Human Papillomavirus (HPV) Infection And Erythropoietin Receptors (EpoR) Expression As Prognostic Indicators In Oropharyngeal Cancer

Hani Almarzouki, MBBS, FRCSC.

Department of Otolaryngology Head and Neck Surgery
McGill University
Montreal, Quebec, Canada
July 2013

A Thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Masters of Science

© Hani Almarzouki 2013
1 DEDICATION

To my loving mother and father and to my precious wife.
2 **Acknowledgements**

I would like to thank Dr. Karen Kost for all the support and guidance throughout my Master's degree and my residency training. Words are not enough to express how privileged I am to have her as a mentor and a role model to whom I will always look up to.

I am also honored to work with a world figure in cancer epidemiology, Dr. Eduardo Franco. I would like to thank him for his continuous guidance and support throughout my Master's to achieve high scientific standards.

I am also deeply grateful to Dr. George Shenouda for all of his insightful ideas and indispensable guidance. I have learned a lot from him and I will always remember his words: “Hani... Research is the future”.

A special thanks to Dr. Lily Shakibnia for her countless help throughout the project. Many thanks to Dr. Agnihotram V Ramanakumar for all the time and effort he has put in helping with the statistical analysis.

I would also like to express my thanks to Dr. François Coutlèe, for performing the HPV analyses in his laboratory, and Dr. Peter Chauvin for performing the EpoR staining. Performing the project would not have been possible without their work. I would like to thank Dr. James Hanley for his help setting up the study protocol.
I would like to thank the McGill Head and Neck Cancer fund for funding the EpoR testing and Dr. Eduardo Franco’s Division of Cancer Epidemiology for funding the HPV laboratory testing.

Furthermore, I would like to extend my thanks to my parents, Zohair and Aisha, for all the love they gave me and for always believing in me. I will never thank them enough for their unconditional support and love.

A special thanks to my wife Nada, my daughter Maya and my son Zuhair for being there for me specially during the difficult times of my residency and trying their best to put a smile in my face to get me through the stressful times.
# TABLE OF CONTENTS

1  DEDICATION .................................................................................................................. 2

2  ACKNOWLEDGEMENTS ................................................................................................. 3

3  LIST OF ABBREVIATIONS: ............................................................................................. 7

4  LIST OF TABLES: ............................................................................................................. 8

5  LIST OF FIGURES: ........................................................................................................... 8

6  ABSTRACT ........................................................................................................................ 9

7  RESUME .......................................................................................................................... 11

8  INTRODUCTION .............................................................................................................. 14

9  LITERATURE REVIEW: .................................................................................................. 16

9.1 OROPHARYNGEAL CANCER: ......................................................................................... 16
  9.1.1 EPIDEMIOLOGY OF HEAD AND NECK AND OROPHARYNGEAL CANCERS: .......... 16
  9.1.2 RISK FACTORS OF OROPHARYNGEAL CANCER: ................................................. 16

9.2 HUMAN PAPILLOMAVIRUS (HPV) AND OROPHARYNGEAL CANCER .......... 20
  9.2.1 HUMAN PAPILLOMAVIRUSES ............................................................................. 20
  9.2.2 MECHANISM OF HPV CARCINOGENESIS .......................................................... 21
  9.2.3 HPV DETECTION METHODS: ............................................................................... 22
  9.2.4 PREVALENCE AND INCIDENCE OF HPV IN OPC ............................................ 24
  9.2.5 DIFFERENCES BETWEEN HPV-POSITIVE AND HPV-NEGATIVE OPC: ............ 26
  9.2.6 HPV AND SURVIVAL ............................................................................................. 29

9.3 ERYTHROPOIETIN AND ERYTHROPOIETIN RECEPTORS (EpoR): .................. 30
  9.3.1 RATIONALE: ......................................................................................................... 30
  9.3.2 MECHANISM OF GROWTH STIMULATION ............................................................ 32
  9.3.3 ERYTHROPOIETIN /EpoR AND SURVIVAL ......................................................... 33

10 METHODS: ...................................................................................................................... 37

10.1 STUDY DESIGN ........................................................................................................... 37

10.2 PATIENTS AND SETTING: ......................................................................................... 37

10.3 SAMPLE PROCESSING: ............................................................................................... 38

10.4 HPV DNA TESTING .................................................................................................... 39
  10.4.1 HPV TYPING ANALYSIS .................................................................................... 41

10.5 EpoR TESTING ............................................................................................................ 41
  10.5.1 HEMATOXYLIN AND E OSM (H&E) STAINING ................................................. 41
10.5.2  EPO RECEPTOR IMMUNOHISTOCHEMISTRY ................................................................. 42
10.5.3  EPO ANALYSIS ........................................................................................................... 43
10.6  STATISTICAL ANALYSIS ............................................................................................... 44

11  RESULTS: ......................................................................................................................... 46

11.1  DESCRIPTIVE ANALYSIS .............................................................................................. 46
  11.1.1  HPV DNA & TYPING AND EPO STATUS RESULTS ......................................................... 46
  11.1.2  PATIENT SOCIODEMOGRAPHIC CHARACTERISTICS ............................................... 46
  11.1.3  PATIENT CLINICAL AND TREATMENT CHARACTERISTICS ..................................... 48

11.2  SURVIVAL ANALYSIS .................................................................................................... 50
  11.2.1  HPV DNA STATUS AND SURVIVAL ........................................................................... 50
  11.2.2  EPO STATUS AND SURVIVAL .................................................................................... 53
  11.2.3  HPV/EPO AND SURVIVAL ....................................................................................... 55
  11.2.4  AGE AND SURVIVAL .................................................................................................. 56
  11.2.5  SMOKING, DRINKING, SITE OF CANCER, STAGE AND USE OF CHEMOTHERAPY ....... 57

12  DISCUSSION ....................................................................................................................... 59

12.1  HUMAN PAPILLOMAVIRUS (HPV) .................................................................................. 59
  12.1.1  RATIONALE: ............................................................................................................... 59
  12.1.2  HPV-DNA STATUS AND SOCIODEMOGRAPHIC CHARACTERISTICS .................... 59
  12.1.3  HPV AND SURVIVAL .................................................................................................. 60

12.2  AGE AND SURVIVAL ....................................................................................................... 61

12.3  ERYTHROPOIETIN RECEPTORS (EPO) ......................................................................... 61
  12.3.1  RATIONALE: ............................................................................................................... 61
  12.3.2  EPO EXPRESSION IN OROPHARYNGEAL SCC .......................................................... 62
  12.3.3  EPO EXPRESSION AND SURVIVAL ......................................................................... 62

12.4  HPV/EPO AND SURVIVAL .............................................................................................. 63

13  CONCLUSION ...................................................................................................................... 65

14  REFERENCES: ..................................................................................................................... 67
3 **List of Abbreviations:**

- HPV: Human papillomavirus
- EpoR: Erythropoietin receptor
- H&NC: Head and Neck Cancer
- OPC: Oropharyngeal Cancer
- SCCs: Squamous Cell Carcinomas
- PCR: Polymerase Chain Reaction
- HR: Hazard Ratio
- CI: Confidence Interval
- RB: Retinoblastoma Protein
4 **LIST OF TABLES:**

Table 1: Patient sociodemographic characteristics .......................... 47

Table 2: Patient clinical characteristics ........................................... 49

Table 3: Patient treatment characteristics ........................................ 50

Table 4: Disease Free Survival (DFS) and Overall Survival (OS) according to socioeconomic, clinical and treatment characteristics ...................... 51

Table 5: Overall survival and disease free survival by HPV and EpoR using Cox regression ................................................................. 52

Table 6: Survival by HPV/EpoR groups using cox regression .......... 55

5 **LIST OF FIGURES:**

Figure 1: EpoR positive immune staining ........................................ 43

Figure 2: Kaplan-Meier survival analysis for HPV DNA status ............ 53

Figure 3: Kaplan-Meier survival analysis for EpoR status ................. 54

Figure 4: Risks classification according to HPV/EpoR groups .......... 56

Figure 5: Kaplan-Meier survival analysis for patients’ age ............... 57
ABSTRACT

Background: Human papillomavirus (HPV) infection is recognized as an independent risk factor for squamous cell carcinomas (SCCs) of the head and neck, and the presence of HPV DNA is associated with a better prognosis. Recent evidence indicates a broader role for erythropoietin via binding and activation of its receptor (EpoR), which is present in many neoplastic non-hematopoietic cells. The expression of EpoR in cancer may represent a selection process that permits cancer cells to survive in an unfavorable microenvironment and may indicate aggressive cancer cell behavior. EpoR is variably expressed in head and neck cancer cells and could independently predict a poorer treatment response.

Objectives: 1. To determine HPV status and the frequency of EpoR expression in archived biopsy specimens obtained from patients with oropharyngeal SCCs. 2. To evaluate whether the EpoR status affects survival and whether this putative effect is influenced by HPV status.

Methods: A retrospective cohort study was conducted by reviewing the charts of 97 patients with oropharyngeal SCCs treated with primary curative intent radiation therapy at the McGill University Health Center, from 2000 to 2009. Eligible patients had to have archived tissue samples available for HPV and EpoR analysis. HPV DNA testing and typing was done using a standard polymerase chain reaction (PCR) protocol. EpoR status was determined by immunohistochemistry using rabbit polyclonal
anti-EpoR staining. Stained sections were analyzed by 2 independent examiners by conventional light microscopy. A score product of 0-300 was determined for each patient by multiplying the percentage of neoplastic cells staining for EpoR (0-100%) and the intensity of the EpoR staining expressed from 0 to 3.

