratosis, nuclear pleomorphism (white arrow) and hyperchromatism (black arrow) in the lower third of the epithelial thickness (H&E, x200).

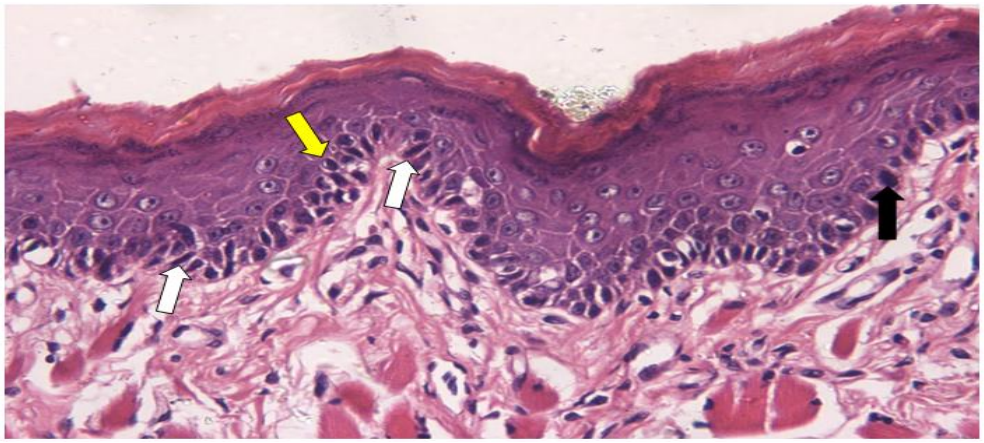

**Fig (10):** Higher magnification of the previous figure showing nuclear pleomorphism (white arrows), increased N/C ratio (black arrow) and hyperchromatism (yellow arrow) in the lower one third of the epithelial thickness (H&E, x400).

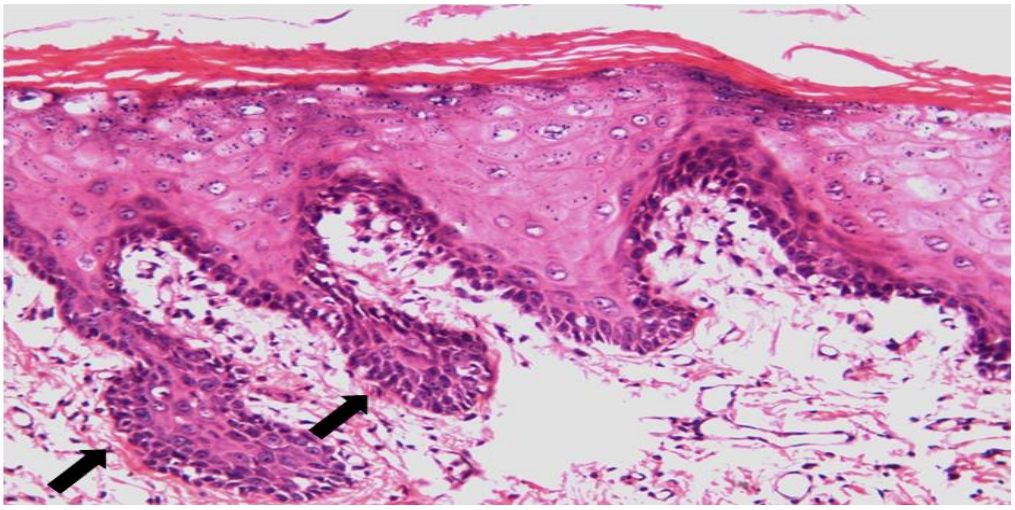

**Fig (11):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing excessive surface hyperkeratosis and club shaped rete ridges (tear drop) (black arrows) (H&E, x200).

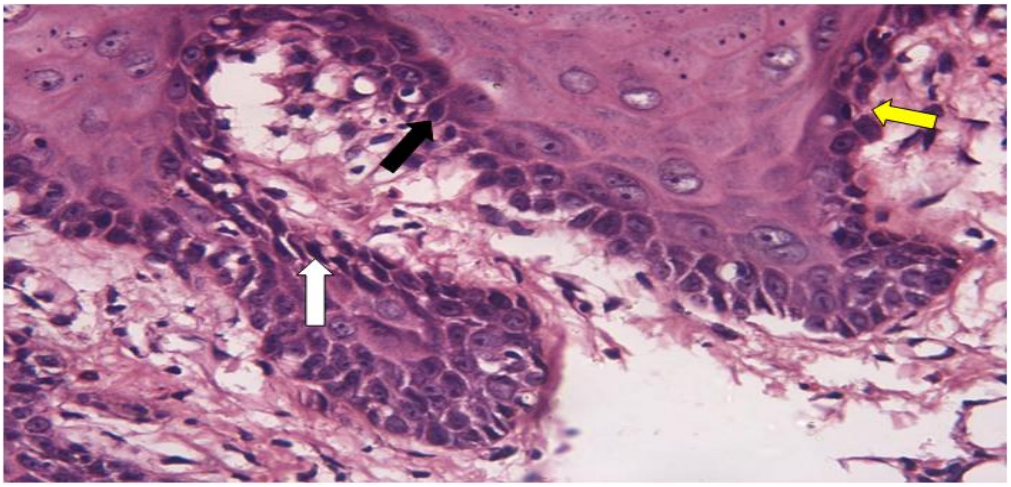

**Fig (12):** Higher magnification of the previous figure showing nuclear pleomorphism (white arrow) and incresed N/C ratio (black arrow) in basal and parabasal layers as well as loss of basal cell polarity (yellow arrow) (H&E, x400).

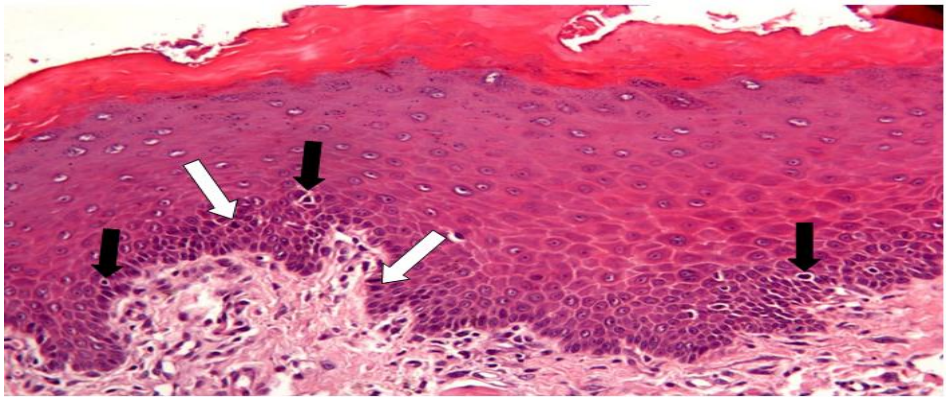

**Fig (13):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing nuclear hyperchromatism (black arrows), increased N/C ratio (white arrows) as well as nuclear and cellular pleomorphism in the lower one third of the epithelium (H&E, x200).

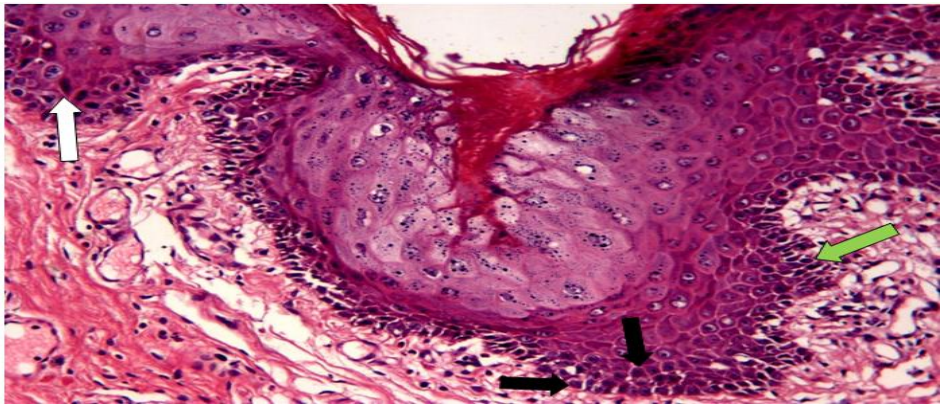

**Fig (14):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing pronounced acanthosis, surface hperkeratosis nuclear pleomorphism (white arrow) as well as increased N/C ratio (black arrows) and hyperchromatism (green arrow) in the lower one third of the epithelium. There are few inflammatory cells in the underlying connective tissue (H&E, x200).

ii. Immunohistochemical examination of CD163 stained sections of low risk OED group (group B1)

Immunostained sections of the buccal mucosa of low risk OED group (group B1) showed some CD163 positive macrophages infiltrating the connective tissue (figs.15,16,17,18,19and 20). Macrophages were seen around blood vessels (figs.15 and 16), under the dysplastic epithelium (figs.17 and 20) and sometimes diffusely scattered in the connective tissue (figs.18 and 19)

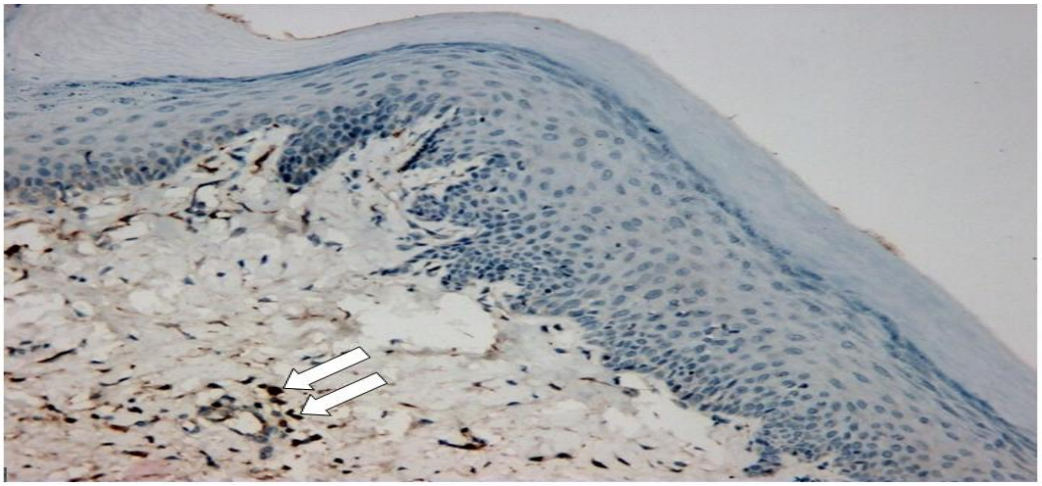

**Fig (15):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing CD163+ macrophages (white arrows) around blood vessels in the connective tissue (CD163 x 200).

