"IMMUNOPROFILING OF NATURAL KILLER CELLS IN NORMAL INDIVIDUALS, TOBACCO CHEWERS AND PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA -A FLOW CYTOMETRY STUDY"

By

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LIST OF ABBREVATION

SL.NO	ABBREVATION	FULL FORM
1	%	Percentage
2	NKCs	Natural Killer cells
3	OSCC	Oral Squamous Cell Carcinoma
4	HNSCC	Head and Neck Squamous Cell Carcinoma
5	TIL	Tumor-infiltrating lymphocytes
6	ACTH	Adrenocorticotropic Hormone
7	TME	Tumor Microenvironment
8	ECM	Extracellular Matrix
9	TAM	Tumor Associated Macrophages
10	CAF	Cancer Associated Fibroblasts
11	Tregs	Regulatory T cells
12	DC	Dendritic Cell
13	MDSC	Myeloid derived suppressor cells
14	DNA	Deoxyribo Nucleic Acid
15	TSA	Tumor-Specific Antigens
16	TAA	Tumor-Associated Antigens
17	IFN	Interferon
18	TNF	Tumor Necrosing Factor
19	CTL	Cytotoxic T lymphocyte
20	FDA	Food and Drug Administration
21	COPD	chronic obstructive pulmonary disease
22	CRP	C-reactive protein
23	IL	Interleukin

24	SLT	Smokeless Tobacco
25	ROS	Reactive Oxygen Species
26	RNS	Reactive Nitrogen Species
27	nAChRs	Nicotine acetylcholine receptors
28	CLPs	Common lymphocyte progenitors
29	ILC	innate lymphoid cell
30	MHC	Major Histocompatibility Complex
31	KIR	killer cell immunoglobulin
32	HLA	Human Leukocyte Antigen
33	PBMC	Peripheral Blood Mononuclear Cells

ABSTRACT

Background-

Smokeless tobacco poses a significant global health risk by suppressing the immune system and promoting cancer development, especially oral squamous cell carcinoma (OSCC). NK cells, key components of innate immunity, play a important role in early immunity and tumor surveillance. Tobacco toxins and nicotine impair NK cell function, aiding tumor immune evasion. Immunoprofiling, particularly via flow cytometry, helps assess these immune alterations. This study aimed to evaluate peripheral NK cell immunoprofiles in healthy individuals, tobacco chewers, and OSCC patients to understand immune changes linked to tobacco exposure and cancer.

Materials and Method-

A total of 75 participants were enrolled, including 25 healthy individuals, 25 tobacco chewers (12 without lesions and 13 with lesions), and 25 OSCC patients. Peripheral blood samples were collected and analyzed for NK cell percentages and surface marker expression (CD56, CD16) using flow cytometry. Statistical analysis was performed using non-parametric tests due to data distribution.

Results-

Immunoprofiling using flow cytometry revealed no statistically significant differences in total NK cell percentages or subsets ($CD16^+$, $CD56^+$, $CD16^+CD56^+$) across the three groups ($p > 0.05$). Although not significant, $CD16^+CD56^+$ and $CD56^+$ NK cells were lesser in tobacco users & OSCC patients as compared to healthy individuals. Among tobacco chewers, those with lesions showed slightly higher mean NK cell

values than those without, suggesting potential immune alterations associated with lesion development.

Conclusion-

While quantitative NK cell levels remained relatively stable with slight changes across groups, phenotypic shifts in NK cell subsets may indicate early immune alterations in tobacco users and possible functional exhaustion in OSCC. NK cell immunoprofiling, especially of functional markers, holds promise as a biomarker in oral precancerous and cancerous conditions.

Key Words-

Natural killer cells, CD56, CD16, Oral squamous cell carcinoma, Tobacco chewers, Flow cytometry, Immune profiling

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INTRODUCTION

Tobacco use is one of the most significant risk factors for early mortality worldwide. More than 60 harmful substances contained in tobacco are capable of reaching the body's different systems[1]. The global cancer burden continues to rise, owing primarily to an increase in tobacco intake, in both smoked and smokeless forms. All tobacco products in both smokeless and smoking forms (cigars, cigarettes, chewing tobacco, pipe tobacco, and snuff) contains toxins, carcinogens, and nicotine (an addictive substance) that may lead to Oral cancer that accounts for 2-4% of all cancers.[2]

There are a lot of studies that suggest nicotine present in smoked cigarette and/or smokeless tobacco is the major immunosuppressive. Nicotine stimulates the release of catecholamines, which supresses the immune system via promoting ACTH secretion.[3].

The body's immune system response is vital for destruction of cancer cells. Because tumor-infiltrating lymphocytes (TILs) undergo apoptosis, tumors such as oral squamous cell carcinoma (OSCC) can evade detection by host immune cells. Immunocompromised people are 100 times more likely to develop cancer than healthy ones.