**Results:** The median age was 62 years (range: 43–83). HPV status was positive in 74% of patients and this was significantly higher in patients aged ≤65 years, p=0.023. Patients with a significant smoking history (>10 pack-years) and drinking history (>4 drinks/week) were significantly more likely to be HPV negative, p= 0.041 and 0.0001 respectively. On Cox regression analysis, HPV positivity was associated with a 29% reduction in risk of death (Hazard Ratio (HR)=0.71; 95% Confidence Interval (CI): 0.34-1.49), albeit non-significantly. EpoR status was positive in 27% of patients and was associated with a non-significant 23% increase in risk of death (HR=1.23; 95%CI: 0.59-2.56). Patients who were HPV positive and EpoR negative had a non-significant 33% reduction in risk of death (HR=0.67; 95%CI: 0.29-1.56).

**Conclusion:** This study demonstrates a trend indicating that HPV and EpoR status correlate with survival. This trend persists when patients are divided into low, intermediate and high-risk groups based on their HPV/EpoR status. The lowest risk group appears to consist of patients, which are HPV positive and EpoR negative. To the best of our knowledge
this is the first study to discuss the relation of EpoR and survival in head and neck cancer.

7 RÉSUMÉ

Contexte: Le virus du papillome humain (VPH) est reconnu comme un facteur de risque indépendant pour les carcinomes épidermoïdes (CE) de la tête et du cou. De plus, la présence de l'ADN du VPH est associée à un meilleur pronostic. Des recherches récentes indiquent un plus grand rôle de l'érythropoïétine via une une activation de son récepteur (EpoR), qui est présent dans de nombreuses cellules non-hématopoïétiques néoplasiques. L'expression de EpoR dans un cancer peut représenter un processus de sélection qui permet aux cellules cancéreuses de survivre dans un environnement défavorable et peut aussi indiquer un comportement agressif des cellules cancéreuses. EpoR est variablement exprimé dans les cancers de la tête et du cou et pourrait prédire de façon indépendante une réponse plus faible aux traitements offerts aux patients.

Objectifs: 1. Pour déterminer le statut VPH des cellules et la fréquence de l'expression du récepteur EpoR dans des biopsies obtenues de patients atteints du cancer épidermoïde oropharyngé. 2. Pour évaluer si la présence d'EpoR affecte la survie et si cet effet négatif est influencé par le statut VPH des cellules.

Méthodes: Une étude rétrospective a été menée en examinant les dossiers de 97 patients atteints du cancer épidermoïde oropharyngé
traités par radiothérapie comme intention curative au Centre universitaire de santé McGill, de 2000 à 2009. Les patients éligibles devaient avoir archivé des échantillons de tissus disponibles pour l'analyse VPH et EpoR. L'analyse de la présence du VPH a été effectuée en utilisant une réaction standard en chaîne par polymérase (PCR). La présence du récepteur EpoR a été déterminée par immunohistochimie en utilisant des marqueurs polyclonaux colorants anti-EpoR obtenus chez les lapins. Les sections de pathologie ont été analysées par 2 examinateurs indépendants par la microscopie optique conventionnelle. Un score de 0 à 300 a été déterminé pour chaque patient en multipliant le pourcentage de cellules néoplasiques contenant l'EpoR (0 à 100%) et l'intensité de la coloration de l'EpoR exprimée de 0 à 3.

**Résultats:** L'âge médian était de 62 ans (extrêmes: 43-83). Le statut VPH a été positif chez 74% des patients. Ceci était significativement plus élevé chez les patients âgés ≤ 65 ans, p = 0,023. Les patients ayant des antécédents de tabagisme importants (> 10 paquet-années) et d'alcoolisme (> 4 verres / semaine) étaient significativement plus susceptibles d'être VPH négatif, p = 0,041 et 0,0001 respectivement. En analyse de régression de Cox, la présence de VPH était associé à une réduction de 29% du risque de décès (Hazard Ratio (HR) = 0,71, Intervalle de confiance (IC) 95%: 0,34 à 1,49), mais de façon non significative (p >0.05). Le statut EpoR était positif chez 27% des patients et a été associé à une augmentation non significative de 23% du risque de
décès (RR = 1,23, IC 95%: 0,59 à 2,56). Les patients qui étaient positifs pour le VPH et négatifs pour l’EpoR avaient une réduction non significative de 33% du risque de décès (RR = 0,67, IC 95%: 0,29 à 1,56).

**Conclusion:** Cette étude démontre une tendance qui indique que le VPH et le statut EpoR ont une corrélation avec la survie. Cette tendance persiste lorsque les patients sont divisés en sous-groupes (faible, intermédiaire et haut risque) en fonction de leur statut VPH / EpoR. Le groupe avec le plus faible risque de décès sont les patients avec le statut de VPH positif et EpoR négatif. Au meilleur de nos connaissances, cette étude est la première à examiner la relation entre l’EpoR et la survie chez les patients avec le cancer de la tête et du cou.
8 **INTRODUCTION**

Head and Neck Cancers (H&NCs) are cancers of the upper aerodigestive tract, which includes the oral cavity, the pharynx and the larynx. The majority of those cancers are squamous cell carcinomas (SCCs) in nature (1-4). Oropharyngeal cancers (OPCs) are part of this group and include cancers occurring in the tonsils, the base of tongue, the soft palate and the posterior pharyngeal wall.

In recent years, there has been an increasing interest in oropharyngeal cancer; it is becoming a major public health problem after the discovery of its relationship with Human Papillomavirus (HPV) infection (5-9) and evidenced by its increasing incidence over the last 30 years, specially in younger patients (10-12).

Erythropoietin has been used in head and neck cancer patients as a radiotherapy sensitizer (13). There is, however, increasing concern that Erythropoietin use in H&NCs may negatively affect prognosis especially in association with Erythropoietin receptor (EpoR) expression in cancer cells (14, 15).

So far, however, there have been no studies evaluating EpoR expression and its relationship to outcomes in OPC specifically. In addition, the epidemiological association of EpoR and HPV together and its effect on survival have not, to our knowledge, been assessed.
The rationale of this study is to better understand the factors, especially EpoR and HPV, associated with Oropharyngeal cancers in order to improve patient survival.

As a result, the Objectives of this study is 1- to verify what has been documented in the literature regarding improved outcomes in HPV-related OPC and 2- to determine the effect of EpoR expression on survival and whether this is influenced by HPV status.
9 LITERATURE REVIEW:

9.1 OROPHARYNGEAL CANCER:

9.1.1 EPIDEMIOLOGY OF HEAD AND NECK AND OROPHARYNGEAL CANCERS:

In 2008, it was estimated that approximately 550,319 new H&NC cases are diagnosed every year, and 305,096 deaths occur, making it the eighth most common cancer worldwide (16). The age-adjusted incidence and mortality rates for H&NCs are 8.1 and 4.4 respectively (16). The majority of these cases and deaths occur in less developed regions (n= 352,914) and (n= 223,789) respectively (16). The incidence and prevalence of OPC will be discussed later in the HPV section (Section: 8.2).

9.1.2 RISK FACTORS OF OROPHARYNGEAL CANCER:

OPC has a multifactorial etiology (1), with tobacco smoking and alcohol consumption being the main risk factors (2-4, 17-21). These two habits account for about 75% of OPC (22). Although smoking and drinking are usually practiced together (22), each of these habits behaves as an independent risk factor for OPC (22). In addition, the combined (synergistic) effect of these habits together has been shown to lead to a multiplicative (20, 22) or supra multiplicative impact of their effects (17). Some have found this multiplicative effect to be specific to pharyngeal cancers and not other H&NCs sub-sites (19).
The risk of acquiring these cancers is known to decrease after smokers stop smoking (20, 23). However, the length of time since smoking cessation necessary to observe a marked reduction in risk was different in different studies. Some studies found that the risk decreases markedly 5 years after smoking cessation (23), while others noted a diminution in risk only 10 years after quitting (17, 18, 20, 22). Similarly, the risk of these cancers has been shown to decline after stopping alcohol drinking (17).

Dietary habits constitute another risk factor for OPC. A plethora of research on the relationship of diet and OPC consistently demonstrates the protective effect of fruit and vegetable consumption in reducing OPC (17), especially when eating citrus fruits (18, 20), and carotene-rich vegetables (18).

Poor oral health conditions as well as certain behaviors are linked to an increased OPC risk (18, 20, 24, 25). A higher number of missing teeth (18, 20, 25), inadequate oral hygiene (18, 20, 24, 25), and absent or insufficient dental checkups (20, 25), have been shown to be associated with a higher risk of OPC (17). Although denture use itself is not associated with an increased risk of H&NCs (20), wearing faulty and defective dentures (25) causing mouth sores is (24).

OPC occurs more frequently in males (1, 4), with a male: female ratio of 4:1 (4). Higher male percentages are usually reported in case-control (C-
C) studies (18, 22, 23). In addition, these cancers tend to occur in older age groups (≥ 50 years) (1).

Some socioeconomic indicators, such as low education levels (26), have been found to be associated with an increased risk of these cancers (20). Occupation also seems to play a role on the risk of these cancers (27), where more cases tended to be farmers (20), with blue collar and farmer occupations showing a slightly elevated risk of these cancers (17, 28). A study was published recently by the ARCAGE (Alcohol Related Cancers and Genetic Susceptibility in Europe), which assessed the role of different occupations on the risk of H&NCs, and the most consistent findings were of an increased risk for those working in the construction industry (27).