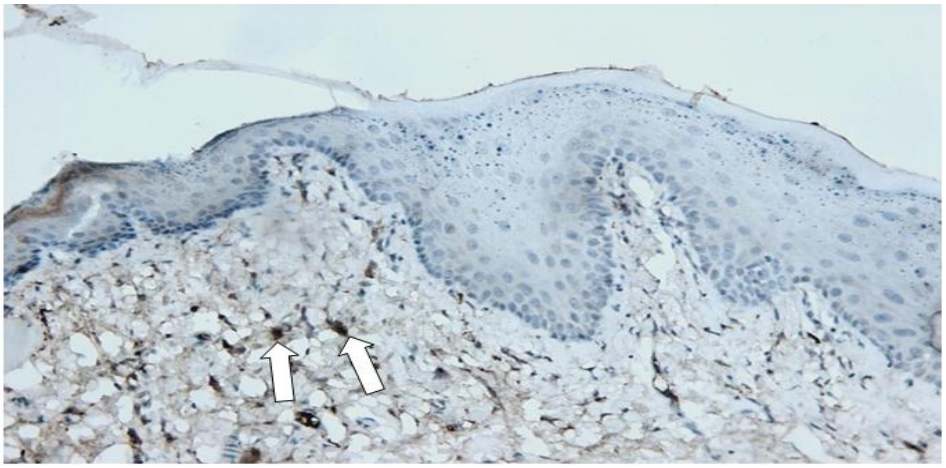

**Fig (16):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing few CD163+ macrophages (white arrows) deep in the connective tissue (CD163 x 200).

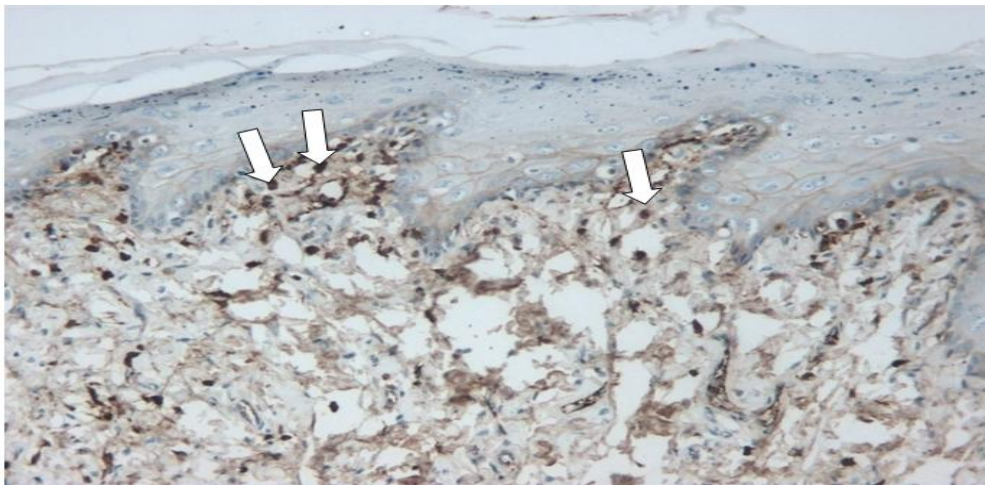

**Fig (17):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing CD163+ macrophages (white arrows) localized mainly under the dysplastic epithelium (CD163 x 200).

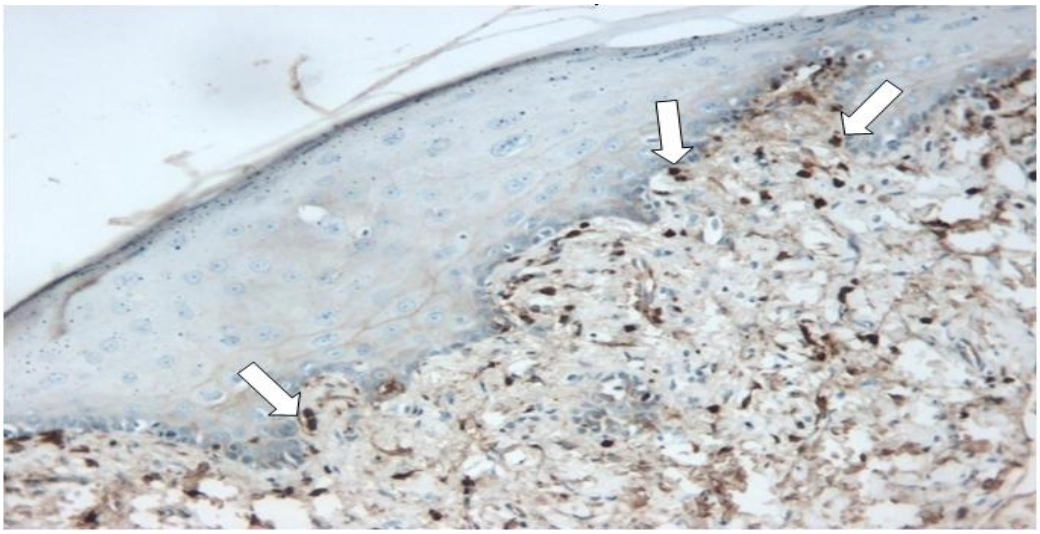

**Fig (18):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing prodominantely rounded CD163+ macrophages (white arrows) diffusely infiltrating the connective tissue (CD163 x 200).

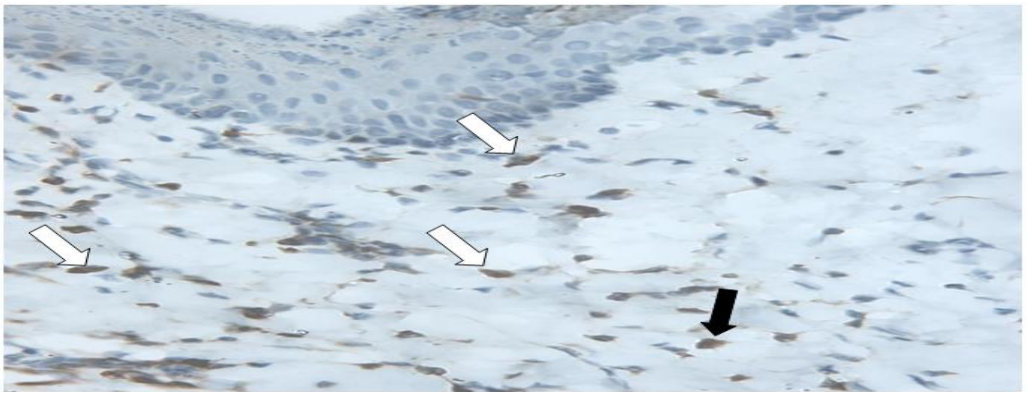

**Fig (19):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing spindle shaped (white arrows) and rounded (black arrows) CD163 positive macrophages arranged in a diffuse pattern in the connective tissue (CD163 x 400).

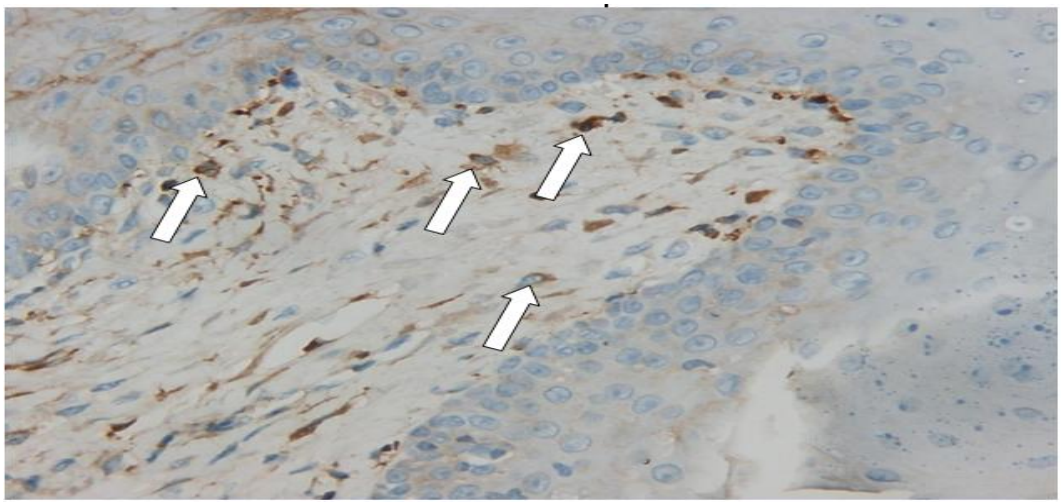

**Fig (20):** Photomicrograph of buccal mucosa of low risk OED group (group B1) showing CD163+ macrophages localized mainly under the dysplastic epithelium. Membranous and cytoplasmic expression are obvious in some cells (white arrows) (CD163 x 400).

iii- Immunohistochemical examination of CD34 stained sections in low risk OED group (group B1)

CD34 expression appeared in endothelial cells surrounding the newly formed vessels which appeared

small in size and had a nearly rounded lumen. there were few newly formed blood vessels in low risk OED group (group B1) and in most cases they were localized just underneath the dysplastic epithelium (figs. 21,22,23 and 24).

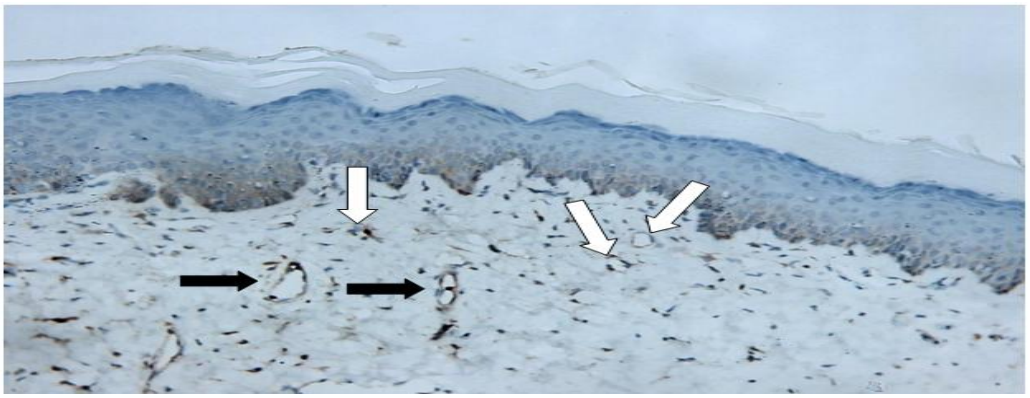

**Fig (21):** Photomicrograph of low risk OED group showing CD34 expression in endothelial cells lining the newly formed vessels (white arrows) as well as the well formed mature blood vessels (black arrows) (CD34 ×100).

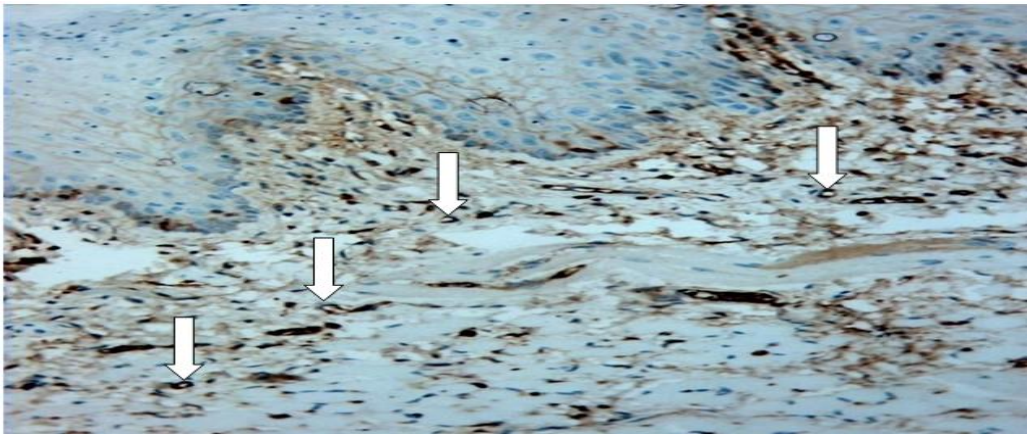

**Fig (22):** Photomicrograph of low risk OED group showing CD34 expression in endothelial cells lining the newly formed vessels (white arrows) which had a narrow and nearly rounded lumen (CD34 ×200).