OSCC found to be a highly immunogenic tumor due to its significant immune cell infiltration. Tumor microenvironment (TME) is made up of complex mixture of extracellular matrix with a range of stromal cells, and immune cells that coordinate and interacts with the tumour cells. Thus, it includes tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), regulatory T cells (Tregs) and endothelial cells, Neutrophils, Dendritic cells (DCs), Eosinophils, Macrophages,

myeloid derived suppressor cells (MDSCs) & natural killer cells (NKs). T cells and B cells make up the adaptive immune cells, while the innate lymphoid cells constitute the innate immune cells. The formation of tumors is mostly driven by cross-talk between the TME, extracellular matrix, and tumor cells. Furthermore, inflammatory cytokines, chemokines, and growth factors are involved in cancer-associated inflammation, specifically chronic inflammation, which can lead to genomic instability, DNA damage, tumor angiogenesis, and conflict between suppression of immunity and promotion may result in tumorigenesis.[4]

Cellular and humoral processes, such as antibodies, make up the immunological response to foreign antigens. The majority of humoral reactions are unable to impede tumor progression. Effector cells with comparatively strong tumoricidal properties include T cells, macrophages, and NK cells. Cells known as antigen-presenting cells—which have tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs) on their surface—induce effector cell activity, which is aided by cytokines like interleukins and interferons.

NK cells- cytotoxic lymphocytes, belong to the innate lymphoid cell (ILC) family and function at the crossroads of innate and adaptive immunity. NK cells play an important role in tumor monitoring, atopic diseases, and many autoimmune/inflammatory disorders, as well as viral pathogen defense.[5] NK cells use a variety of intrinsic receptors to sense their surroundings and respond to diseases, cellular stress, and transformations. The subsequent NK cell activation, which includes cytotoxicity and cytokine generation, is an important part of the early immune response[6]. They can affect the outcome of adaptive responses and promote immune homeostasis.

As important rapid-acting immune receptors, NK cells are a major producer of IFN during early-phase immunological responses. IFNγ transforms naive CD4+ T cells into Th1 cells and increases the immunogenicity of tumor cells, both of which strengthen cell-mediated anticancer responses. IFNγ facilitates communication between effector memory CD4+ T cells, naïve CD8+ T cells, and myeloid cells (DCs and macrophages). In order to improve antitumor immunity, NK cells can also produce the chemokines CCL5, XCL1, and Flt3L, which stimulate naive effector CD8+ T cells and conventional type I DCs (cDC1s)[9].

By expressing cytotoxic granules or death-receptor ligands, NK cells can directly kill tumor cells in addition to generating cytokines. In general, the TNF family of receptors and ligands plays a major role in tumor eradication.[4]

NK cells are $CD3^-$ lymphocytes that lack antigen specific receptors on their surface. CD56 functions as a tag to identify NK cells from other non-T lymphocytes in humans [7]. Effective anti-tumor immunity is by CD8 positive (CD8+) cells, which are a significant subset of cytotoxic T lymphocytes and natural killer (NK) cells. [8]

According to research, smoked tobacco products are frequently linked to serious immune system effects, such as decreased immunoglobulin levels, helper/suppressor T cell ratios, and NK cell activity.[9] Chronic smoke exposure impairs natural killer (NK) cell-mediated immunity by suppressing NK cell activation and cytotoxic T lymphocyte (CTL) activity, despite unchanged NK cell mobilization or numbers. Smoke exposure also decreases dendritic cell function, which is vital for NK cell activation. Interestingly, smoking cessation reversed these detrimental effects, restoring NK cell function and reducing tumor burden. This highlights the reversibility of smoke-induced immune suppression and underscores the importance of quitting smoking. [10]

Studies have shown that use of smokeless tobacco is also practiced worldwide which can cause similar effects to those of smoking in the association with the development of oral squamous-cell carcinoma.[11]

Furthermore, studies have shown immunophenotypical disarrangements in peripheral natural killer cells in alcoholics leading to immunosuppression[12], whereas immunoprofiling of peripheral natural killer cells in tobacco chewers and its role in oral squamous cell carcinoma initiation remains unexplored. Immunoprofiling – a way to measure the state of an individual's immune systems at a given point of time.Flow cytometry is the technique used commonly for immunoprofiling as this identifies and quantifies the population of cells in a heterogenous sample which can be usually blood, bone marrow or lymph.

Therefore, considering the significance of NKCs in immunosurveillance and antitumor immunity, smokeless tobacco may be linked to a change in their immunoprofile that could indicate a disruption in their dynamic balance, and consequently to aberrant activation, trafficking, or cytotoxicity that results in tumor cells evading the immune system.

As there is limited literature on this aspect, the aim of the present study is to evaluate the immunoprofile of peripheral natural killer cells in normal, tobacco chewers and oral squamous cell carcinoma patients, using flow cytometry.

AIMS AND OBJECTIVES

AIM OF THE STUDY:

Evaluation of immunoprofile of peripheral natural killer cells in tobacco chewers and oral squamous cell carcinoma patients by flow cytometry.

OBJECTIVES:

- To evaluate the expression of peripheral natural killer cells in normal individuals using flow cytometry
- To evaluate the expression of peripheral natural killer cells in tobacco chewers using flow cytometry.
- To evaluate the expression of peripheral natural killer cells in Oral squamous cell carcinoma patients using flow cytometry
- To corelate the expression of peripheral natural killer cells in normal, tobacco chewers and oral squamous cell carcinoma patients.