Familial history and genetic factors seem to play a role on H&NC as well (29, 30). Individuals with a first degree relative who had H&NC were 3 times more likely to get this cancer, with a relative risk (RR) of 3.65 (95% CI: 1.97- 6.76) (29). Higher risks of these cancers were also demonstrated for those who have siblings or a father with H&NC history (29).

More recently, a relationship between sexually transmitted Human Papilloma Virus (HPV) infection and OPC has been noted (5, 8, 9, 31, 32) and will be discussed in more detail in the next section. This discovery led researchers to study the relationship between sexual behaviors and OPC with varying results; some studies found no relation between sexual activity indicators and these cancers (17, 20), while others reported a
direct relationship between the number of lifetime vaginal and/or oral sexual partners and OPC (5, 31). A recent pooled analysis of 8 studies from “The International Head and Neck Cancer Epidemiology Consortium” (INHANCE) supported these relationships (33). A significant reduction in risk among those who practiced oral sex compared with those who didn’t was also reported (31). Some studies found a relationship between risky sexual behaviors and these cancers among males only (32). It seems that the sexual behaviors of HPV-positive H&NC cases are different from the HPV-negative cases, with the former group having riskier sexual behaviors, including a higher number of oral sex partners (34). The actual mechanism of transmission of HPV to the oral cavity is still largely unknown (31, 35).

In summary, the main risk factors for OPC are tobacco smoking and alcohol consumption. While other risk factors seem to play roles, results in the literature are not as strong as those for tobacco and smoking. Differences in results, could be explained by differences in risk factor distributions among different populations (18). Also, some information such as dietary habits is hard to estimate very accurately. Finally, methodological limitations of epidemiological studies, such as recall bias, should always be considered.
9.2 Human Papillomavirus (HPV) and Oropharyngeal Cancer

9.2.1 Human Papillomaviruses

Human papilloma viruses are a group of DNA viruses which are known to cause cervical and anogenital cancers (36). Recently, HPV was found to be associated with a subset of H&NC; specifically that occurring in the oropharynx (5, 9, 37), which includes the tonsils and base of tongue (4, 9). The main risk factor for HPV infection is sexual behavior, i.e. a high number of sexual partners. Immunocompromised individuals, such as those who are HIV positive, are more likely to be infected with HPV. Genetic factors also play a role in some HPV-related diseases (38).

HPVs are tissue and host specific, and usually infect keratinocytes in stratified squamous epithelia (38). There are more than 100 types of HPVs (38), with some infecting the skin (38); and others infecting the mucous membranes of the anogenital tract and oral cavity (38). Mucosal HPVs can lead to a number of diseases, ranging from benign warts or papillomas to invasive cancer (39). The majority of HPV infections are asymptomatic and resolve on their own, with about 10% persisting and leading to significantly increased risk of developing cancer (38).

HPVs have been classified according to their carcinogenic potential into: High risk (HR) and Low risk (LR) HPVs. As the names indicate, the former types have a tendency to lead to malignancy, while the latter have a much
lower potential for malignant transformation. HPV-16 and 18 are examples of HR-HPVs, while HPV-6 and 11 are examples of LR-HPVs (40).

As with cervical cancer, the most common type of HPV type found in OPC is HPV-16 (4, 35, 37, 41, 42).

9.2.2 Mechanism of HPV Carcinogenesis

HPVs are characterized by small capsids, and their genome is a double stranded circular DNA molecule (38). The viral genome is composed of three regions: (1) a long control region (LCR); (2) The early region (E) which contains three oncoproteins, E5, E6 and E7. E5 has growth promoting properties, while E6 and E7 help regulate the cell cycle. There are also two regulatory proteins; E1 and E2 which help in replication and transcription of the viral genome; and E4 which is usually released late in the cell life cycle; (3) the late region, which encodes two structural proteins, L1 and L2 which form the viral capsid (9, 38, 39).

The role the oncoproteins play in carcinogenesis is thought to occur as follows; In the early stages of infection, the oncoprotein E5 promotes cell proliferation by binding to epidermal growth factor receptor (EGFR), platelet derived growth factor β receptor, and colony stimulating factor 1 receptor (39, 43, 44).

However, the important role in carcinogenesis, and in the maintenance of the malignant phenotype is played by E6 and E7 (39). Oncoproteins E6 and E7 bind with and deactivate the tumor suppressors P53 and
Retinoblastoma (pRB) respectively (9). One of the functions of p53 is to trigger cell apoptosis (45), and eliminating cells that have irreparably damaged DNA (46). Therefore, when E6 is expressed, this mechanism is not possible, and genetic instability results (39, 47), which leads to carcinogenesis and cancer progression (47). When E7 deactivates pRB, the result is cell-cycle disruption and malignant changes (43).

9.2.3 HPV DETECTION METHODS:

Originally electron microscopy (EM), and immunohistochemical staining were used for HPV detection; however, these methods lead to inconsistent and irreproducible results (36).

Later, hybridization techniques such as, Southern blot, dot blot, and in situ hybridization were developed (36). These techniques allow for improved detection of viral genetic sequences in cells (36).

Southern blot hybridization technique was long considered the gold standard for HPV detection (38), and genotyping (36). Unfortunately, it has a low sensitivity, is labor intensive, and inter-laboratory variation in results are frequent (36, 38). While dot blot hybridization is faster, only requires small quantities of DNA, and can be used to test several specimens, and it is a faster technique (36, 48), the technique suffers from false positive results. (48). With in situ hybridization, the location of HPV in the tissue can be detected; the tissues can be directly probed for HPV detection.
Unfortunately, the technique is labor intensive and false positive results cannot be distinguished (36, 48).

Amplification of viral DNA sequences using the polymerase chain reaction (PCR) is currently used, and allows the detection of most types of cutaneous or mucosotropic HPV infections, whether they cause asymptomatic or clinically-evident disease (38). PCR protocols have a higher sensitivity and specificity than the other hybridization techniques (36). PCR only requires small amounts of DNA to be tested (36), and different HPV types can be detected using type-specific or broad spectrum primers (38, 49). The type-specific primers allow detection of individual HPV genotypes, while the broad-spectrum primers uses a wide range of primers to detect one or more HPV types simultaneously (49). Some of the primer systems commonly used include, SPF10, GP5+/6+, MY09/11, and PGMY (49). The principal disadvantage of PCR is the possibility of getting false positive results, from amplification of spurious HPV DNA originating from laboratory or pre-testing contamination (4, 36, 48). PCR is also of limited value in parts of the oral cavity that are heavily keratinized and therefore contain low numbers of nucleated cells (4).

Another method that is used for HPV detection, albeit indirectly, is immunohistochemical (IHC) staining of tumors for p16; a protein that acts as a marker of high-risk HPV E7 expression, which in turn acts as a surrogate marker for the presence of HPV (5, 39, 49).
HPV serology can also be used to detect HPV L1 antibody using ELISA or Luminex protocols, and it indicates current or past HPV infections in the oral or anogenital regions, i.e. cumulative exposure of all sites (38, 49). Therefore, it is not very helpful in clinical diagnostic or prognostic procedures (49).

When HPV infection occurs, the virus doesn’t go through the blood, therefore epithelial samples are required to test for HPV presence (49). These samples can consist of biopsy specimens or exfoliated epithelial cells, which can be collected by direct swab sampling or by a mouth rinse (49).

9.2.4 Prevalence and Incidence of HPV in OPC

The reported prevalence of HPV in H&NC varies widely in different studies, surprisingly ranging from 0% to 100% (49). More specifically, HPV-16 has been noted in 20% to 90% of cases of OPC (4). A systematic review of HPV prevalence and type distribution in H&NC was conducted in 2005 (42); The results indicated the overall HPV prevalence in H&NC was 25.9% [95% Confidence Interval (CI): 24.7-27.2]. The prevalence was significantly higher in OPC at 35.6% (95%CI: 32.6-38.7), compared with 23.5% (95%CI: 21.9-25.1) for oral cancers, and 24.0% (95%CI: 21.8-26.3) for laryngeal cancers (42). Another multicenter case-control study coordinated by the International Agency for Research on Cancer (IARC) reported a much lower HPV prevalence in oral and oropharyngeal cancer.
at 3.9% (95%CI: 2.5% to 5.3%) and 18.3% (95%CI: 12.0% to 24.7%) respectively (35).

Worldwide estimates of HPV in OPC are variable, in some studies ranging between 12-22% (50-52). However, in recent studies it has increased to 40-78% (11, 53-55). The reason for the widely varying prevalence in different studies could be attributed to different sampling techniques or different HPV detection methods used in the different studies (4). In addition, the type and quality of the samples tested for HPV presence may affect the results (49).

In a recent systematic review and meta analysis by Mehanna H et al performed in 2012; the overall HPV prevalence in OPC detected was 47.7% (95% CI, 42.9–52.5%) (12). It was also noted that the HPV prevalence in OPC increased over the years; it was 40.5% (95% CI, 35.1–46.1) before 2000, increased to 64.3% (95% CI, 56.7–71.3) between 2000 and 2004, and further increased to 72.2% (95% CI, 52.9–85.7) between 2005 and 2009 (12). However, the prevalence of HPV in non-OPC (21.8%; 95% CI, 18.9–25.1%) did not increase over time (12).