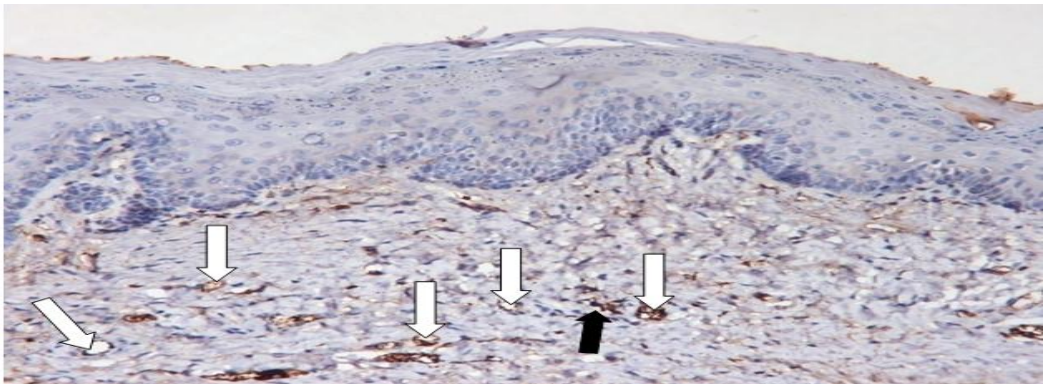

**Fig (23):** Photomicrograph of low risk OED group showing CD34 expression in endothelial cells lining the newly formed vessels (white arrows) as well as in groups of endothelial cells (black arrow) (CD34 ×200).

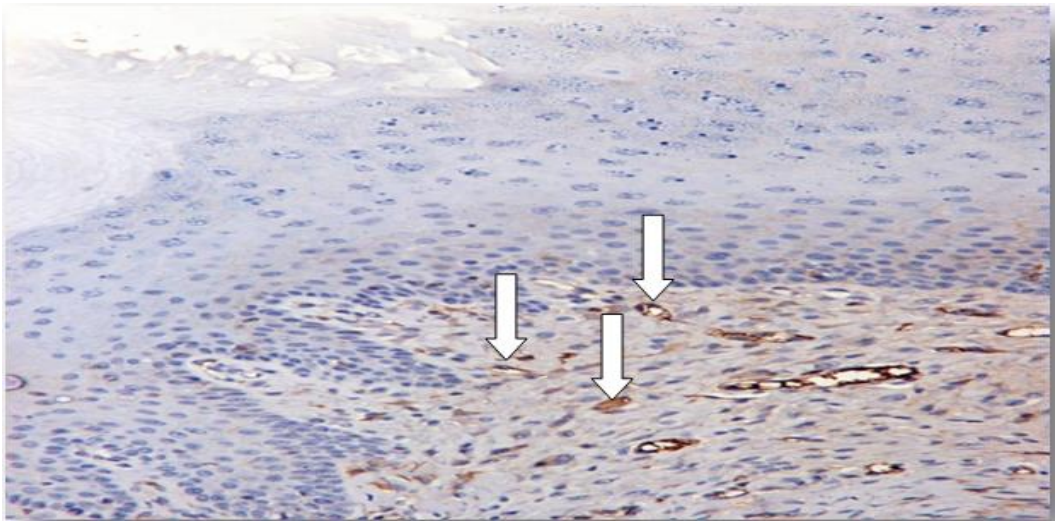

**Fig (24):** Photomicrograph of low risk OED group showing CD34 expression in endothelial cells lining the newly formed vessels (white arrows) which were localized just underneath the dysplastic epithelium (CD34 ×200).

**C- High risk OED group (group B2)**

i- Histopathological examination of H&E stained sections in high risk OED group (group B2)

Examination of H&E stained sections of high risk OED group (group B2) revealed epithelial dysplastic changes (cellular and architectural) in the basal cell layer and extending beyond the middle half of the epithelium. Cellular changes were characterized by abnormal variation in nuclear and cellular shape and size, increased N/C ratio and hyperchromatism. Architectural changes were characterized by irregular epithelial stratification, loss of polarity of basal cells, bulbous or drop shaped rete ridges and cell nest formation within the epithelium. The connective tissue showed intense inflammatory cell infiltration (figs.25,26,27,28,29,30 and 31).

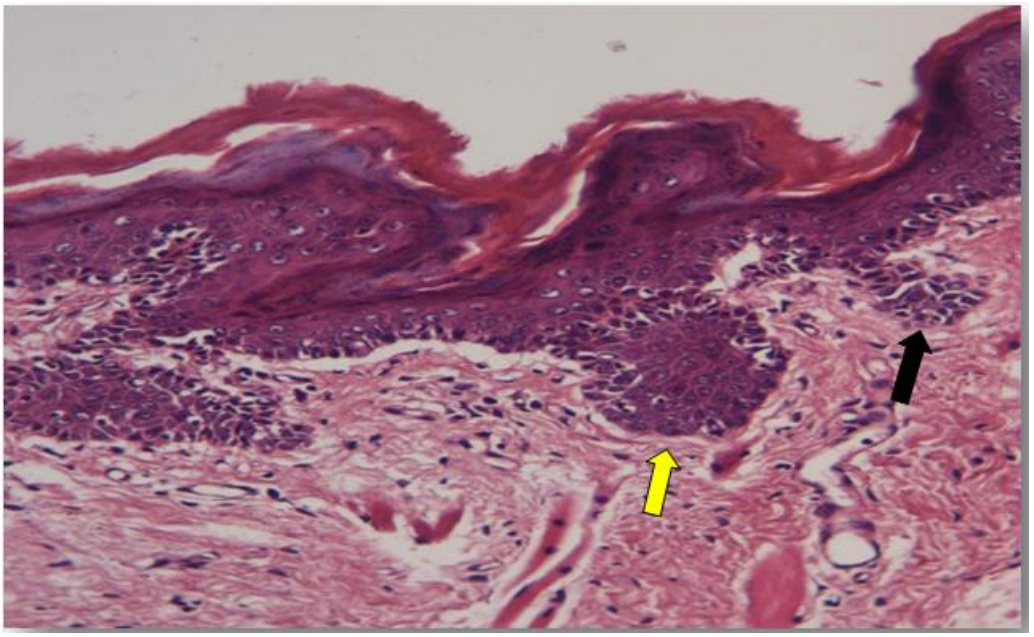

**Fig (25):** Photomicrograph of buccal mucosa of high risk group (B2) showing excessive surface hyperkeratosis, tear drop rete ridge (yellow arrow) and loss of basal cell polarity (black arrow) (H&E, x200).

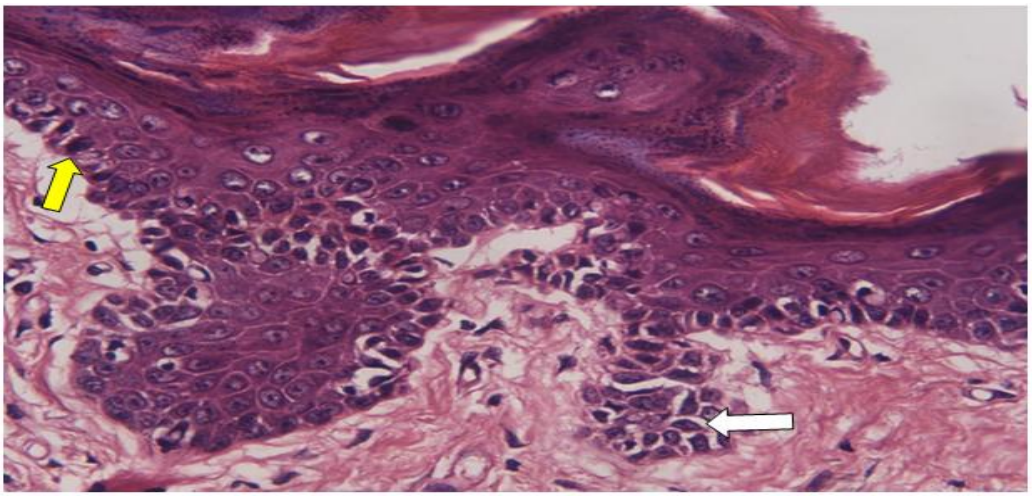

**Fig (26):** Higher magnification of the previous figure showing nuclear pleomorphism (white arrow), increased N/C ratio and hyperchromatism (yellow arrow) (H&E, x400).

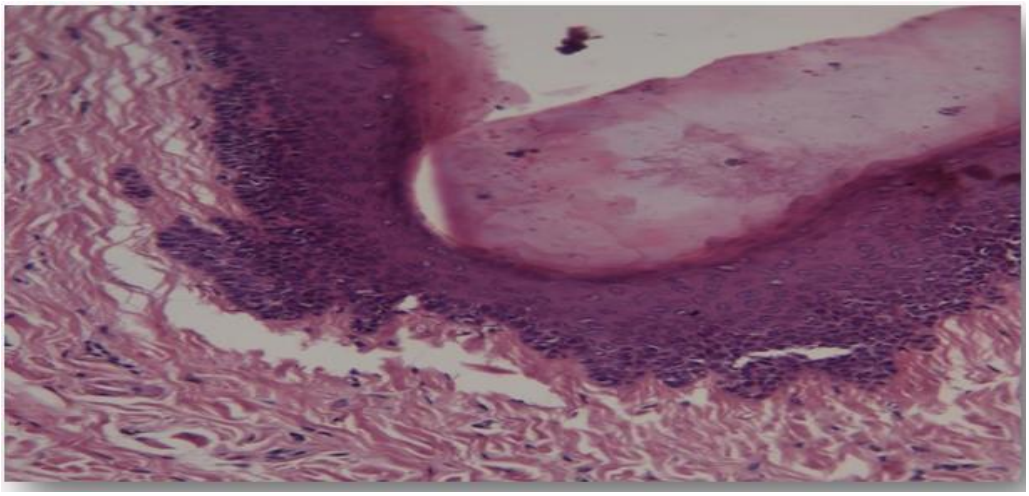

**Fig (27):** Photomicrograph buccal mucosa of high risk group (B2) showing nuclear hyperchromatism, pleomorphism and increased N/C ratio affecting the lower half of the epithelium (H&E, x200).