REVIEW OF LITERATURE

As stated by World Health Organization, about one-third of world's adult population uses tobacco. According to the Global Adult Tobacco Survey-2 (GATS-2), 28.6% of people worldwide consumes tobacco in some form, 10.7% smokes, and 21.4% uses smokeless tobacco. Men use SLT at a higher rate (27%-37%) than women (10%-15%).[13]

There are about 8,000 compounds in cigarette smoke, including 93 that the FDA has classified as hazardous or possibly hazardous. Smoking has been directly linked to numerous diseases, particularly those affecting the pulmonary and cardiovascular systems, such as cancer, chronic obstructive pulmonary disease (COPD) and coronary heart disease, [14] Extensive research has examined the health impacts of cigarette smoking at organ, cellular, and molecular levels. Mechanistically, smoking disrupts various molecular pathways, including those related to oxidative stress and immune responses. Oxidative compounds produced during cigarette combustion cause lipid and DNA damage and activate lung epithelial cells and macrophages.[15] This activation triggers immune cell responses, causing inflammatory mediators release, systemic inflammation, and increased circulating leukocyte levels in chronic smokers. Alterations in inflammatory mediators, like cytokines, TNF-α and IL-6, along with acute phase proteins like C-reactive protein (CRP) and fibrinogen, have also been observed in smokers.[16]

The term "smokeless tobacco" signifies tobacco products that are used without smoking. These products are usually chewed, inhaled, or inserted between the gums and the cheek or lips. Such as chewing tobacco, snuff, snus, and soluble tobacco products. Smokeless tobacco is especially popular in South Asia, and it makes up for

over 80% of global usage. There is no safe level of smokeless tobacco usage, which contributes to around 650,000 fatalities worldwide each year.[17]

In nations such as India, smokeless tobacco (SLT) is consumed in a variety of ways, including pan (betel quid) with tobacco, slaked lime concoctions, pan masala, snuff, zarda, khaini areca nut, mishri, mawa, and gutkha. Besides locally manufactured tobacco products, various commercially sold tobacco products have recently been made available at low prices, which makes them widely accessible to everyone, especially the young and underprivileged.[17]

National Cancer Institute have reported that Smokeless tobacco contains over 25 carcinogenic compounds, including tobacco-specific nitrosamines (TSNAs), polonium, cadmium, formaldehyde, lead, and benzo[a]pyrene. TSNAs, particularly N-nitrosonornicotine (NNN) and 4-methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK), which are the primary cancer-causing agents in smokeless tobacco.

The carcinogenic risk of smokeless tobacco varies depending on product type and production methods. Dry snuff has a higher associated risk for oral cancer, while moist snuff and chewing tobacco carry a lower risk. Factors such as fermentation, curing processes, and the addition of preservatives influence the concentration of harmful and antioxidant compounds in these products. Epidemiological studies have linked smokeless tobacco use with an increased risk of oral cancer, which varies based on the product's composition and usage patterns.[17]

Xiaoge Jiang et al. reviewed how tobacco use causes oral squamous cell carcinoma (OSCC) through multiple complex mechanisms. It highlighted that tobacco contains over 60 toxic and carcinogenic compounds that can cause epigenetic alterations in oral epithelial cells**,** such as mutations in p53, GLUT-1, p16, DAPK, and

PI3K**,** all of which are associated with tumor progression. Tobacco also leads to immune system suppression**,** notably altering CD4+ and CD3+ T-cell function and affecting cytokines like IL-2 and IL-4**,** thus aiding tumor evasion. Additionally, tobacco-induced oxidative stress from free radicals (e.g., ROS, RNS) damages DNA and cellular structures, promoting carcinogenesis. The authors concluded that tobacco as a major carcinogen for OSCC**,** and understanding its diverse carcinogenic pathways is utmost important for early detection, prevention, and interventions.[18]

EFFECTS OF NICOTINE IN IMMUNE SYSTEM:

Nicotine is a key component in smokeless tobacco that contributes to addiction. Nicotine, a cholinergic drug activates nicotinic or muscarinic receptors either by inhibiting cholinesterase or inducing acetylcholine release. It appears to suppress immunological activity and diminish the release of different cytokines. Nicotine's effects appear to vary depending on the concentration and route of administration. Nicotine significantly alters immunological function, increasing the total number of leukocytes and TCD8+ cells while decreasing TCD4+ cells and NK cells. It also causes tumor invasion, cell migration, and carcinogenesis in a7-receptor and Src-dependent pathways.[19]

Along with immune and nerve cells, Nicotine acetylcholine receptors (nAChRs) are found on the surface of various cells. Long term exposure of nicotine stimulates these receptors, which are ion channels also triggered by neurotransmitters like acetylcholine. α1-α7, α9, α10, β1-β4, γ, δ, and μ are 17 subunits of nAChRs found in mammals, encoded by 17 distinct genes. A specific subunit of nAChRs is present in immune cells, while diverse combinations of nicotine receptor subunits may be expressed in other cells that leads to varied responses to nicotine.[19] Nicotine activates intracellular signaling cascades that result in significant changes to the

immune system by affecting the alpha subunit of the nicotinic receptor on the phospholipid bilayer membrane. These cascades decrease inflammation by decreasing NF-κB and lowering cytokine production. Furthermore, nicotine inhibits the polarization of Th0 lymphocytes into Th1 and Th17 cells, supporting a shift to the less inflammatory Th2 pathway. Nicotine also decreases the expression of IRAK4, IRF4, and RIG1, which reduces antiviral responses. Furthermore, increased expression of the FASL receptor and caspase-3 on immune cells leads to widespread apoptosis.[20]