Interestingly, the incidence of H&NC has been declining over the past few decades, while the incidence of OPC and in particular, HPV- related H&NC has been on the rise (9, 56), without a parallel increase in smoking and alcohol drinking levels (57). Several countries including the USA (56, 58), Europe (57, 59) and Canada, (60), have been consistent in noting the
same disease patterns. In the United States, the incidence of HPV-unrelated H&NC decreased from 1983-2004, while the incidence of HPV-related H&NC increased significantly between 1972-2004 particularly in young Caucasian males (56). Similarly, a 2.9-fold increase in HPV-related tonsillar cancer was observed in Sweden between 1970-2002 (57). A recent 2012 Canadian article, reported an increase in the annual percent change (APC) of 1.5% for men and 0.8% for women for OPC between the years 1992-2007 (60), while the other H&NC showed a decrease over the same time period (60). These trends were accompanied by a decrease in the age of diagnosis over the same period, and an improved OPC survival in the period 1992-2001, specifically in males (60). It has been suggested that the increase in OPC may be attributable to sexually transmitted HPV infection (8, 9) from changing sexual behaviors over time (9).

9.2.5 **Differences between HPV-positive and HPV-negative OPC:**

H&NCs have traditionally been treated as a relatively homogeneous group in studies (49). Increasing research and intense interest in HPV as a causative agent strongly suggests that these cancers should be stratified according to several factors such as HPV status and anatomical sub-site (49).

HPV-related and HPV-unrelated H&NC have been described as two distinct disease entities (12), because of their different demographic, clinical, histological, and molecular characteristics (34, 61). HPV-related
cancers occur more frequently in younger males (9, 12, 41, 49, 56), in Caucasians (9), and in non-smokers (9). They also occur more in the oropharynx than any other anatomical sub-site (12, 37, 49). The ratio of males to females for oral cancers has decreased to 1.5:1, while the ratio for OPC has increased to 2.8:1 (1). The reason for the higher OPC rate in men is unclear, but it has been speculated that males may share certain characteristics such as riskier sexual behavior that make them more susceptible to HPV-related OPC (1).

On the molecular level, HPV-positive OPC has a wild type but deactivated p53 tumor suppressor, rather than a mutated p53 which is found in HPV-negative OPC (9). In addition, E7 deactivates RB in HPV-positive tumors, and thus RB levels decrease which leads to p16 overexpression (9). In contrast, higher levels of RB and lower expression of p16 are usually found in HPV-negative OPC (9).

From a histological point of view, HPV-related H&NC is often described as “poorly differentiated”, or “basaloid” (9, 49), and is usually a non-keratinizing SCC (9).

For reasons that are unclear, HPV-positive OPC are usually diagnosed at later stages (41), are of advanced grades (41), and are more likely to have nodal involvement (41). Despite the above findings, these patients tend to respond well to treatment and have a better prognosis (9, 37, 49), and improved survival (4, 9, 12, 37, 56). However, the prognosis is worse in
those patients with a history of tobacco smoking (9). These findings were supported by a number of systematic reviews and meta analyses (37); A systematic review by Ragin et al in 2007, revealed that patients with HPV-positive OPC had a 28% decreased risk of death, and a 49% reduced risk of disease failure when compared to HPV-negative OPC (62). A more recent, 2010 systematic review and meta analysis by Dayyani et al, demonstrated a Hazard Ratio (HR) of 0.42 (95%CI: 0.27-0.57) for HPV positive cancers compared with HPV negative cancers, and a better response to treatment in the former group (37). Genetic differences between HPV-positive and HPV-negative have also been suggested but further study is needed in this area (61).

The risk factor profiles of HPV-positive versus HPV-negative H&NCs are different (34). Risky sexual behaviors (41), and marijuana consumption are more common in HPV-positive H&NC (34). On the other hand, smoking, alcohol drinking and poor oral hygiene are more frequently associated with HPV-negative H&NC (34).

In view of the cumulative evidence presented, it is reasonable to recognize HPV-positive OPC and indeed HPV-positive H&NC as a new emerging disease which is rapidly increasing in incidence (49). Further study continues into the etiology of HPV-related H&NC, as well as methods permitting early diagnosis and treatment. Early data suggest that these cancers may respond differently to treatment, and consequently, should have a separate management approach(37, 62).
Human papillomavirus (HPV) related oropharyngeal squamous cell carcinoma (OPC) and its relation to survival has become an increasingly important topic in the past few years. A considerable amount of literature from Europe, USA and Canada has been published on the HPV-DNA status in OPC and its relationship to survival. These studies have shown a significant improvement in prognosis and survival in HPV positive OPC compared to HPV negative OPC (10, 50, 51, 53, 54, 63-66).

In 2006, Licitra et al. demonstrated that HPV-related OPC patients had a better survival when treated surgically (51). In another major study, Lassen (2009) studied 156 patients with Head and Neck SCC (HNSCC) and again found that HPV-positive HNSCC was associated with a better overall and disease-free survival and on a multivariate analysis where HPV positivity remained as an independent prognostic factor for survival (50). Surprisingly, in the latter two studies, only 19-22% of subjects were HPV positive. These numbers are significantly lower than the much higher percentages of HPV positive cancers especially in the oropharynx found in the literature (50, 51, 53, 64).

In a previous major study, Ang et al (2010) studied 720 patients retrospectively and found that 63.8% of the OPCs were HPV positive and their overall and progression-free survival were significantly better than the HPV negative group. They also confirmed its significance as an
independent risk factor for survival after adjustment for age, race, stage, tobacco exposure, and treatment protocol (53). In a recent systematic review and meta-analysis by O’Rorke et al (2012), pooled results from 42 studies were analyzed and the relationship between HPV-related OPC and improved outcomes was confirmed with a 53% reduction in risk of death (HR=0.47, 95%CI: 0.35–0.62) and 74% reduction in risk of recurrences (HR=0.26, 95%CI: 0.17–0.39) (54).

9.3 ERYTHROPOIETIN AND ERYTHROPOIETIN RECEPTORS (EpoR):

9.3.1 RATIONALE:

Erythropoietin is a glycoprotein that is produced in response to hypoxia and/or anemia from the cortex of the kidneys. Its primary mechanism of action is by stimulating erythropoiesis in the bone marrow by inhibiting apoptosis of erythrocyte precursors (67-69). Erythropoietin was previously administered more frequently to cancer patients to reduce anemia and decrease the need for blood transfusion (70).

Radiation therapy works either by directly damaging the DNA of cells or by forming free radicals that can then damage cellular DNA. Oxygen is a radiosensitizer which increases the efficacy of a given dose of radiation therapy. Hypoxia adversely affects both radiation therapy and chemotherapy outcomes, including survival in head and neck cancers (71).
A relationship exists between the presence of anemia and hypoxia or anoxia of solid tumors. Increasing the concentration of hemoglobin is directly related to an increase in oxygenation in advanced breast and gynecological tumors (72, 73), which may lead to a better response to treatment and better outcome. This raised the possibility that correcting the anemia with Erythropoietin could improve oxygen supply to the tumor tissue and increase sensitivity to radiotherapy (74). Concurrently, it has also been noted that the expression of EpoR is greater in hypoxic cancer cells (75).

In 2001 Littlewood et al. demonstrated that Erythropoietin treatment in cancer patients receiving radiation therapy might improve their survival (13). It was thought that Erythropoietin growth stimulation was restricted to erythrocyte precursors in the bone marrow, but recent evidence suggests that this Erythropoietin stimulation effect extends to Erythropoietin receptors (EpoR) throughout their distribution on other cells in the body including tumor cells (75-79). EpoR has been detected in many neoplastic cells e.g. breast cancer, embryonal carcinoma, hepatocellular and cervical carcinoma (80-83), and in 96% of non-small cell lung cancer cells (77). Recently, in vivo and in vitro studies have shown that EpoR can be found in endothelial cells and that the Erythropoietin /EpoR complex promotes angiogenesis (67, 84, 85).

The first serious discussions and analyses of EpoR expression and its possible contribution in tumor growth emerged from studies on breast
cancer (86-88). In recent years, there has been an increasing amount of literature on EpoR expression in head and neck cancer cells (14, 15, 76, 89). It has been demonstrated that exogenous erythropoietin therapy in the presence of EpoR is an indicator of bad prognosis and poor survival in head and neck cancer (14, 15).

9.3.2 MECHANISM OF GROWTH STIMULATION

Hypoxia of cancer cells may stimulate endogenous Erythropoietin production and that in conjunction with stimulation of EpoR on cancer cells may stimulate tumor growth and increase tumor resistance to treatment by stimulating vascular endothelial cells, inhibiting apoptosis of cancer cells and favoring the formation of neovascularization of cancer cells (80, 90-92).

Previous studies have shown that EpoRs are stimulated by Erythropoietin, which in turn stimulate JAK2 kinase that activates cytoplasmic singling proteins e.g. STAT5, AKT, and ERK1/2. The cytoplasmic singling proteins may lead to the stimulation of cellular proliferation and invasion and inhibition of apoptosis (69, 93, 94). Therefore, the presence of EpoR on cancer cells by itself might be an indication of more aggressive behavior, which may be related to the stimulation of these receptors by endogenous Erythropoietin (80, 95).

In vitro studies have shown that EpoR positive tissues are resistant to radiation therapy and chemotherapy. The administration of a specific JAK2
kinase inhibitor increases the sensitivity to radiation and cisplatin therapy (96). JAK2 kinase inhibitors and other drugs, which antagonize the effect of endogenous Erythropoietin in head and neck cancer, may play a role in the eventual development of molecular targeted therapies against these types of cancers (94, 97).

9.3.3 **ERYTHROPOIETIN /EpoR AND SURVIVAL**

EpoR expression in cancer cells is associated with increased cancer cell survival and proliferation (86, 88). The presence of EpoR in patients with head and neck cancer may relate inversely to radiotherapy response and even more so when associated with exogenous Erythropoietin administration (95). Several studies have revealed that using Erythropoietin in the treatment of head and neck cancer patients with anemia did not improve radiotherapy response. By comparison, control groups not receiving Erythropoietin showed better loco-regional control and survival (98, 99). These results support the hypothesis that the Erythropoietin /EpoR complex stimulates tumor proliferation.