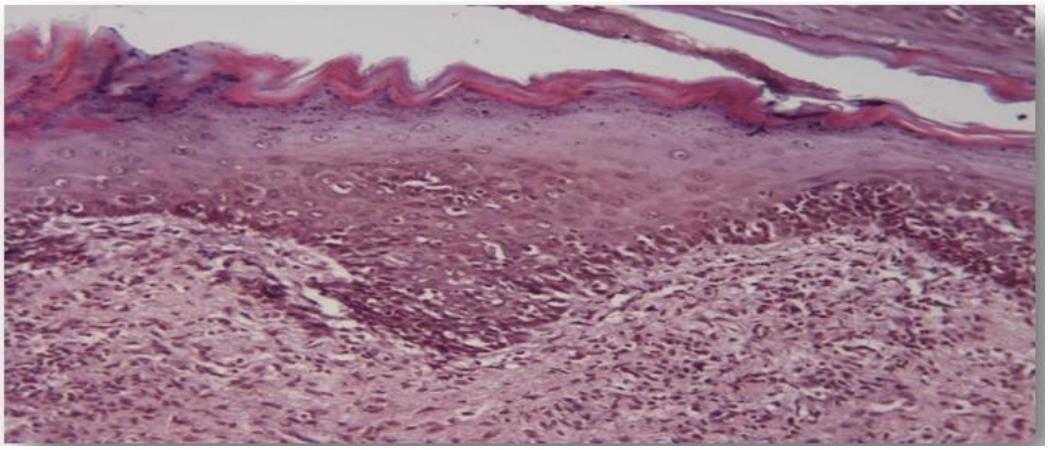

**Fig (28):** Photomicrograph buccal mucosa of high risk group (B2) showing excessive hyperkeratosis with papillary surface projections. The underlying connective tissue shows numerous chronic inflammatory cells (H&E, x200).

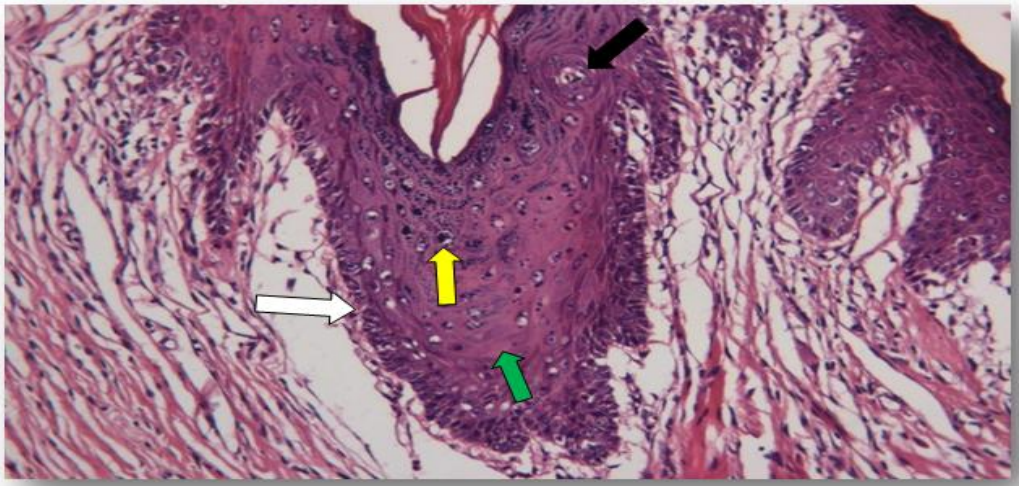

**Fig (29):** Photomicrograph buccal mucosa of high risk group (B2) showing loss of basal cell polarity (white arrow), nuclear hyperchromatism and increased N/C ratio (yellow arrow) involving the whole epithelial thickness. cell nest (black arrow) is formed in the upper layers of the epithelium. Notice the loss of stratification of prickle cell layer (green arrow) (H&E, x200).

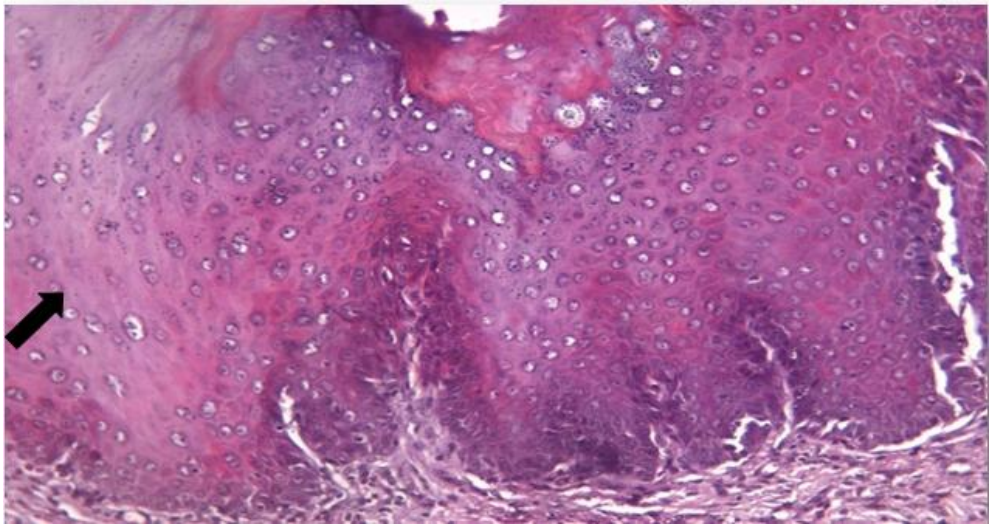

**Fig (30):** Photomicrograph buccal mucosa of high risk group (B2) showing severe acanthosis, club shaped rete ridges and abnormal stratification of epithelial layers (black arrow) .The connective tissue is heavily infiltrated with chronic inflammatory cells (H&E, x200).

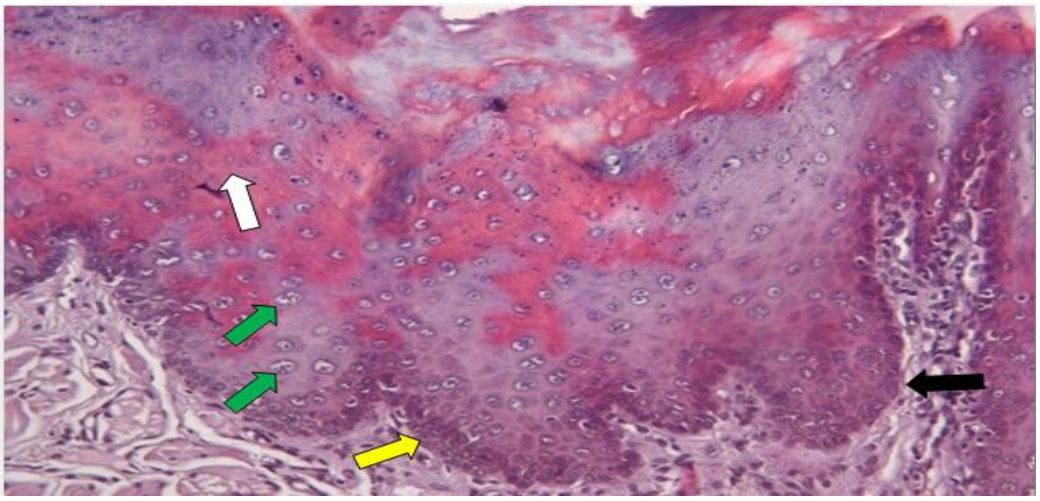

**Fig (31):** Photomicrograph buccal mucosa of high risk group (B2) showing abnormal stratification (white

arrow), loss of polarity of basal cells (black arrow) and basilar hyperplasia (yellow arrow). abnormal mitosis (green arrows) are seen within the epithelium (H&E, x200).

ii- Immunohistochemical examination of CD163 stained sections in high risk OED group (group B2)

Immunostained sections of the buccal mucosa of high risk OED group (group B2) showed numerous CD163 positive macrophages infiltrating the connective tissue. Membranous and cytoplasmic expression are obvious and localized mainly underneath dysplastic epithelium (figs.32,33,34and 35).

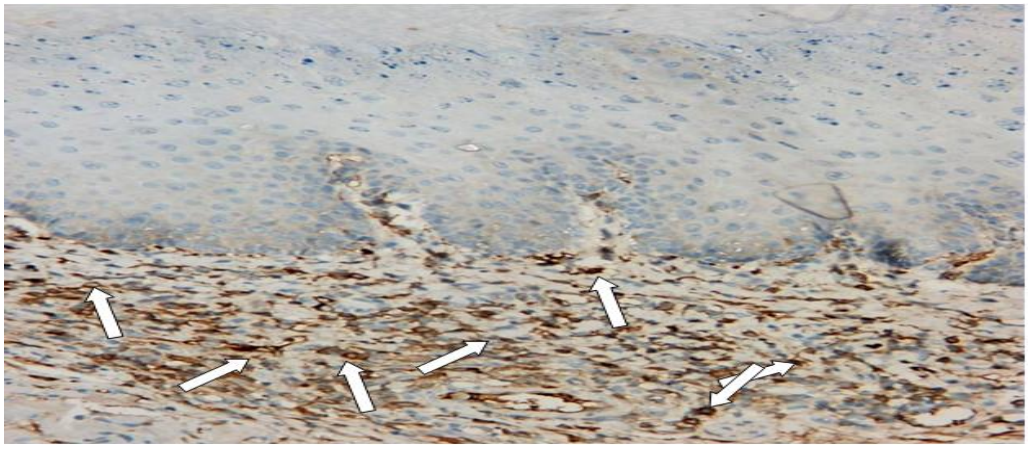

**Fig (32):** Photomicrograph showing many CD163 positive macrophages infiltrating the connective tissue in the high risk OED group. Macrophages were localized under the dysplastic epithelium (white arrows) (CD163 x 200).

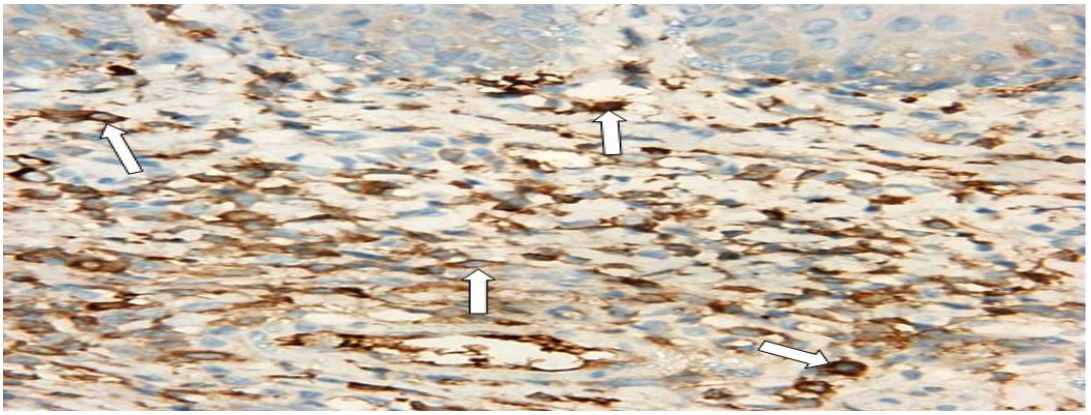

**Fig (33):** Higher magnification of the previous figure showing obvious membranous and cytoplasmic macrophages CD163 (white arrows) immunoexpresion (CD163 x 400).