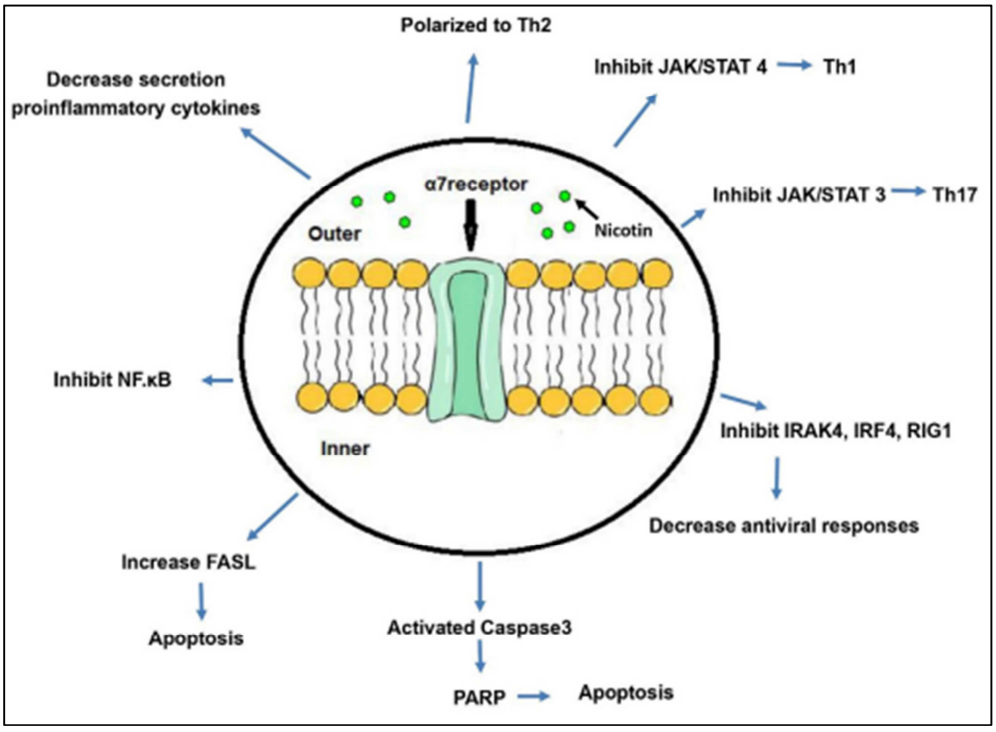


Figure 1: Effects of nicotine on various components of immune system

Abbreviations: JAK/STAT, Janus kinase/signal transducer and activator of transcription; Th, T helper; IRAK4, Interleukin-1 receptor associated kinase 4; IRF, Interferon regulatory factor 4; RIG1, retinoic-acid inducible gene I; PARP, poly-ADP ribose polymerase; FASL, Fas cell surface death receptor ligand; NF-κB, Nuclear factor kappa B.

DERIVATION, DISTRIBUTION AND CLASSIFICATION OF NATURAL KILLER CELLS

NK cells are found in a variety of tissues, including bone marrow, lungs, liver, spleen peripheral blood and lymph nodes. They have also been found in many organs, including kidneys, thymus, pancreas, brain, tonsils, bladder, adipose tissue, skin, intestines and uterus.[44]CD56dim NK cells are the most common subset in peripheral blood, while CD56bright NK cells are more abundant in lymph nodes.[45]

In comparison to T and B cells, NK cells are bigger and possess specific cytoplasmic granules. They were found in 1975 by Kiessling et al. and Herberman et al. [46] Common lymphocyte progenitors (CLPs) gradually change from CD34+ hematopoietic progenitor cells to NK cells by downregulating CD34 and upregulating CD56, which leads to NK cell maturation and differentiation.[44]

All human innate lymphoid cell (ILC) subpopulations, including NK cells, are produced by a pluripotent progenitor near CLPs. Originating from CLP, the common ILC precursor (CILCP) subsequently differentiates into NK-restricted progenitors. NK lineage commitment is characterized by the expression of CD122 as well as the deletion of CD34 and CD127.