In a recent in vitro study by Abhold et al (2011), head and neck cancer cell lines demonstrated significant EpoR expression and Erythropoietin exerted a significant proliferative effect on tumor progression in EpoR positive cancer cells (100). However, another study by Sasaki et al (2009), from Sweden showed that Erythropoietin did not have a significant stimulatory effect on tumor proliferation in head and neck cancer cells
except in high concentrations where Erythropoietin might have had some proliferative effect through stimulation of receptors other than EpoR on the tumor cells (101). The lack of cellular proliferation in this study might be explained by the apparent absence of EpoR expression in the head and neck experimented cell lines.

A number of studies have reported EpoR expression in head and neck cancer cells but did not examine any possible association with survival (76, 102). Arcasoy et al in 2005 studied twenty-one patients with primary SCC of the head and neck for the expression of EpoR, endogenous Erythropoietin and their relationship to hypoxia. EpoR was present in 97% of the patients and was significantly related to hypoxia, but its possible association with survival was not evaluated (76). In the same year, Mohyeldin et al. also demonstrated an increased expression of EpoR in head and neck cancer tissues and diminished expression in normal tissues of the head and neck, but again did not assess its effect on prognosis (102). Previous studies have reported poor survival in cancer patients who receive Erythropoietin treatment (98, 103). A large multicenter randomized study by The Breast Cancer Erythropoietin Trial (BEST) group was terminated because of a significant disease progression in the arm that received Erythropoietin compared to the placebo group in patients with metastatic breast cancer (103). In another major study, Henke et al (2003) studied 351 different types of head and neck cancer patients and found that survival was significantly worse in
patients given Erythropoietin therapy to correct anemia (98).

This was confirmed in studies by Henke et al in 2006 and Miller et al in 2009, which found that survival is significantly higher in EpoR positive patients who received placebo compared with those who received Erythropoietin therapy (14, 104). In these same studies, patients with negative expression of EpoR demonstrated no significant difference in survival between whether or not they received Erythropoietin (14, 104). However, when Henke et al (2006) compared all EpoR positive and negative patients the survival results did not reach significance but showed a trend to better survival in the EpoR negative group and by looking at the placebo groups only, the negative expression of EpoR by itself was protective; although it didn’t reach statistical significance, but might explain the possible effect of the endogenous Erythropoietin in stimulating EpoR (14). In 2009, Miller and co workers demonstrated that patients who were EpoR positive had a trend to worse locoregional progression-free survival than EpoR negative patients when both received Erythropoietin treatment (104).

Recent studies by Li HG et al (2009), Roh JL et al (2009) and Lin YT et al (2012) suggest that EpoR negative oral cavity cancer patients have significantly higher survival than EpoR positive ones and that EpoR expression is higher in advanced stages of cancer (89, 105, 106). The former two studies involved only tongue cancer patients and concluded that EpoR is an independent prognostic factor for survival (89, 106). Of
note, in the study by Lin et al, which included all oral cavity cancer patients, EpoR expression was associated with a poorer survival, but in multivariate analysis, stage was found to be the only independent prognostic factor for survival (105).

As a result of the above literature review, the following sections of this thesis will focus on oropharyngeal cancer patients - looking at HPV and EpoR as prognostic indicators for survival. Understanding the relationship between EPOR and the prognosis of cancer may open the door for the development of molecular-targeted therapy that might improve the efficiency of treatment in head and neck cancer patients.
10 METHODS:

10.1 STUDY DESIGN

The study protocol was reviewed and approved by an institutional review board committee at the McGill University Health Center (MUHC). A retrospective cohort study was designed to investigate HPV and EpoR as prognostic markers for survival in head and neck cancer patients. Biological samples from a consecutive group of head and neck cancer patients were collected and subjected for analysis.

10.2 PATIENTS AND SETTING:

All patients with squamous cell carcinoma of the oropharynx diagnosed, treated and followed at the McGill University Health Center (MUHC), Montreal, Quebec, Canada from 2000 to 2009 were included in the cohort (N=163). Patients who did not receive radiation therapy or those with missing archived paraffin blocks were excluded from the study (N=66).

A chart review of these patients was conducted to determine the following parameters: age, gender, risk factors (tobacco and alcohol), site of lesion, TNM staging, treatment protocol, treatment response and outcome.

Archived tissue samples and paraffin blocks from these patients were processed, and then were subjected to HPV and EpoR testing. Prognosis
was then looked at as a function of presence/absence of HPV and/or EpoR.

10.3 Sample Processing:

Most of the paraffin blocks were processed in McGill Centre for Translational Research in Cancer (MCTRC) at the Jewish General Hospital. Approximately 10-15 5-um sections were cut with the microtome from portions of the paraffin block representing active tumor growth. The middle one or two sections were separated for IHC to determine the EpoR status. The remaining section was placed in a plastic tube labeled with the patient's study number and sent to the laboratory for HPV DNA analysis. The first and last sections of each specimen were stained with Hematoxylin and Eosin (H&E) stain. Because of the exquisite sensitivity of the PCR assay, meticulous precautions to prevent and monitor inter-specimen contamination were taken throughout the processing of the specimens. These procedures included frequent changing of gloves between specimens, careful cleaning of the microtome, blade replacement between specimens, and the use of disposable material to transfer tissue ribbons to their storage vials. After each specimen was processed, all tubes were capped and the microtome blades were thoroughly cleaned with alcohol. Any instrument touching the sections (fine brush or forceps) was either discarded or washed with alcohol. A separate glass slide was used for laying out the sections belonging to the specimens prior to transferring them into the tubes. Sectioning was performed at a facility.
that does not perform HPV testing.

10.4 HPV DNA Testing

The samples, contained in plastic tubes were sent to Dr. Francois Coutlee’s laboratory at Notre Dame Hospital for HPV testing and typing using accepted and standardized PCR methods (for the status of DNA and viral load). Each paraffin section was deparaffinized using octane at room temperature after shaking the solubilized section for fifteen to thirty minutes. A pellet of tissue was formed after centrifugation at 13,000 x g for 15 minutes and washed with 100% ethanol. Then the tissue was dried by the addition of 10 µl acetone in a 55°C heating block. After that, the tissue was re-suspended in Tris-EDTA buffer with a pH 7.5 and the DNA was purified with a Master pure extraction kit (Epicentre, Madison, WI) (107). The extracted DNA (500 ng of DNA) was tested for HPV DNA by PCR based on the Linear Array HPV genotyping assay (LA-HPV) (Roche Molecular Systems). LA-HPV was used to test the extracted DNA by polymerase chain reaction (PCR) using a PGMY primer system for amplification of a 450 bp segment in the HPV L1 gene. Using this technique allowed for testing of thirty-six HPV genotypes: types 6, 11, 16, 18, 26, 31, 33, 34 (previously known as type 64), 35, 39, 40, 42, 44 (previously known as type 55), 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82 (including subtype IS39), 83, 84, and 89 (previously known as CP6108). At the same time ß-globin amplification was performed on the extracted DNA by performing PCR using PC04 and
GH20 primers to determine if a 268 bp fragment could be amplified from the DNA extracted from the paraffin blocks i.e. to assess adequacy of the sample for HPV-DNA testing. Those samples that were HPV-negative with PGMY were retested with a primer pair (GP5+/GP6+) using AmpliTaq gold that amplifies a shorter segment of the L1 HPV-DNA gene of 145 bp. This process increased the likelihood of a positive result despite the fragmentation of the DNA (108-110). PGMY primer amplifies only long segments of the DNA so using it alone would increase the chance of false negative results specially in paraffin-embedded biopsies, so this protocol using GP5+/GP6+ would increase the chances of detecting HPV-DNA as it is more sensitive than PGMY on picking up damaged DNA in our paraffin biopsy specimens (111). After gel electrophoresis, a DNA sequencing procedure was carried on in which, 145 bp amplicons were identified and extracted for sequencing. The fluorescent cycle-sequencing method (BigDye terminator ready reaction kit, Perkin-Elmer) was used on 20 ng of the purified amplicons for direct PCR-sequencing of the amplicons using the same primers described above (112, 113). Sequence analysis was performed on an ABI Prism 3100 Genetic Analyser system. Amplicon sequences were compared with known HPV sequences using BLAST. To exclude any possibility of false negative results for HPV detection because of fragmentation of DNA in the old paraffin-fixed biopsy specimens, 2 µl of extracted DNA from samples that tested negative for HPV with GP5+/GP6+ primers were retested for the presence of β-globin
using the primer pair PC03/PC04 that amplifies a shorter fragment of DNA of 110 bp (Saiki, R. 1990). HPV-negative specimens that also tested negative for β-globin with PC03/PC04 were considered inadequate for HPV DNA detection and analysis.

10.4.1 HPV TYPING ANALYSIS

Patients who tested positive for high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and probable high-risk (HPV 26, 53, and 66) were considered HPV positive while those who tested negative for HPV or tested positive for low-risk HPV types (6, 11, 40, 42, 54, 55, 61, 62, 50, 64, 67, 69, 70, 71, 72, 81, 83, and 84) were considered HPV negative (53, 114-117).