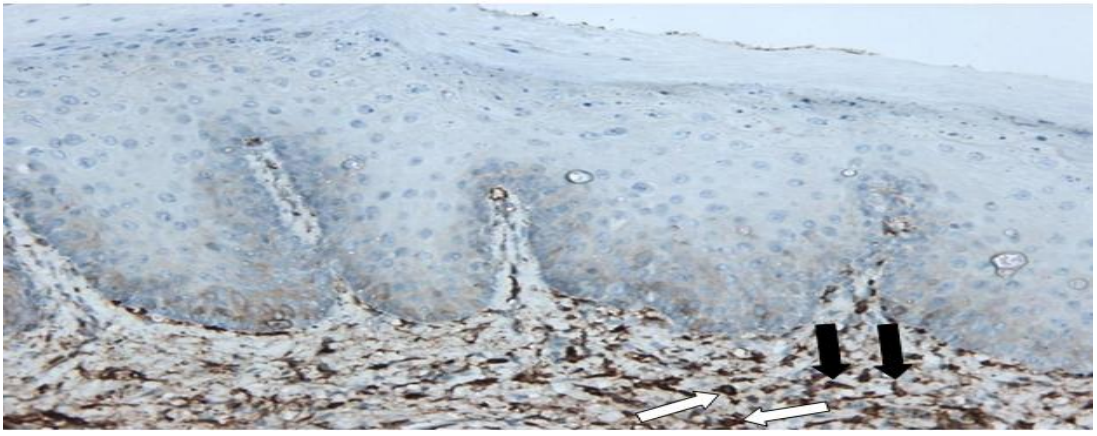

**Fig (34):** Photomicrograph showing many CD163 positive macrophages infiltrating the connective tissue in the high risk OED group. Some macrophages appeared rounded (white arrows) while others were spindle (black arrows) (CD163 x 200).

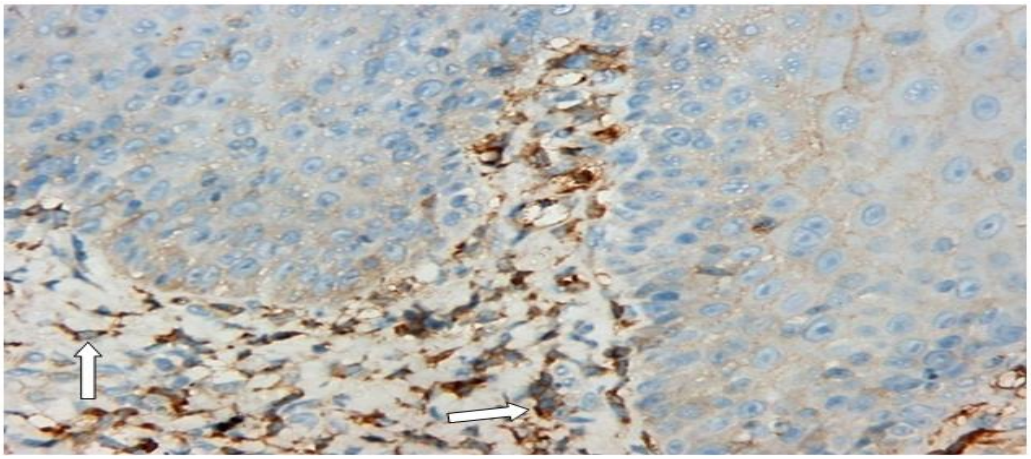

**Fig (35):** Photomicrograph showing cytoplasmic and membranous CD163 expression in most of the macrophages with negative nuclear expression (CD163 x 400).

iii- Immunohistochemical examination of CD34 stained sections in high risk OED group (group B2)

Immunostained sections of the buccal mucosa of high risk OED group (group B2) showed numerous CD34+ new formed vessels in the connective tissue which were localized under the dysplastic epithelium (figs.36,37 and 38).

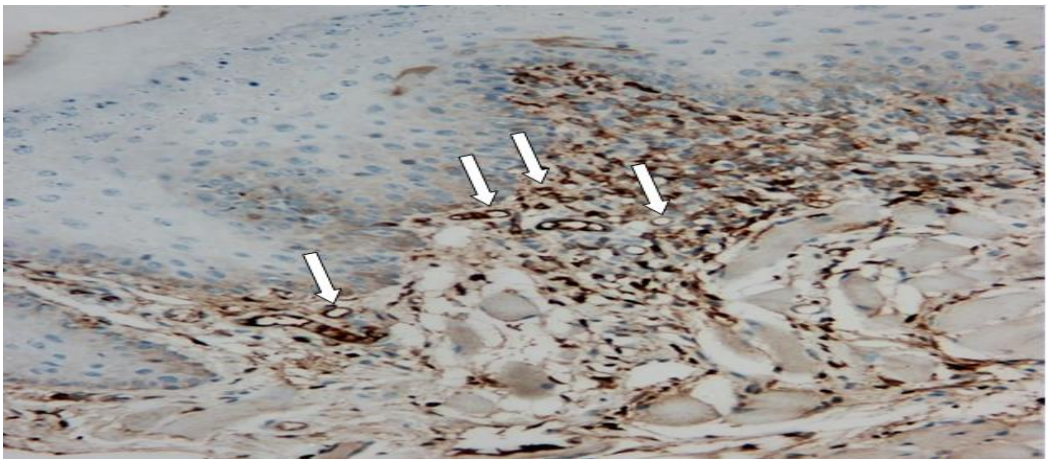

**Fig (36):** Photomicrograph of buccal mucosa of high risk OED group showing many CD34 positive newly formed vessels in the connective tissue under the dysplastic epithelium (white arrows) (CD34 ×200).

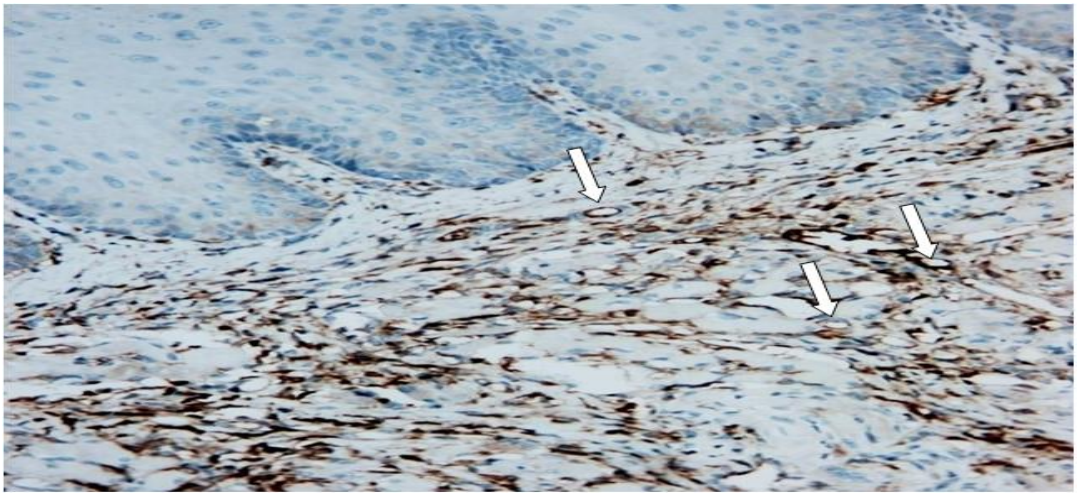

**Fig (37):** Photomicrograph buccal mucosa of high risk OED group showing many CD34 positive newly formed vessels in the connective tissue. They appeared small in size and had a narrow rounded lumen (white arrows) (CD34 ×200).

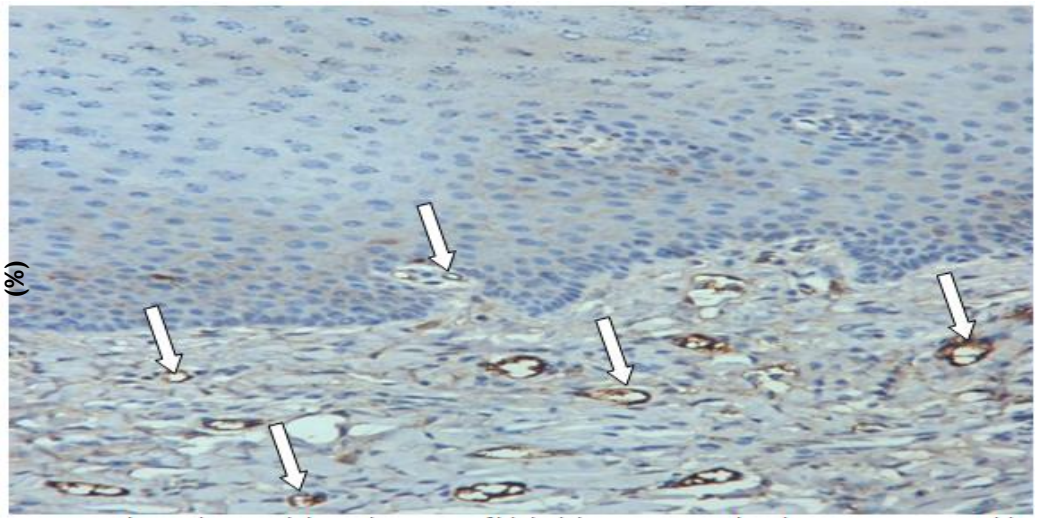


**Fig (38):** Photomicrograph buccal mucosa of high risk OED group showing many CD34 positive newly formed vessels in the connective tissue (white arrows) (CD34 ×200).

## IV. Statistical analysis:

**I- Comparison between low risk group and control group A-Area percentages of CD163 immunoexpression**

It was found that the low risk group (group B1) recorded a statistically significant (P<0.0001) higher mean area percentage value of CD163 immunoexpression when compared to the control group (group A1) as indicated by student t-test. (Table 1, figure 39).

**Table no 1:** Shows Mean area percentage of CD163 immunoexpression in control group ( group A1 ) and low risk group (group B1)

| | group | Mean | SD | P value |
|---|---|---|---|---|
| Area percentage | Control | 2.85 | ±1.09 | <0.0001* |
| | low risk | 20.80 | ±3.83 | |

SD= standard deviation
P value <0.05 are statistically significant

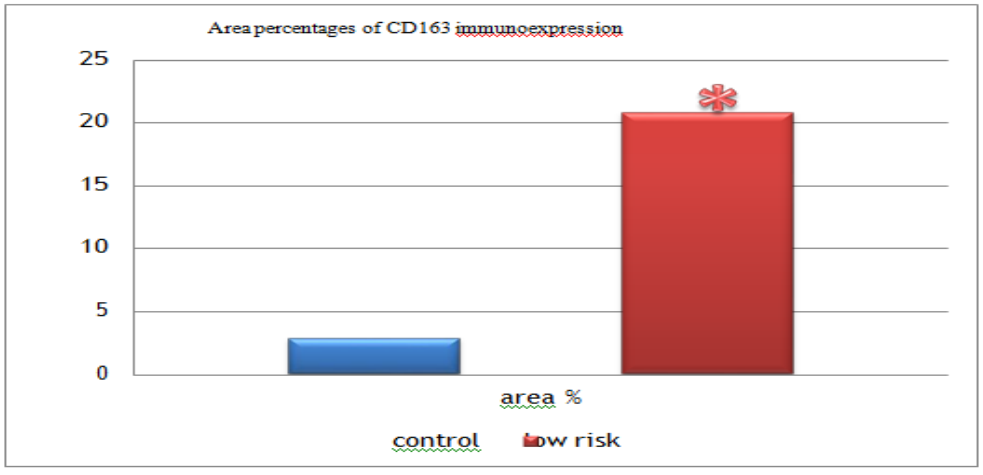


**Figure (39):** Bar chart showing mean area percentage of CD163 immunoexpression in control group (group A1) and low risk group (group B1)

**B-Mean number of CD34 positive blood vessels**

It was found that the low risk group (group B1) recorded a statistically significant (P<0.0001) higher mean number of CD34 positive blood vessels when compared to the control group (group A1) as indicated by Student t-test. (Table 2, figure 40).