Also, the transcription factors Eomes and T-bet are essential for the functional differentiation of natural killer cells. CLPs in mice produce CILCPs, which in turn produce helper-like ILCs and NK cells.[44]

NK cells are classified into different subsets based on surface membrane marker expression. In humans, they are primarily categorized by CD56 expression into $CD56^{dim}$ and $CD56^{bright}$ subsets. $CD56^{dim}$ NK cells are known for their potent cytotoxic activity against tumors, while both subsets can secrete cytokines. Both

populations exhibit activating receptors like NKp46 and NKp80. $CD56^{bright}$ NK cells can differentiate into $CD56^{dim}$ NK cells through expression of PEN5 and CD16. In mice, NK cell subsets are recognized based on CD11b, CD226 (DNAM-1), CD27, and KLRG1 expression; however, direct parallels between human and murine NK cell subsets have yet not been established.[47]

ROLE OF CD56 AND CD16 NATURAL KILLER CELLS

CD56dimCD16dim NK cells, are relatively overlooked subclass in immunological research. Jacques Zimmer et al., comments on a study by Hofland et al., which examined NK cell activation in chronic lymphocytic leukemia (CLL) using in vitro model, demonstrated that NK cells exhibit enhanced degranulation and cytotoxic functions when stimulated via the CD16a receptor (FcgRIIIa), leading to increased granzyme B release and interferon-gamma (IFN-γ) production. Also the study noted that NK cell activation through NKG2D was negatively affected. Moreover, CLL patients had a significantly higher proportion of mature NK cells (NKG2C+CD57+ILT2+) compared to healthy individuals, whereas the inhibitory receptor KLRG1, a marker of senescence, showed similar expression in both groups.[49]

Zimmer points out that the study categorized NK cells into three primary subsets: CD56brightCD16−, CD56dimCD16+, and CD56−CD16+. However, it excluded three additional subsets: CD56brightCD16dim, CD56dimCD16−, and CD56dimCD16dim, despite their presence in the dataset. The CD56dimCD16dim subset, previously identified in a 2017 study, appears to be a distinct NK cell population, located adjacent to the CD56dimCD16bright subset in flow cytometry dot plots.[50] This exclusion is significant because the CD56dimCD16dim subset displays distinct phenotypic features that suggest it might represent an immature precursor of the CD56dimCD16bright NK cells. Supporting this claim, the subset has a higher

expression of NKG2A and CD27, but a lesser presence of KIR, CD57, and CD62L compared to its CD56dimCD16bright counterpart.

Other research groups, such as those led by Stabile et al.[51] and Béziat et al.,[52] have previously described related NK cell subsets, though some have used inconsistent nomenclature. The CD56dimCD16dim subset, in particular, has been shown to have unique functional and developmental characteristics, which are often overlooked when it is either gated out or misclassified within the CD56dimCD16bright population. Zimmer argues that standardizing NK cell nomenclature based on leading immunology research groups would help improve data consistency across studies.

It is important to include the CD56dimCD16dim NK cell subset in immunological analyses, as it provides valuable insights into NK cell maturation and function, particularly in the context of leukemia, squamous cell carcinomas and other immune-related disorders.

THE SIX NK CELL SUBPOPULATIONS PRESENT IN HUMAN PERIPHERAL BLOOD

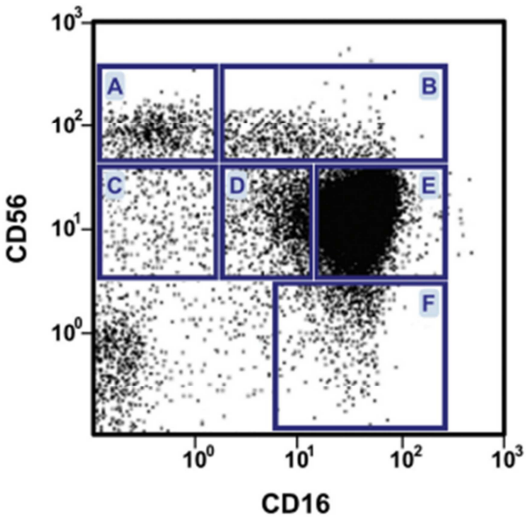


Figure 2: Dot plot of flow cytometry experiment after peripheral blood mononuclear cells been stained with fluorescent anti-CD16 (X axis) and anti-CD56 (Y axis) antibodies and the monocytes ($CD14^+$), B ($CD19^+$), T ($CD3^+$) lymphocytes were gated out. A)CD56brightCD16,B)CD56brightCD16dim, C)CD56dimCD16-, D)CD56dimCD16dim, E)CD56dimCD16+ ,F)CD56-CD16+. [50]

STIMULATION AND INHIBITION OF NK CELLS:

i) Inhibitory and activatory signals-

Natural killer (NK) cells control their activity utilising a combination of inhibitory and activating signals. In a resting state, inhibitory receptors suppress NK cell activation, preventing unintended cytotoxicity. Unlike T cells, NK cells acknowledge target cells in a manner that is not restricted by MHC molecules. When encountering cells that express MHC-I, NK cells receive inhibitory signals that prevent them from attacking healthy host cells.[53] However, under conditions of cellular stress, infected or malignant cells downregulate MHC-I expression, leading to

a loss of inhibitory signaling and triggering NK cell activation through the "missing-self recognition" mechanism. In addition to MHC-dependent regulation, NK cells use inhibitory receptors such as NKR-P1B, 2B4, and NKR-P1A to identify non-MHC molecules such as Clrb, CD48, and LLT-1. Other inhibitory receptors include C-type lectin receptors (CD94/NKG2A/B) and killer cell immunoglobulin-like receptors (KIRs) like KIR2DL and KIR3DL.[54]