10.5 EpoR TESTING

10.5.1 Hematoxylin and Eosin (H&E) Staining

Sections were deparaffinized in xylene and rehydrated through graded alcohols to water. For routine histology, sections were stained with hematoxylin and eosin (H&E). H&E staining is a general stain to visualize cell morphology in tissue sections, so sections are stained for 1 minute with hematoxylin (Gill Method, Fisher) followed by another 5 seconds incubation in eosin (Fisher) and finally, dehydrated in graded alcohols, cleared in xylene and cover slipped. Sections were analyzed by conventional light microscopy.
10.5.2 EpoR Immunohistochemistry

Immunohistochemistry was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital). Tissue samples were cut at 4-µm, placed on Super Frost/ Plus slides (Fisher), and dried overnight at 37°C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical System). All solutions used for automated immunohistochemistry were from Ventana Medical System unless otherwise specified. Immunostaining for EpoR was performed online using a heat protocol. Briefly, rabbit polyclonal anti-EpoR (Santa Cruz Biotechnology, Inc.) diluted 1:200 in Antibody diluent solution was manually applied for 32min, and then followed by the appropriate detection kit (Omnimap anti-Rabbit HRP) to virtually eliminate background and non-specific staining. A negative control was performed by the omission of the primary antibody. Positive control consisted of human liver tissue. Slides were counterstained with hematoxylin for four minutes, blued with Bluing Reagent for four minutes, removed from the autostainer, washed in warm soapy water, dehydrated through graded alcohols, cleared in xylene, and mounted with Permount. Sections were analyzed by conventional light microscopy.
10.5.3 **EpoR Analysis**

A designated MUHC pathologist determined the EpoR status. For each specimen, the first and last slides were examined under the microscope to confirm the presence of cancer cells in between. Then the percentage of positively stained neoplastic cells, varying from 0% to 100%, was determined using low power on the microscope. The intensity of EpoR expression was determined by comparing staining of the slide under high power to a positive control, and assigning it 0 for complete absence of staining or as +3 for strongly positive staining (Figure 1).

**Figure 1: EpoR positive immune staining**

A staining intensity of 0 was considered negative, and mildly stained cells with the intensity of 1 were considered positive only if staining 60% or more of the neoplastic cells, moderately stained cells with intensity of 2 and strongly stained cells with intensity of 3 were considered positive with at least 30% and 20% stained neoplastic cells, respectively. The score product of the percentage of positive stained cells, and the intensity score
was calculated for each specimen. The score product that results from the percentage of positive stained cells multiplied by the intensity score was calculated for each specimen, and was referred to as the Weighed staining intensity (WSI) (= Intensity score X Percentage of cells). A WSI above 60 was considered positive.

10.6 Statistical analysis

A total of 97 patients with oropharyngeal cancers between 2000-2009 were included. Basic descriptive statistics were performed to compare patient characteristics including socioeconomic, clinical and treatment characteristics with HPV and EpoR status. Univariate and multivariate statistical analyses were performed to identify whether the results of HPV testing and EpoR expression were significant predictors of outcome in the cohort, over and above the prognostic information currently used. Kaplan-Meier curves (118) and log-rank tests (119) were used to assess significance of HPV and EpoR status on overall survival, disease-free survival, mean survival and 2-year survival rates in relation to age, sex, stage, smoking, drinking, HPV status and EpoR status. The Cox proportional-hazards model was used to assess the incremental prognostic and predictive values of the EpoR expression and HPV status over and above the other standard and potential prognostic features of the tumor and the patient (age of patient, smoking status, and stage of disease).
The disease-free survival was analyzed according to the patterns of failure in relation to the HPV-EpoR status. Patterns of failure may be: local, regional, distant metastasis, local and regional, and local, regional and distant metastatic spread. In order to understand patient relationships with overall and disease-free survivals, exposure groups were divided into 4 groups according to their HPV/EpoR status as follows: 1. HPV-/EpoR-, 2. HPV-/EpoR+, 3. HPV+/EpoR- and 4. HPV+/EpoR+. These groups were then evaluated using logistic regression.
11 **RESULTS:**

11.1 **DESCRIPTIVE ANALYSIS**

11.1.1 **HPV DNA & TYPING AND EPOR STATUS RESULTS**

HPV status was positive in 74% (n=72/97) of the patients. 92% (n=66/72) of the HPV positive patients were HPV-16 positive, 6% (n=4/72) were HPV-18 positive, 1% (n=1/72) was positive for HPV-33 and 1% (n=1/72) was positive for HPV-35. None of the patients tested positive for probable high-risk HPV (HPV 26, 53, 66). Only 2 patients (3%) tested positive for low-risk HPV (HPV-69) but both were also positive for HPV-16. The rest of the patients n=25/97 (26%) were HPV negative. EpoR status was positive in 26.80% (n=26).

11.1.2 **PATIENT SOCIODEMOGRAPHIC CHARACTERISTICS**

A total of 97 patients with oropharyngeal cancers between 2000-2009 were enrolled in the study after excluding patients who did not meet the study protocol criteria. All the patients were treated with radiation therapy with or without surgery. Sociodemographic characteristics are shown in Table 1.
### Table 1: Patient sociodemographic characteristics

<table>
<thead>
<tr>
<th></th>
<th>According to HPV status</th>
<th>According to EpoR status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV neg (n=25)</td>
<td>HPV positive (n=72)</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤65</td>
<td>61</td>
<td>11 (44.0)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>36</td>
<td>14 (56.0)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>73</td>
<td>20 (80.0)</td>
</tr>
<tr>
<td>F</td>
<td>24</td>
<td>5 (20.0)</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10 pack-years</td>
<td>32</td>
<td>4 (18.2)</td>
</tr>
<tr>
<td>&gt;10 pack-years</td>
<td>56</td>
<td>18 (81.8)</td>
</tr>
<tr>
<td><strong>Drinking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3 drinks/week</td>
<td>46</td>
<td>4 (16.0)</td>
</tr>
<tr>
<td>&gt;4 drinks/week</td>
<td>37</td>
<td>16 (64.0)</td>
</tr>
</tbody>
</table>

The average age of our oropharyngeal cancer patients was 62 years (range: 43–83) and most of them were younger than 65 (62.89%). HPV status was positive in 74% (n=72) of all patients. Patients’ ≤65 years of age accounted for the majority, or 69.4% of all HPV-positive patients, and this was significant with a p-value of 0.023. EpoR status was positive in 26.80% (n=26). There was no statistical difference between the two age groups in regards to their EpoR status. Most patients were males n=73 (75.25%), with no statistical significance in relation to HPV or EpoR status.
and gender. 58% (n=56) of the patients were heavy smokers (>10 pack-years). Heavy smokers, accounted for 82% of all HPV-negative patients and this was statistically significant with a p-value of 0.041. Furthermore, 64% of the HPV-negative patients were heavy alcohol drinkers (>4 drinks/week) and this was also statistically significant with a p-value of 0.0001. EpoR status did not show any statistically significant association with smoking or drinking.

11.1.3 Patient Clinical and Treatment Characteristics

All of the patients in this study were diagnosed with primary squamous cell carcinoma of the oropharynx. The most commonly involved subsite was tonsillar, in 60% (n=58), followed by the base of tongue in approximately 25%. More than 80% of the patients were diagnosed in advanced stages (stage III & IV) of the disease. More than 90% of the patients were treated primarily with radiation therapy and more than 60% of the patients were treated with concurrent chemotherapy and radiotherapy (Table 2 and 3).
<table>
<thead>
<tr>
<th>Site</th>
<th>All Patients n= 97</th>
<th>According to HPV status</th>
<th>According to EpoR status</th>
<th>p value</th>
<th>EpoR neg (n= 71 ) n (%)</th>
<th>EpoR positive (n= 26) n (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HPV neg (n= 25) n (%)</td>
<td>HPV positive (n= 72) n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsils</td>
<td>58</td>
<td>12 (48.00)</td>
<td>46 (63.89)</td>
<td></td>
<td>39 (54.93)</td>
<td>19 (73.08)</td>
<td></td>
</tr>
<tr>
<td>Soft Palate</td>
<td>6</td>
<td>3 (12.00)</td>
<td>3 (4.17)</td>
<td>0.293</td>
<td>5 (7.04)</td>
<td>1 (3.85)</td>
<td>0.022</td>
</tr>
<tr>
<td>Base of Tongue</td>
<td>25</td>
<td>9 (36.00)</td>
<td>16 (22.22)</td>
<td></td>
<td>23 (32.39)</td>
<td>2 (7.69)</td>
<td></td>
</tr>
<tr>
<td>Posterior Pharyngeal Wall</td>
<td>2</td>
<td>0 (0.00)</td>
<td>2 (2.78)</td>
<td></td>
<td>2 (2.82)</td>
<td>0 (0.00)</td>
<td></td>
</tr>
<tr>
<td>Other Oropharynx</td>
<td>6</td>
<td>1 (4.00)</td>
<td>5 (6.94)</td>
<td></td>
<td>2 (2.82)</td>
<td>4 (15.38)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>11</td>
<td>5 (20.00)</td>
<td>6 (8.33)</td>
<td>0.279</td>
<td>6 (8.45)</td>
<td>5 (19.23)</td>
<td>0.046</td>
</tr>
<tr>
<td>III</td>
<td>23</td>
<td>5 (20.00)</td>
<td>18 (25.00)</td>
<td></td>
<td>21 (29.58)</td>
<td>2 (7.69)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>63</td>
<td>15 (60.00)</td>
<td>48 (66.67)</td>
<td></td>
<td>44 (61.97)</td>
<td>19 (73.08)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Patient treatment characteristics