**Table no 2:** Shows Mean number of CD34 positive blood vessels in control group ( group A1) and low risk group (group B1).

| | group | Mean | SD | P value |
|---|---|---|---|---|
| Mean blood vessel count | control | 4 | ±0.89 | <0.0001* |
| | low risk | 9.33 | ±2.25 | |

SD= standard deviation
P value <0.05 are statistically significant

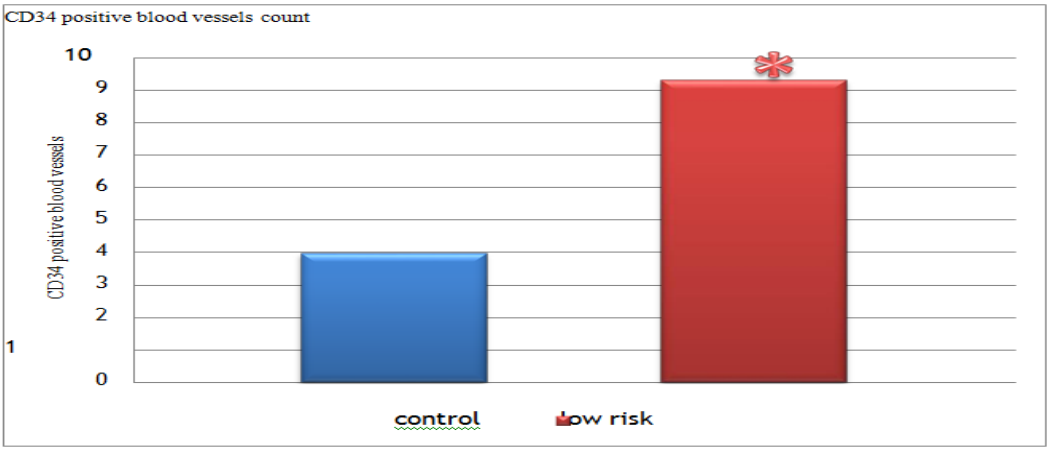


**Figure (40):** Bar chart showing mean number of CD34 positive blood vessels in control group ( group A1) and low risk group (group B1).

**II- Comparison between control and high risk groups A- Area percentages of CD163 immunoexpression**

It was found that the high risk group (group B2) recorded a statistically significant (P<0.0001) higher mean area percentage of CD163 immunoexpression when compared to control group (group A2) as indicated by Student t-test. (Table 3, figure 41).

**Table no 3:** Shows Mean area percentage of CD163 immunoexpression in control group (group A2 ) and high risk group (group B2)

| | group | Mean | SD | P value |
|---|---|---|---|---|
| Area percentage | control | 2.85 | 1.09 | <0.0001* |
| | High risk | 43.92 | 5.26 | |

SD= standard deviation
P value <0.05 are statistically significant

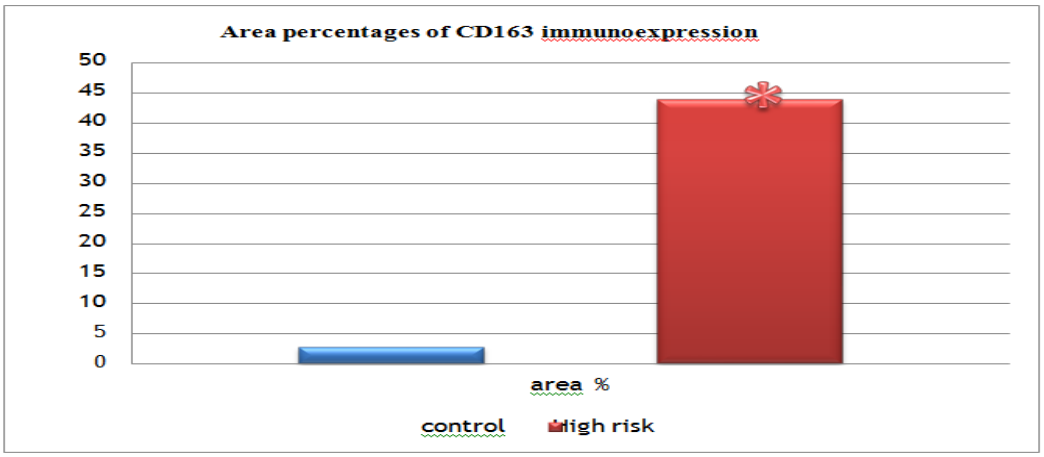


**Figure (41):** Bar chart showing mean area percentage of CD163 immunoexpression in control group ( group A2 ) and high risk group (group B2)

**B- Mean number of CD34 positive blood vessels**

It was found that the high risk group (group B2) recorded a statistically significant (P<0.0001) higher mean number of CD34 positive blood vessels when compared to control group (group A2) as indicated by student t-test. (Table4, figure 42).

**Table no 4:** Shows Mean number of CD34 positive blood vessels in control group ( group A2) and high risk group (group B2).

| | Group | Mean | SD | P value |
|---|---|---|---|---|
| CD34 positive blood vessels count | control | 4 | ± 0.89 | <0.0001* |
| | High risk | 29.16 | ± 0.98 | |

SD= standard deviation

P value <0.05 are statistically significant

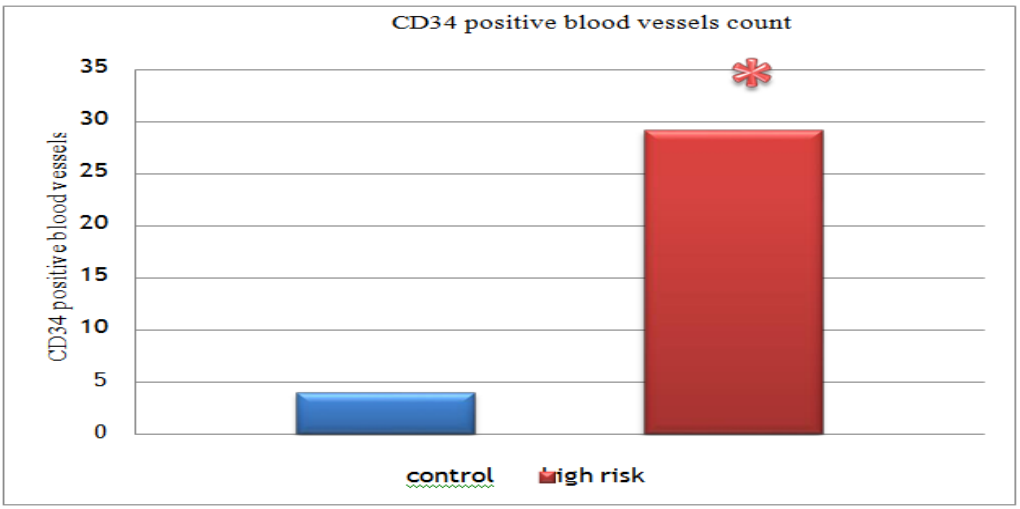


**Figure (42):** Bar chart showing mean number of CD34 positive blood vessels in control group ( group A2) and high risk group (group B2).

**I I I - Comparison between low and high risk groups**

**A- Area percentages of CD163 immunoexpression**

It was found that the high risk group (group B2) recorded a statistically significant (P<0.0001) higher mean area percentage of CD163 immunoexpression when compared to the low risk group (group B1) as indicated by Student t-test. (Table 5 , figure 43).

**Table no 5:** Shows Mean area percentage of CD163 immunoexpression in low risk group (group B1 ) and high risk group (group B2)

| (%) | group | Mean | SD | P value |
|---|---|---|---|---|
| Area percentage | Low risk | 20.80 | ± 3.83 | <0.0001* |
| | high risk | 43.92 | ± 5.26 | |

SD= standard deviation

P value <0.05 are statistically significant

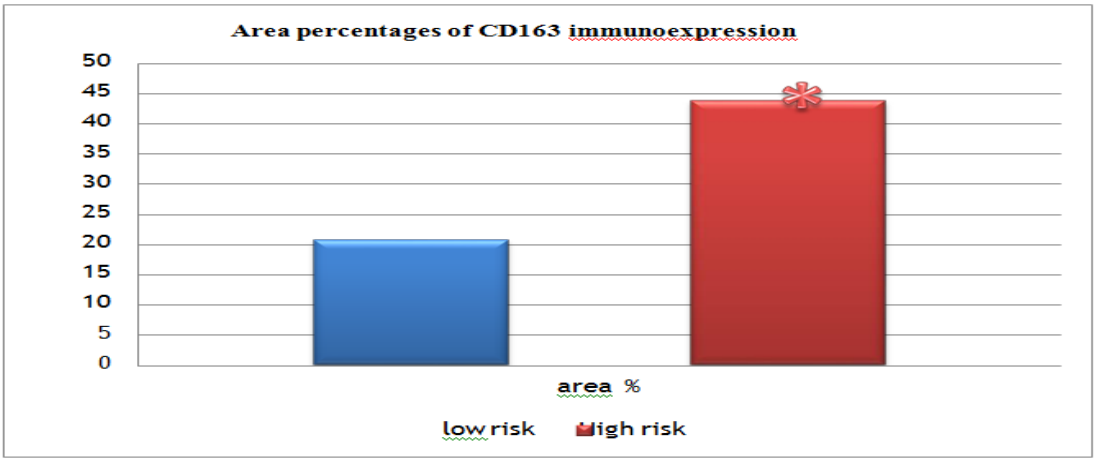


**Figure (43):** Bar chart showing mean area percentage of CD163 immunoexpression in low risk group ( group B1 ) and high risk group (group B2)

**B- Mean number of CD34 positive blood vessels**

It was found that the high risk group (group B2) recorded a statistically significant (P<0.0001) higher mean number of CD34 positive blood vessels when compared to low risk group (group B1), as indicated by Student t-test. (Table 6 , figure 44).

**Table no 6:** Shows Mean number of CD34 positive blood vessels in low risk group ( group B1) and high risk group (group B2).

| | Group | Mean | SD | P value |
|---|---|---|---|---|
| CD34 positive blood vessels count | Low risk | 9.33 | ± 2.25 | <0.0001* |
| | high risk | 29.16 | ± 0.983 | |

SD= standard deviation

P value <0.05 are statistically significant

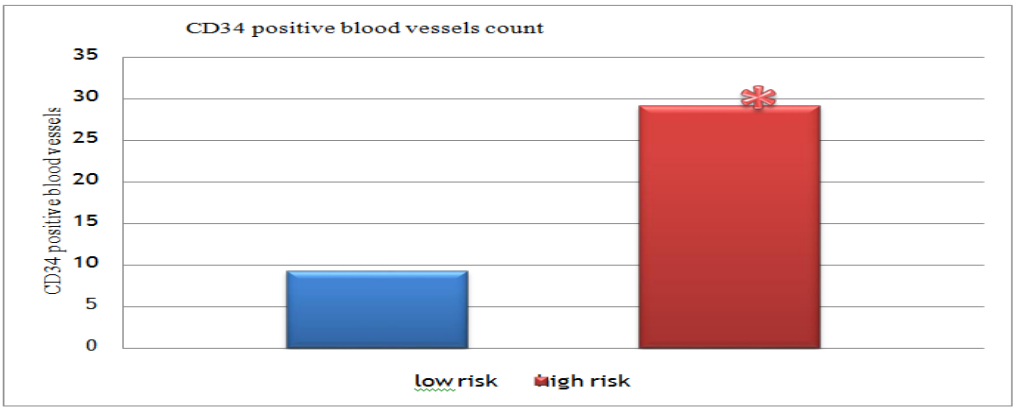


**Figure (44):** Bar chart showing mean number of CD34 positive blood vessels in low risk group ( group B1) and high risk group (group B2).