Emerging research indicates that NK cells also exhibit activating receptors capable for recognizing both pathogen-derived molecules and self-expressed proteins. Normally, host cells do not produce pathogen-encoded molecules, a recognition process referred to as "non-self recognition." Conversely, diseased or transformed cells may upregulate stress-induced self-proteins, leading to "stress-triggered self-recognition." Cytotoxicity receptors like NKp30, NKp44, and NKp46, as well as C type lectin receptors like NKG2E/H, CD94/NKG2C, NKG2F, and NKG2D, are the activating receptors.[55]

Additionally, stimulatory KIRs such as KIR-2DS and KIR-3DS contribute to NK cell activation, while inhibitory KIRs, including KIR-2DL and KIR-3DL, play a role in suppressing NK function. Ultimately, NK cells determine their response by integrating various activating and inhibitory signals, with the final effect depending on the specific characteristics of target cells.[53]

ii) Increase in NK cells in the tumor microenvironment by regulatory cytokines:

Contemporary research studies continues to highlight the significance of NK cells in the primary regulation of viral diseases, hematopoietic stem cell transplantation (HSCT) outcomes such as enhanced grafting, graft versus tumor

effects, graft versus host disease and in cancer immune surveillance. Various regulatory cytokines enhance NK cell functions against tumors. Several cytokines, including IL-12, IL-2, IL-18, IL-21, IL-15 and Type I interferons, have utilized for in vitro augmentation and NK cell activation prior to adoptive transfer.[56] Notably, naïve NK cells exhibit limited cytotoxicity when stimulated by individual activating receptors, but cytokine exposure significantly enhances their preactivation. IL-12, in particular, amplifies NK cell receptor signaling, and when combined with IL-18 and IL-15, provokes a memory like NK cell population which is able to expand in the immune-deficient mice treated with exogenous IL-2. IL-2 is frequently administered to patients receiving NK cell therapy to support in vivo expansion. While low-dose IL-2 was handled without adverse effects in clinical studies, no significant clinical benefits were observed in comparative assessments.[57]

The individual anti-tumor effects of IL-12 and IL-18 are restricted, whereas IL-21 has shown strong potential, especially when combined with monoclonal antibodies targeting cancer cells. In NK cells, among these cytokines, IL-15 stands out as the most promising, with ongoing research uncovering its signaling process. However, another study found that IL-15-stimulated NK cells provoked a clinical response in four out of six pediatric patients with refractory solid tumours. Currently, IL-15 is being assessed in clinical trials for the treatment of both solid tumors and hematologic malignancies.[58] A modified IL-15 super agonist, ALT-803 (IL15N72D:IL15RαSu/IgG1 Fc complex), has demonstrated enhanced biological activity compared to native IL-15 and is undergoing clinical trials as a potential NK cell activator against metastasis. Additionally, genetic engineering strategies aimed at ectopic IL-15 expression offer another promising avenue for boosting NK cell function. Type I interferons, as proinflammatory cytokines, takes part in priming NK

cells for activation through their activating receptors. Collectively, regulatory cytokines are essential for stimulating NK cells in tumor eradication, with several cytokine-based NK cell therapies currently being evaluated in clinical and preclinical studies.[59]

OVERVIEW OF NATURAL KILLER CELL ACTIVITY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC):