<table>
<thead>
<tr>
<th></th>
<th>All Patients n=97</th>
<th>According to HPV status</th>
<th>According to EpoR status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HPV neg (n= 25)</td>
<td>HPV positive (n= 72)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>90</td>
<td>23 (92.0)</td>
<td>67 (93.06)</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>7</td>
<td>2 (8.00)</td>
<td>5 (6.94)</td>
</tr>
<tr>
<td>Surgery</td>
<td>Biopsy</td>
<td>83</td>
<td>21 (84.0)</td>
</tr>
<tr>
<td>Neck Dissection</td>
<td>7</td>
<td>1 (4.00)</td>
<td>6 (8.33)</td>
</tr>
<tr>
<td>Ablative</td>
<td>7</td>
<td>3 (12.00)</td>
<td>4 (5.56)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>No Chemo</td>
<td>33</td>
<td>12 (48.0)</td>
</tr>
<tr>
<td></td>
<td>Concurrent chemo</td>
<td>61</td>
<td>12 (48.00)</td>
</tr>
</tbody>
</table>

11.2 Survival analysis

11.2.1 HPV DNA Status and Survival

The 2-year overall survival rate was 83% (95% CI, 71.54 - 89.80) in HPV-positive patients, compared to 75% (95% CI, 53.01-88.06) for the HPV-negative group. The 2-year disease-free survival was also higher in the HPV positive group (77%; 95% CI, 0.651 - 0.852) compared to the HPV negative group (59%; 95% CI, 0.373 - 0.756) (Table 4).
### Table 4: Disease Free Survival (DFS) and Overall Survival (OS) according to socioeconomic, clinical and treatment characteristics

<table>
<thead>
<tr>
<th>Variable and categories</th>
<th>OS</th>
<th>DFS</th>
<th>Sex</th>
<th>HPV</th>
<th>Site</th>
<th>Stage</th>
<th>Radiation</th>
<th>Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean 2yr Survival 95% CI Mean 2yr Survival</td>
<td>Mean 2yr Survival 95% CI</td>
<td>Mean 2yr Survival 95% CI</td>
<td>Mean 2yr Survival 95% CI</td>
<td>Mean 2yr Survival 95% CI</td>
<td>Mean 2yr Survival 95% CI</td>
<td>Mean 2yr Survival 95% CI</td>
<td>Mean 2yr Survival 95% CI</td>
</tr>
<tr>
<td>Age</td>
<td>113.7 0.848 0.7293-0.9183</td>
<td>112.3 0.748 0.617-0.839</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>&gt;65 61.5 0.740 0.5591-0.8558</td>
<td>57.3 0.682 0.498-0.811</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sex</td>
<td>M 78.6 0.7856 0.6694-0.8649</td>
<td>76.4 0.686 0.562-0.781</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F 108.2 0.8750 0.6608-0.9579</td>
<td>106.7 0.833 0.614-0.933</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Smoking</td>
<td>≤10 pack-years 118.9519 0.8438 0.6646-0.9318</td>
<td>115.9 0.781 0.595-0.889</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>&gt;10 pack-years 80.5555 0.8279 0.6946-0.9067</td>
<td>77.1 0.715 0.571-0.818</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drinking</td>
<td>≤3 drinks/week 90.8209 0.8674 0.7284-0.9382</td>
<td>89.7 0.777 0.624-0.874</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4 drinks/week 69.1858 0.7720 0.5949-0.8790</td>
<td>63.4 0.662 0.401-0.792</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EpoR</td>
<td>Negative 101.8574 0.8409 0.7309-0.9087</td>
<td>99.1 0.738 0.616-0.826</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Positive 75.3614 0.7200 0.5009-0.8555</td>
<td>74.8 0.682 0.464-0.827</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HPV</td>
<td>Negative 97.04692 0.7527 0.5301-0.8806</td>
<td>95.1 0.591 0.373-0.756</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Positive 84.02068 0.8271 0.7154-0.8890</td>
<td>81.7 0.769 0.651-0.852</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Site</td>
<td>Tonsils 90.81475 0.8595 0.7384-0.9271</td>
<td>89.2 0.789 0.658-0.874</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Soft Palate 119.4047 1.0000 -</td>
<td>115.9 0.833 0.273-0.974</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Base of Tongue 71.75069 0.7980 0.5806-0.9106</td>
<td>64.3 0.671 0.448-0.821</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Poste. Pharyngeal Wall 14.87 0 -</td>
<td>12.0 0 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Oropharynx NOS 40.664 0.4000 0.0520-0.7528</td>
<td>40.7 0.400 0.052-0.753</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stage</td>
<td>I/II 115.9342 0.8182 0.4474-0.9512</td>
<td>113.7 0.727 0.371-0.903</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>III 77.79725 0.8169 0.5818-0.9273</td>
<td>74.2 0.773 0.536-0.899</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IV 78.30646 0.8029 0.6789-0.8830</td>
<td>73.7 0.704 0.571-0.802</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Radiation</td>
<td>Primary 101.8167 0.8039 0.7035-0.8734</td>
<td>100.4 0.736 0.630-0.816</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Adjuvant 72.60857 0.8571 0.3341-0.9786</td>
<td>64.6 0.571 0.172-0.837</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Surgery</td>
<td>Biopsy 97.32164 0.8002 0.6945-0.8726</td>
<td>95.7 0.726 0.814-0.811</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Neck Dissection 106.4925 1.0000 -</td>
<td>105.6 0.857 0.334-0.979</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ablative 66.82286 0.7143 0.2582-0.9198</td>
<td>63.5 0.571 0.172-0.837</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>No Chemo 107.5108 0.8182 0.6394-0.9139</td>
<td>104.6 0.697 0.510-0.824</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Concurrent chemo 80.88174 0.8097 0.6824-0.8900</td>
<td>80.4 0.757 0.623-0.848</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| DFS P-Value | 0.0308 | 0.4868 | 0.0586 | 0.6440 | 0.1943 | 0.0039 | 0.9180 | 0.8083 | 0.3137 | 0.5013 | 0.041 | 0.567 | 0.110 | 0.868 | 0.204 | 0.017 | 0.939 | 0.620 | 0.342 | 0.236 |

| OS P-Value | 0.041 | 0.394 | 0.567 | 0.110 | 0.868 | 0.204 | 0.017 | 0.939 | 0.620 | 0.342 | 0.236 |

51
Table 5 shows the details for the overall and disease-free survival by HPV DNA status using Cox regression analysis.

Table 5: Overall survival and disease free survival by HPV and EpoR using Cox regression

<table>
<thead>
<tr>
<th>Predictor</th>
<th>OS</th>
<th></th>
<th>DFS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>HR (95% CI)</td>
<td>Adjusted HR</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>HPV</td>
<td>0.64 (0.32-1.26)</td>
<td>0.71 (0.34-1.49)</td>
<td>0.64 (0.32-1.27)</td>
<td>0.76 (0.36-1.57)</td>
</tr>
<tr>
<td>EpoR</td>
<td>1.36 (0.66-2.80)</td>
<td>1.23 (0.59-2.56)</td>
<td>1.26 (0.61-2.59)</td>
<td>1.14 (0.55-2.37)</td>
</tr>
</tbody>
</table>

Adjusted for age and stage. HRs refer to the negative status for each of the two variables.

HPV positivity was associated with a 29% reduction in risk of death (HR 0.71; 95% CI, 0.34-1.49) when adjusted for age and stage. HPV DNA positive patients also had a better overall survival in Kaplan-Meier Graph.
analysis than HPV DNA negative patients, but the \( p \)-value using Log-rank test did not reach statistical significance (\( p=0.2 \)) (Figure 2).

**Figure 2: Kaplan-Meier survival analysis for HPV DNA status**

11.2.2 **EPOR STATUS AND SURVIVAL**

The 2-year disease-free survival was higher in patients with EpoR status negative (74%; 95% CI, 61.6 - 82.6) compared to the EpoR positive group (68%; 95% CI, 46.4 - 82.7) and the 2-year overall survival rates were also higher in EpoR negative patients versus EpoR positive patients with the rates of 84% (95% CI, 73.09 - 90.87) and 72% (95% CI, 50.09 - 85.55), respectively (Table 4).
Table 5 shows the details for the overall and disease-free survival by EpoR status using Cox regression analysis. EpoR positivity was associated with a 23% increase in risk of death (HR 1.23; 95% CI, 0.59-2.56) when adjusted for age and stage. EpoR positive patients had also worse overall survival in Kaplan-Meier Graph analysis than EpoR negative patients, but $p$-value using Log-rank test was non-significant ($p=0.6$) (Figure 3).

**Figure 3: Kaplan-Meier survival analysis for EpoR status**
11.2.3 HPV/EpoR and Survival

The patients were divided into 4 groups according to their HPV/EpoR status together as follows: 1. HPV-/EpoR- (n=17), 2. HPV-/EpoR+ (n=8), 3. HPV+/EpoR- (n=54) and 4. HPV+/EpoR+(n=18). Using Cox regression analysis it was noted that patients who were HPV positive and EpoR negative (HPV+/EpoR-) had a 35% reduction in risk of death (HR 0.65; 95% CI, 0.29 -1.48) and patients who were HPV-/EpoR+ had a 15% increased risk of death (HR 1.15; 95% CI, 0.34 - 3.88) (Table 6).