## IV. Correlation between area percentage of CD163 and number of CD34 positive blood vessels in control, low risk and high risk OED groups

It was found that there was strong significant correlation between area percentage of CD163 and number of CD34 positive blood vessels as indicated by Pearson test (R= 0.94 and P >0.0001) (fig. 45).

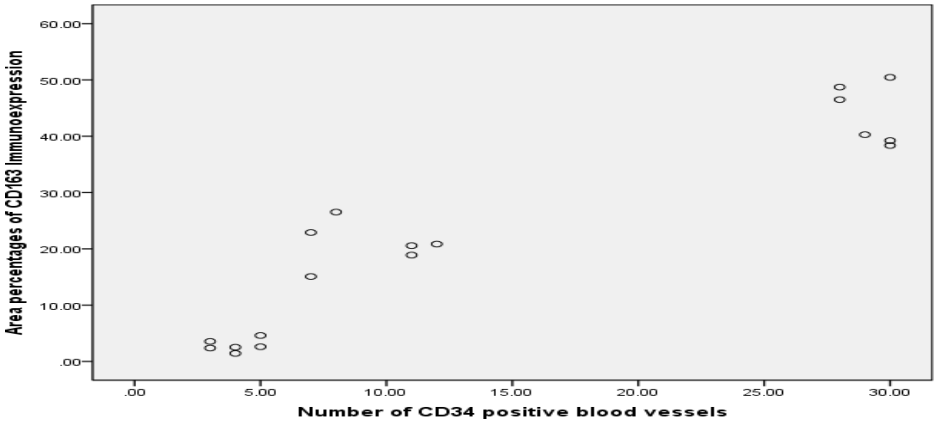


**Figure (45):** Correlation between area percentage of CD163 and number of CD34 positive blood vessels

## IV. Discussion

Evidence from clinical and experimental studies indicates that macrophages promote solid-tumour progression and metastasis. Macrophages are educated by the tumor microenvironment, so that they adopt a trophic role that facilitates angiogenesis, matrix breakdown and tumour-cell motility. All of which are elements of the metastatic process (Pollard, 2004). CD163, a marker of M2 macrophages, has been studied in several aggressive tumors and the increased expression of CD163 was significantly associated with a poor overall survival in various cancers (He et al., 2014). TAMs are now considered as a promising target for tumour therapy and reduction of their tumor-promoting activities has become a hot study area (Weigert & Sekar, 2009). Although the phenotypes of TAMs in various types of solid tumors have been extensively characterized, the phenotypes and functional properties of the TAMs that infiltrate oral premalignant lesions of solid tumors remain to be determined (Mori et al., 2015). In this respect, the present study attempted to illustrate the role of macrophges in oral premalignant lesions by evaluating CD163 immunoexpression in oral premalignancy induced in buccal mucosa of experimental rats in comparison to normal mucosa. Moreover, studies in literatures have revealed an important role for macrophages in facilitating angiogenesis (Koh etal., 2014, Li et al., 2015 and Lewis et al., 2016 ). Therefore, this study aimed to correlate CD163, the macrophage marker, with CD34,an endothelial cell marker, in oral premalignant lesions induced in experimental rats. This work was carried out on forty eight rats. Sacrifice of rats was done at 6 weeks (group B1) and at 9 weeks (group B2) depending on a previous study (Kasem et al., 2014). Rats showing hyperplasia or mild dysplasia were considered as group B1 (low risk group) while rats showing moderate to severe dysplasia were considered group B2 (high risk group) (Kujan et al., 2006). In this study, area percent of CD163 immunoexpression and number of CD34 positive blood vessels (MVD) were measured by using the image analyzer computer system which is an accurate and reproducible method that avoids human subjectivity (Decaestecker et al., 2009). In this work, CD163

immunoexpression was observed in the cell membrane and the cytoplasm of most macrophages. This is consistent with Bracaglia et al. (2008) who declared that CD163 was primarily localized to the cytoplasm or cytoplasm and cell membrane of macrophages. Moreover, Yafei et al. (2016) demonstrated that CD163 immunostainning was cytoplasmic in human macrophages. This result could be explained according to Nielsenet al. (2006) who reported that CD163 appears to be involved in intracellular signaling where it delivers cargo to endosomes then recycles again to the plasma membrane for a new round of endocytosis. This signaling function is triggered by ligand binding to CD163 at the cell surface and results in a protein tyrosine kinase-dependent signal and secretion of interleukin-6 (IL-6) and IL-10. In this work, the shape of CD163 positive macrophages in control, low risk and high risk groups did not vary. They varied from spindle to round cells. This is in accordance with Wehrhan et al., (2014) who showed that CD163 positive macrophages appeared spindle-shaped and occasionally round-shaped cells. Results of the present work revealed that little or no CD163 positive macrophages were observed in the control groups. This is consistent with Fujii et al., (2012) who reported that CD163 positive macrophages were rarely observed in the subepithelial stroma of the normal mucosa specimens. In the present study, the low risk group showed more macrophages compared to the control group but did not show a specific distribution pattern for CD163 positive macrophages. Some cases showed CD163 positive macrophages surrounding blood vessels, while others showed their presence either deep in the connective tissue or just underneath the dysplastic epithelium. This is in consistent with Lewis et al. (2016) who declared that a distinct subset of PV macrophages has been shown to correlate with increased tumor angiogenesis. This can also be explained by Chanmee et al. (2014) who showed that TAMs secrete a wide range of pro-angiogenic mediators, including basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator (uPA), and adrenomedullin (ADM), which facilitate tumor angiogenesis. The variability in the consistent distribution pattern of CD163 positive macrophages in low risk group could be explained by the weak role of macrophages in early dysplastic changes which is to be magnified in high risk OED group and OSCC. This is in accordance with Gannot et al. (2002) who revealed that severe pathological changes in oral epithelial tissues (i.e. moderate and severe dysplasia or SCC) are accompanied by a higher level of infiltrating lymphocytes and macrophages, when compared to lesions with milder changes (i.e. hyperkeratosis or mild dysplasia). Results of the present work illustrated that CD163 positive macrophages were mainly distributed in the subepithelial region of the high risk group. This finding is consistent with Ye et al. ( 2016) who declared that the majority of the macrophages were located in the subepithelial stroma of oral leukoplakia (OLK) and OSCC. This can be explained by Mori et al. (2015) which stated that CD163 positive macrophages have a role in the breakdown of the basement membrane that contribute to the infiltration of intraepithelial CD4+ T cells. The authors added that CD163 positive macrophages and the infiltration of T cells into the epithelial lesion may contribute to the early architectural disturbance of the epithelium during the development of dysplasia. Statistical analysis in the present work illustrated that the mean area percent of CD163 immunoexpression in low risk OED was significantly greater than that of control group. This finding is consistent with Ye et al. (2016) who detected that the expression of CD163 in low risk oral leuokoplakia (OLK) was gradually increased compared to normal epithelium. Moreover the present work revealed that the mean area percent of CD163 immunoexpression in high risk OED was significantly greater than that of low risk and control groups. This is in consistent with Mori et al. (2015) who observed a significant increase in CD163 positive macrophages in moderate dysplasia (included in the high risk group) compared to samples without dysplasia. This work also revealed a significant increase in mean area percent of CD163 immunoexpression in high risk OED compared to low risk OED. The previous results can be explained by Gannot et al. (2002) who observed an increased infiltration of mononuclear cells (T cells, B cells and macrophages) in oral premalignant lesions and OSCC. They revealed that the amount of the infiltrating cells was significantly higher in moderate and severe dysplasia and SCC compared to hyperkeratosis and mild dysplasia. These results suggest that the tumor microenvironment of oral premalignant lesions and OSCC creates a Type 1 helper T cells (Th1) -dominated microenvironment that polarizes TAMs toward the M2 phenotype that have reduced anti-tumor activities and increase the production of angiogenic mediators that include VEGF and IL-10, in addition to M2- specific genes known to be involved in the promotion of cell proliferation (Leyva-Illades et al., 2012) .On the contrary, Bdelaziz et al. (2016) demonstrated that in normal and dysplastic epithelia, macrophages were sporadically seen beneath the epithelial layer, and most of them were CD68+. There was only a very small number of CD163+ cells. In the present experiment, CD34 immunoexpression was observed in endothelial cells. This is in accordance with Sidneyet al. (2014) who reported that CD34 stains endothelial cells and is thought to be involved in cell migration and adhesion. Moreover, Nielsen & McNagny (2008) hypothesized that CD34 has a general role in enhancing cell proliferation and/or blocking differentiation thus the expression of CD34 in endothelial cells is important in understanding

the process of angiogenesis in oral cancer and premalignant lesions (Desai et al., 2010). Results of the present work illustrated that CD34 was expressed in endothelial cells lining the newly formed blood vessels. They were few and sporadically distributed in the connective tissue of the control group. This is consistent with

Hegde & Marla (2015) who revealed that in normal oral mucosa, the endothelial cells lined capillaries and were observed in the lamina propria which were evenly distributed. Results of the present work also illustrated that the newly formed microvessels were mainly distributed in the subepithelial region of the dysplastic groups especially the high risk group. This finding is consistent with Michailidou et al., (2008) who detected that microvessels are mainly located just underneath the epithelium and were stained as brown spots, lines or lumens. The results could be explained by Mohtasham et al. (2010) who declared that angiogenesis results from angiogenic factors released from the overlying tumor cells. In this work, examination of tissue specimens from OED both low and high risk groups revealed that CD34+ blood vessels varied in size. However most of the blood vessels were small sized rounded blood vessels. This is consistent with Yang et al. (2006) who illustrated a high number of small and immature newly formed vessels in DMBA induced OED unlike the large and mature vessels of the normal oral mucosa. This work illustrated that the mean number of CD34+ blood vessels was significantly greater in low and high risk OED groups compared to the control groups. This finding is consistent with Sathyakumar et al. (2012) who declared a significant increase of CD34 immunostaining among low and high risk OED when compared to normal cases. This can be explained by Mohtasham et al. (2010) who observed an increase in vascularization during transformation from normal oral mucosa, through dysplasia, to in-situ and infiltrating carcinoma supporting the pivotal role of angiogenesis in malignancy progression. Statistical analysis in the present experiment revealed that the mean number of CD34+ blood vessels immunoexpression in high risk OED was significantly greater than that of low risk OED. This finding is consistent with Michailidou et al. (2008) who revealed that the number of microvessels was found to be increased significantly from leukoplakia with mild dysplasia to leukoplakia with severe dysplasia and concluded that an angiogenic switch seemed to be turned on in the later stages of dysplasia indicating a transformation into malignancy. Therefore, this significant difference in mean number values of CD34+ blood vessels allows us to conclude that microvessel density can be used as an indicator of disease progression which will have an important impact regarding the management of these lesions. This study, showed a highly significant correlation between area percentage of CD163 and number of CD34 positive blood vessels among the studied groups. In other words the increased number of macrophages associated with oral premalignancy may possibly play role in angiogenesis. This is in consistent with Chanmee et al. (2014) who showed that TAMs secrete a wide range of pro-angiogenic mediators, including basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator (uPA), and adrenomedullin (ADM), which facilitate tumor angiogenesis. In contrast to our results Kawai et al., (2008) showed that on the contrary to their tumor promoting function, TAMs that infiltrated lung cancers have been associated with a better prognosis. Finally, this work points out a novel marker, CD163, whose increased expression in tissues denotes an increase in macrophage infiltration and could be used to detect early dysplastic changes prior to malignant transformation and could be used as a marker for poor prognosis.