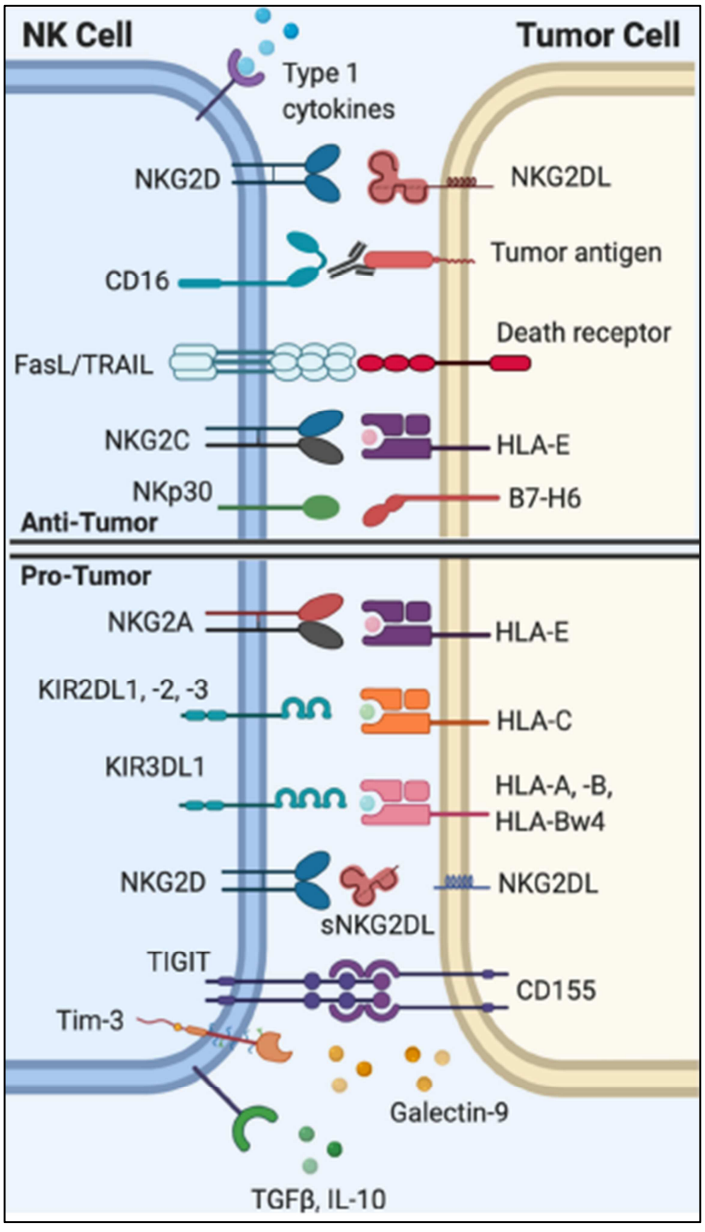


Figure 3: Natural Killer Cells interaction with Tumor Cells

Malignancies of oral cavity, oropharynx, nasopharynx, larynx and hypopharynx which form the upper aerodigestive tract, together are categorized as squamous cell carcinoma of head and neck. The primary cause for HNSCC are environmental, tobacco usage, alcohol intake and also high risk human papillomavirus (HPV) infection.[21]

Immune check point inhibitors, which trigger T cells to combat recurring HNSCC cancers, have shown encouraging benefits in some patients, although they frequently disappoint in the majority of instances. This has sparked interest in studying additional immune effector cells, especially natural killer (NK) cells, as prospective immunotherapy targets.

The secondary lymphoid organs such as tonsils, spleen, and lymph nodes and bone marrow are the origins of NK cells, which are $CD3^{-}CD56^{+}$ innate lymphoid cells. [21] NK cells exhibit traits of both the innate and adaptive immune cells: they can produce long-lasting "memory-like" or "recall" immune responses even if they do not go through genetic recombination to produce a wide variety of antigen-specific receptors.

NK cells plays a key role in eliminating virally infected and transformed cells, and their cytolytic action is faster than that of adaptive lymphocytes, as they do not rely on in situ licensing. These features of NK cells make them a promising candidate for new immunotherapies.

i) Natural Killer Cells interaction with Tumor Cells-

When the total number of activatory signals are more than the number of inhibitory signals, NK cells are activated. To identify "altered-self" or "missing-self" protein expression patterns on the tumor cells, NK cells utilize a collection of

germline-encoded receptors. The variety of activatory and inhibitory receptors found on the NK cells makes them potential targets for immunotherapy. TGFβ (transforming growth factor beta), TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), Tim-3 (T cell immunoglobulin mucin-3), HLA (human leukocyte antigen), IL-10 (interleukin-10), and TIGIT (T cell immunoreceptor with Ig and ITIM domains) are some of these receptors.[22]

ii) NK Cells and Cancer

NK cells are essential for tumor management, according to a wealth of evidence. Their role as quick immune effectors is highlighted by the fact that they are a paramount generator of IFNγ during initial immunological responses. [23] By increasing tumor cells' immunogenicity, IFNγ directly affects them. It also shapes the immune responses by causing naive CD4+ T cells to differentiate into Th1 cells, which support cell mediated anticancer responses. [23] IFNγ also improves the communication between effector memory CD4+ T cells, naive effector CD8+ T cells, and myeloid cells (such as DC and macrophages). [24] To further boost antitumor immunity, NK cells also generate chemokines like XCL1, Flt3L and CCL5, which recruit naive CD8+ T cells and conventional type I DCs (cDC1s).

By releasing cytotoxic granules or expressing death-receptor ligands like TNF-related apoptosis inducing ligand and Fas-ligand , NK cells can directly destroy tumor cells in addition to generating cytokines. The TNF family of receptors and ligands plays a major role in tumor elimination.[25] One typical mechanism of NK cell activation in response to malignancies is the loss of inhibitory signals. The surface MHC repertoire of cells is frequently lost during viral infection or neoplastic transformation. Although HLA-A, HLA-B, and HLA-C are required for CD8+ T cell activation, they also suppress NK cells by increasing HLA-E expression and

inhibiting KIR ligands.Thus, loss of HLA allows NK cells to be activated and eliminates tumor cells, even as it evades adaptive immune responses that rely on MHC-mediated antigen presentation.

In other words, conditions that activate NK cells may not be optimal for CD8+ T cells, and vice versa. Although the actual frequency of this in vivo is yet unknown, it has been proposed that NK-mediated tumor eradication involves changing or losing their HLA expression. Loss of HLA alone may not always be sufficient for NK cells to eradicate malignancies, as evidenced by the persistence of some cancers which do not posses HLA. [22]