Table 6: Survival by HPV/EpoR groups using cox regression

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>HR</th>
<th>p-Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-/EpoR-</td>
<td>17</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>HPV-/EpoR+</td>
<td>8</td>
<td>1.15</td>
<td>0.82</td>
<td>0.34-3.88</td>
</tr>
<tr>
<td>HPV+/EpoR-</td>
<td>54</td>
<td>0.65</td>
<td>0.30</td>
<td>0.29-1.48</td>
</tr>
<tr>
<td>HPV+/EpoR+</td>
<td>18</td>
<td>0.73</td>
<td>0.56</td>
<td>0.25-2.11</td>
</tr>
</tbody>
</table>

Although these findings did not reach statistical significance (secondary to our small number of subjects), a trend can definitely be seen and may help health care providers to classify patients into High-risk, intermediate-risk and Low-risk according to their HPV/EpoR status (Figure 4).
11.2.4AGE AND SURVIVAL

The 2-year overall survival rates were significantly higher in the younger ≤ 65 age group than in the > 65 age group at 84% (95% CI, 72.9 – 91.8) compared to 74% (95% CI, 55.9 – 85.5), respectively with a p-value of 0.03 using the log-rank test. The rates for disease-free survival were 75% (95% CI, 61.7 – 83.9) in the ≤ 65 age group and 68% (95% CI, 49.8 – 81.1) in the > 65 age group, with a p-value of 0.04 using the log-rank test (Table 4). In the Kaplan–Meier analysis, patients ≤ 65 years old had a better overall survival than patients > 65 years old (Figure 5).
11.2.5 Smoking, Drinking, Site of Cancer, Stage and Use of Chemotherapy

Patients with a significant smoking history (>10 pack-years), and/or drinking history (>4 drinks/week) had lower rates of survival, but these differences did not reach statistical significance. Patients with a primary cancer of the tonsils had a significantly higher overall and disease-free survival than patients with primary cancer of the base of tongue with p-values of 0.004 and 0.02 respectively. There was no statistically significant
difference in survival with respect to the stage of disease. There was a higher disease-free survival in patients who received concurrent chemo-radiation therapy compared to patients that did not receive chemotherapy, but the differences were not statistically significant (Table 4).
12 DISCUSSION

12.1 HUMAN PAPILLOMAVIRUS (HPV)

12.1.1 RATIONALE:

HPV as a causative agent of oropharyngeal cancer is of increasing interest by the general public as well as by healthcare providers, especially with the increasing number of oropharyngeal cancers seen in younger patients. Although tobacco and alcohol use are the primary risk factors for squamous cell carcinomas (SCCs) of the head and neck, HPV is strongly associated with oropharyngeal SCCs. HPV DNA positivity has been well recognized as an independent risk factor for oropharyngeal cancer with a distinctly better prognosis and survival (53, 54). The present study was designed to clarify the epidemiological association between oropharyngeal carcinoma and HPV infection and its effect on patient survival.

12.1.2 HPV-DNA STATUS AND SOCIODEMOGRAPHIC CHARACTERISTICS

In this study, HPV-DNA was positive in 74% of all the OPC, a finding consistent with the literature, which has documented an increasing number of HPV-related OPCs in recent years with positive HPV-DNA rates in the 64-85% range. In the current study, HPV-16 constituted 92% of the HPV DNA positive cases, which is again consistent with reports in the
literature of HPV-16 in 92-96% of cases (5, 53, 64, 120). The current results were consistent with previously reported studies that HPV related OPC is a distinct disease (121, 122) of younger patients (10, 63, 64, 123). The average age of the HPV positive patients in our study was 60.5 (median= 59) years old compared to ≈ 65 (median=66) in the HPV negative patients. HPV positivity was significantly higher in the younger age group (≤65 years) with a p-value of 0.023. Previous studies have shown that tobacco smoking and alcohol consumption are significantly higher in HPV negative OPC patients (5, 53, 64) and this was confirmed in our earlier observations. Most of our the HPV negative patients were heavy smokers and drinkers and was statistically significant when compared to HPV positive OPC with p-values of 0.041 and p < 0.001 respectively.

12.1.3 HPV AND SURVIVAL

A strong relationship between HPV-related OPC and improvement in survival has been reported in the literature (53, 63). In a recent metanalysis (O'Rorke et al, 2012), showed a significant overall survival improvement in HPV positive-OPC (53% better survival) (54). While the results of this study showed a trend toward better overall and disease free survival in HPV-related OPC when compared to HPV-negative OPC, the findings were not statistically significant. In the current study, the 2-year disease-free survival was higher in the HPV-positive group (77%) (95% CI, 0.651 - 0.852) compared to the HPV-negative group (59%) (95% CI, 0.373
- 0.756). Similarly the 2-year overall survival was also higher in the HPV-positive group of OPC. Another important finding was the 29% better survival (HR=0.71) in HPV-related OPC, a trend that persisted in the multivariate cox-regression analysis after adjustment for age and stage. On Kaplan-Meier analysis, positive HPV status was also associated with better survival.

12.2 AGE AND SURVIVAL

In this study, patients' young age (≤ 65 years) was found to be significantly associated with better overall and disease-specific survivals with a p-value < 0.05. That’s might be related to higher HPV related OPC in younger patients. The results of the current study are consistent with those of N. P. Nguyen et al (2010) who also found that 5-year and 10-year survivals were significantly higher in younger patients with tonsillar cancer (123).

12.3 ERYTHROPOIETIN RECEPTORS (EpoR)

12.3.1 RATIONALE:

As overviewed in the literature review, EpoR was found to be expressed in head and neck squamous cell carcinoma (SCC) and prior studies have noted its association with a poor prognosis and survival. In reviewing the literature, no data was found on the association between EpoR expression and oropharyngeal cancer. The current study aimed to assess the utility of EpoR as a prognostic indicator for overall and disease-free survival in oropharyngeal cancer patients.
12.3.2 **EpoR Expression in Oropharyngeal SCC**

There is no consensus in the literature on prevalence of EpoR expression in head and neck SCC ranging from 0-97% (76, 89, 105, 124). These results might be related to the lack of specificity of the immune staining for EpoR. In this study, EpoR was expressed in 26.80% (n=26) of all oropharyngeal SCC diagnosed in our institution from 2000 to 2009. In this study there was no association between EpoR expression and age, sex, smoking or drinking. In addition, EpoR expression was significantly higher in advanced stages of cancer (stage IV) with a p-value of 0.046. This finding is in agreement with Li et al (2009) and Lin et al (2012) findings which showed a significant correlation of EpoR expression and TNM stage with p values of 0.05 and < 0.001 respectively (89, 105).

12.3.3 **EpoR Expression and Survival**

In the current study, patients with negative expression of EpoR in oropharyngeal SCC showed a trend toward better disease-free survival (DFS) and overall survival (OS) when compared to EpoR positive patients. These results were not statistically significant, possibly because of the small number of patients. However, results are consistent with the literature and several reports of EpoR expression associated with poor survival in head and neck SCC (14, 104). Other studies have considered the relationship between EpoR expression and survival in oral cavity cancers specifically and found that EpoR expression was significantly
associated with poorer survival (89, 105, 106). Another important finding in the current study was that EpoR expression was associated with a 23% decrease in overall (OS) and a 26% decrease in disease-free survival on multivariate cox-regression analysis. Again however, the results did not reach significance, probably because of the relatively small numbers. These findings are consistent with those of Lin et al (2012) who noted that absence of EpoR expression was protective but not as an independent factor for survival in oral cavity SCC (105). Other studies, however, have demonstrated that EpoR expression is an independent factor for survival in tongue cancer (89, 106). In the current study, comparing EpoR status with Kaplan-Meier curves showed that patients with positive EpoR expression had a poorer survival in oropharyngeal cancer. These results are consistent with previous studies on oral cavity SCC (89, 105, 106).

12.4 HPV/EpoR AND SURVIVAL

This was the first study to show the concurrent effect of HPV and EpoR on mortality. The most interesting finding to emerge from the data was the correlation between HPV status and EpoR expression (HPV/EpoR) and its effect on survival. Further analysis showed a possible classification of OPC patients into three risk groups (low, intermediate and high risk) according to their HPV/EpoR status (Figure 4 and Table 6). This is a novel way of classifying OPC patients and may give patients, surgeons and radiation oncologists an idea on the possible risk of death according to their HPV/EpoR status. However, more research on this topic with larger
numbers needs to be undertaken before the association between HPV/EpoR and survival is more clearly understood.
13 Conclusion

This study has investigated HPV status and EpoR expression in oropharyngeal cancer patients treated in a tertiary care institution, studying in detail their individual and combined effect on survival outcome. Regarding the hypothesis/question posed at the beginning of this study, it is now possible to state that in OPC patients:

1. HPV-related OPC is associated with better survival,

2. EpoR expression showed a trend toward worse survival and

3. Age was a significant predictor of survival.

In addition, a new method of classifying OPC patients according to their HPV/EpoR status has been suggested in this study.

The most important limitation of the current study stems from the relatively small sample size available for study. Despite this, the present study makes several noteworthy contributions to the current literature. First, it has verified and supported the epidemiological association between HPV-related oropharyngeal carcinoma (OPC) and EpoR expression and survival. Second, it is the first to study EpoR expression and its association with survival in oropharyngeal carcinoma (OPC). Third, it is the first to speculate a possible HPV/EpoR epidemiological association that might need more research to better understand a biological association.
Finally, this research has raised some interesting questions that will serve as a basis for future studies.
REFERENCES:


23. Schlecht NF, Franco EL, Pintos J, Kowalski LP. Effect of smoking cessation and tobacco type on the risk of cancers of the upper aero-


29. Foulkes WD, Brunet JS, Kowalski LP, Narod SA, Franco EL. Family history of cancer is a risk factor for squamous cell carcinoma of the head


78. Sinclair AM, Todd MD, Forsythe K, Knox SJ, Elliott S, Begley CG. Expression and function of erythropoietin receptors in tumors: implications


84. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M. Erythropoietin has a mitogenic and positive chemotactic effect on


109. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at


114. Bernard HU. The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses. Journal of clinical virology : the