## V. Conclusion

- In respect to the previous results and discussion we can conclude that The infiltrated TAMs in OED exhibited the M2 phenotype (CD163 marker) which were significantly increased with the increasing severity of the OED.
- CD163 immunoexpression could be used for early detection of molecular changes prior to malignant transformation.TAMs can be a promising target for oral premalignant and malignant tumors therapy by reduction of their tumor-promoting activities.CD163 immunoexpression is strongly correlated with angiogenesis and poor prognosis.

## References

[1]. Bánóczy, J., Gintner, Z., & Dombi, C. (2001). Tobacco use and oral leukoplakia. Journal of Dental Education, 65(4), 322–7.
[2]. Bdelaziz, A. H. A., Ssa, M. O. E., Amazaki, M. A. Y., Aruyama, S. A. M., Bé, T. A. A., Abkair, H. A. B.,Aku, T. A.S. (2016). Tumour-associated macrophages are recruited and differentiated inthe neoplastic stroma of oral squamous cell carcinoma.Pathology. 48(3):219-27.
[3]. Bracaglia, C., Devito, R., Insalaco, A., Buonuomo, P. S., Campana, A., Cortis,E., & Ugazio, A. G. (2008). High membrane expression of CD163 by bone marrow cells is not a specific marker of macrophage activation syndrome (MAS). Pediatr Rheumatol Online J.6(Suppl 1): P17.
[4]. Carlile, J., Harada, K., Baillie, R., Macluskey, M., Chisholm, D. M., Ogden, G. R., … Schor, A. M. (2001). Vascular endothelial growth factor (VEGF) expression in oral tissues: possible relevance to angiogenesis, tumour progression and field cancerisation. Journal of Oral Pathology & Medicine.30(8), 449–57.
[5]. Chanmee, T., Ontong, P., Konno, K., & Itano, N. (2014). Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment. Cancers (Basel).13;6(3):1670-90.
[6]. Decaestecker, C., Lopez, X. M., Haene, N. D., & Roland, I. (2009). Requirements for the valid quantification of immunostains on tissue microarray materials using image analysis. Proteomics.9(19):4478-94.
[7]. Desai, R. S., Mamatha, G. S., Khatri, M. J., & Shetty, S. J. (2010). Immunohistochemical expression of CD34 for characterization and quantification of mucosal vasculature and its probable role in malignant transformation of atrophic epithelium in oral submucous fibrosis. Oral Oncology.46(7), 553–558.
[8]. Fujii, N., Shomori, K., Shiomi, T., Nakabayashi, M., & Takeda, C. (2012). Cancer-associated fibroblasts and CD163- positive

macrophages in oral squamous cell carcinoma : their clinicopathological and prognostic significance. J Oral Pathol Med. 41(6):444-51.
[9]. Gannot, G., Gannot, I., Vered, H., Buchner, A., & Keisari, Y. (2002). Increase in immune cell infiltration with progression of oral epithelium from hyperkeratosis to dysplasia and carcinoma. Br J Cancer. 6;86(9):1444-8.
[10]. He, K., Zhang, L., Huang, C., Ma, S., Wang, Y., Wang, W., … Sun, Z. (2014). CD163+ tumor- associated macrophagesCorrelated with Poor Prognosis and Cancer Stem Cells in Oral Squamous Cell Carcinoma.Biomed Res Int. 838632
[11]. Hegde, V., & Marla, V. (2015). Mast cells and angiogenesis in oral epithelial dysplastic lesions and oral squamous cell carcinoma. International Journal of Medical Research & Health Sciences.4(1), 46.
[12]. Iamaroon, A., Pongsiriwet, S., Jittidecharaks, S., Pattanaporn, K., Prapayasatok, S., & Wanachantararak, S. (2003).
[13]. Increase of mast cells and tumor angiogenesis in oral squamous cell carcinoma. J Oral Pathol Med. 32(4):195-9.
[14]. Jaber, M. A. (2010). Oral epithelial dysplasia in non-users of tobacco and alcohol: an analysis of clinicopathologic characteristics and treatment outcome. Journal of Oral Science.52(1), 13–21.Jiménez, B., & Volpert, O. V. (2001). Mechanistic insights on the inhibition of tumor angiogenesis. Journal of Molecular Medicine (Berlin, Germany).78(12), 663–72.
[15]. Kasem, R. F., Hegazy, R. H., Arafa, M. a. a., & AbdelMohsen, M. M. (2014). Chemopreventive effect of Mentha piperita on dimethylbenz[ a ]anthracene and formaldehyde-induced tongue carcinogenesis in mice (histological and immunohistochemical study). Journal of Oral Pathology & Medicine.43(7), 484–491.
[16]. Kawai, O., Ishii, G., Kubota, K., Murata, Y., Naito, Y., Mizuno, T., … Ochia, A. (2008). Predominant infiltration of macrophages and CD8+ T cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer.113(6), 1387–1395.
[17]. Koh, Y. W., Park, C., Yoon, D. H., Suh, C., & Huh, J. (2014). CD163 Expression Was Associated with Angiogenesis and Shortened Survival in Patients with Uniformly Treated Classical Hodgkin Lymphoma. PLoS One. 29;9(1):e87066.
[18]. Krishnan, L., Karpagaselvi, K., Kumarswamy, J., Sudheendra, U., Santosh, K., & Patil, A. (2016). Inter- and intra- observer variability in three grading systems for oral epithelial dysplasia. Journal of Oral and Maxillofacial Pathology.20(2), 261.
[19]. Kujan, O., Oliver, R. J., Khattab, A., Roberts, S. a., Thakker, N., & Sloan, P. (2006). Evaluation of a new binary system of grading oral epithelial dysplasia for prediction of malignant transformation. Oral Oncology.42(10), 987– 993.
[20]. Lewis, C. E., Harney, A. S., & Pollard, J. W. (2016). The Multifaceted Role of Perivascular Macrophages in Tumors.Cancer Cell.30(1), 18–25.
[21]. Leyva-Illades, D., McMillin, M., Quinn, M., & Demorrow, S. (2012). Cholangiocarcinoma pathogenesis: Role of the tumor microenvironment. Translational Gastrointestinal Cancer. Transl Gastrointest Cancer.1(1):71-80.
[22]. Li, W., Liang, R., Zhou, C., Wu, M., Lian, L., Yuan, G., … Tao, M. (2015). The association between expressions of Ras and CD68 in the angiogenesis of breast cancers. Cancer Cell Int. 7;15(1):17.
[23]. Michailidou, E. Z., Markopoulos, a K., & Antoniades, D. Z. (2008). Mast cells and angiogenesis in oral malignant and premalignant lesions. The Open Dentistry Journal. 2, 126–32.
[24]. Mohtasham, N., Babakoohi, S., Salehinejad, J., Montaser-Kouhsari, L., Shakeri, M. T., Shojaee, S., … Firooz, A. (2010). Mast cell density and angiogenesis in oral dysplastic epithelium and low- and high-grade oral squamous cell carcinoma. Acta Odontol Scand.68(5), 300–304.
[25]. Mori, K., Haraguchi, S., Hiori, M., Shimada, J., & Ohmori, Y. (2015). Tumor-associated macrophages in oral premalignant lesions coexpress CD163 and STAT1 in a Th1-dominated microenvironment. BMC Cancer.15(1), 573.
[26]. Mori, K., Hiroi, M., Shimada, J., & Ohmori, Y. (2011). Infiltration of M2 Tumor-Associated Macrophages in Oral Squamous Cell Carcinoma Correlates with Tumor Malignancy. Cancers. 3(4), 3726–3739.
[27]. Nagatsuka, H., Hibi, K., Gunduz, M., Tsujigiwa, H., Tamamura, R., Sugahara, T., … Nagai, N. (2005). Various immunostaining patterns of CD31, CD34 and endoglin and their relationship with lymph node metastasis in oral squamous cell carcinomas. Journal of Oral Pathology and Medicine.34(2), 70–76.
[28]. Nielsen, J. S., & McNagny, K. M. (2008). Novel functions of the CD34 family. Journal of Cell Science.121(24), 4145–4145.
[29]. Nielsen, M. J., Madsen, M., Møller, H. J., & Moestrup, S. K. (2006). The macrophage scavenger receptor CD163: endocytic properties of cytoplasmic tail variants. J Leukoc Biol.79(4):837-45.
[30]. Onofre, G., Koláčková, M., Jankovičová, K., & Krejsek, J. (2009). Review Article Scavenger Receptor CD163 And Its Biological Functions Molecular characterization. Acta Medica (Hradec Kralove).52(2):57-61.
[31]. Pollard JW (2004). Tumour educated macrophages promote tumour progression and metastasis.Nat Rev Cancer.4(1):71-8.
[32]. Sathyakumar, M., Sriram, G., Saraswathi, T. R., & Sivapathasundharam, B. (2012). Immunohistochemical evaluation of mast cells and vascular endothelial proliferation in oral precancerous lesion - leukoplakia. J Oral MaxillofacPathol.16(3):343-8.
[33]. Shieh, Y.-S., Lee, H.-S., Shiah, S.-G., Chu, Y.-W., Wu, C.-W., & Chang, L.-C. (2004). Role of angiogenic and non- angiogenic mechanisms in oral squamous cell carcinoma: correlation with histologic differentiation and tumor progression. Journal of Oral Pathology and Medicine.33(10), 601–606.
[34]. Sidney LE1, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. (2014). Concise review: evidence for CD34 as a common marker for diverse progenitors. Stem Cells.32(6):1380-9.
[35]. Wehrhan, F., Büttner-herold, M., Hyckel, P., Moebius, P., Preidl, R., Distel, L., … Weber, M. (2014). Increased malignancy of oral squamous cell carcinomas ( oscc ) is associated with macrophage polarization in regional lymph nodes – an immunohistochemical study. BMC Cancer.21;14:522.
[36]. Weigert, A., & Sekar, D. (2009). Tumor-associated macrophages as targets for tumor immunotherapy.Immunotherapy.1(1):83-95.
[37]. Yafei, Z., Jun, G., & Guolan, G. (2016). Correlation between macrophage infiltration and prognosis of ovarian cancer-a preliminary study. Biomedical Research.27 (2): 305-312.
[38]. Yang, Y., Ã, Z. T. Z., & Ge, J. P. (2006). Effect of genistein on DMBA-induced oral carcinogenesis in hamster.Carcinogenesis. 27(3):578-83.
[39]. Ye, X., Zhang, J., Lu, R., & Zhou, G. (2016). Signal regulatory protein a associated with the progression of oral leukoplakia and oral squamous cell carcinoma regulates phenotype switch of macrophages. Oncotarget.7(49): 81305– 81321.

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