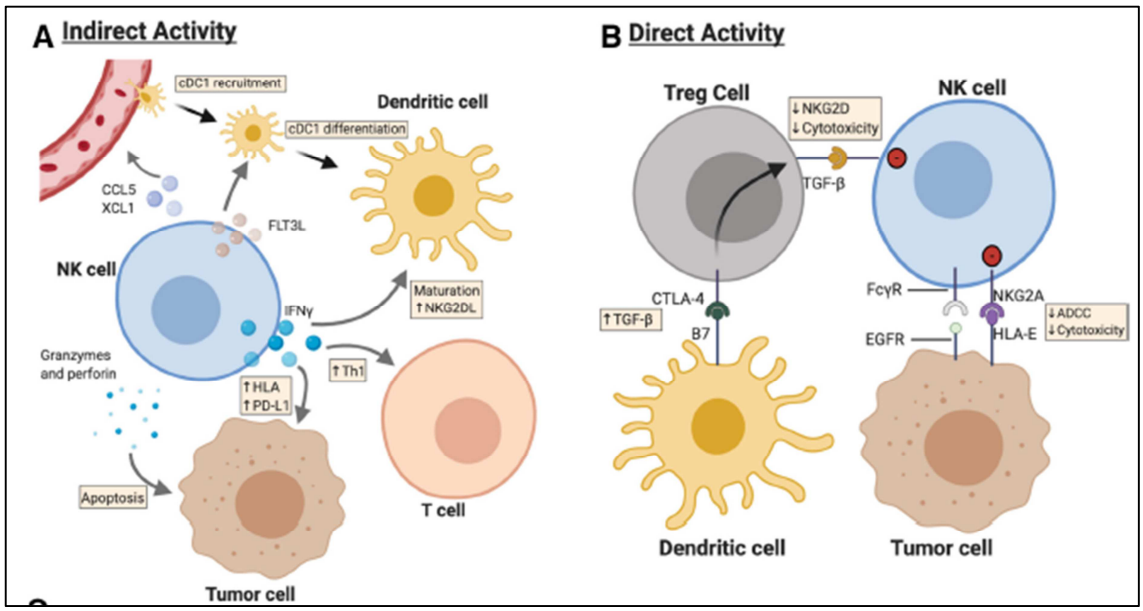


Figure 4: Dynamic Interrelationship of Natural killer cells with immune, Stromal and Tumor microenvironment of head and neck cancer

Within the TME of head and neck cancer, the natural killer (NK) cell forms the complex relationships with the tumor, stromal, and immune cells which involves cDC1 (A) Within the TME, NK cells release interferon (IFN)-gamma, which affects surrounding immune cells and tumor cells to improve antitumor responses.

Additionally, NK cells help cDC1 differentiate and mature within the TME by recruiting them. (B) The interplay between NK cells & their surrounding cells regulate the intensity for their activation.[22]

E. Zancope et al. investigated the differential infiltration of NK cells and CD8+ cytotoxic T cells in lip and oral squamous cell carcinoma to assess their role in tumor progression and prognosis. Using immunohistochemistry, the study analyzed these immune cells in malignant, pre-malignant (leukoplakia, actinic cheilitis), and normal tissues, correlating their presence with tumor size, metastasis, and proliferation index (Cyclin B1 expression). The findings revealed higher CD8+ and NK cell infiltration in LSCC, suggesting a stronger immune response contributing to lower metastasis and better prognosis. In contrast, metastatic OSCC showed reduced NK cell infiltration, indicating a potential role in tumor progression. Higher peritumoral CD8+ density correlated with lower tumor proliferation and improved survival, though not statistically significant. Additionally, pre-malignant lesions exhibited low immune cell infiltration, suggesting early immune evasion during tumorigenesis. The study highlights the importance of immune profiling in oral cancer and suggests that enhancing CD8+ and NK cell activity may improve OSCC outcomes.[8]

S. L. Johansson et al. investigated the impact of smokeless tobacco (oral snuff) on natural killer (NK) cell activity in rats. Over a 15-week period, rats exposed to oral snuff showed a significant reduction in NK cell cytotoxicity in peripheral blood compared to controls, with this suppression evident as early as four days into treatment and maintained throughout the study. The experiment used YAC-1 lymphoma cells as target cells to measure NK activity through standard cytotoxicity assays. The results revealed that snuff exposure led to immunosuppression, likely diminishing immune surveillance against virus-infected or transformed cells,

potentially facilitating the development of oral squamous cell carcinoma. Although no tumors developed during the 15-week period, previous research indicates malignancies often arise after 35 weeks or more of exposure. The study emphasized the immune-suppressive effect of tobacco-specific nitrosamines found in snuff and suggesting that this suppression of NK cell activity could contribute to increased susceptibility to oral cancers. [11]

Janis V. de la Iglesia et al. studied the impact of smoking tobacco on TME in patients with HNSCC). In a cohort of 177 HPV-negative HNSCC patients, the authors analyzed tumor samples for immune markers (CD3, CD8, FOXP3, PD-1, PD-L1) and conducted RNA and whole-exome sequencing. The findings indicated that, in comparison to former and never-smokers, current smokers had significantly lesser infiltration levels of CD8+ cytotoxic T cell and PD-L1 expression in tumor areas. Importantly, interferon-alpha (IFN-α) and interferon-gamma (IFN-γ) signaling pathways were downregulated in current smokers, alongside reduced expression of CXCR3 chemokines (CXCL9, CXCL10, CXCL11), which are critical for immune cell migration to tumors. These immunosuppressive changes correlated with poorer survival outcomes and may explain the reduced response of smokers to immune checkpoint inhibitors, such as anti-PD-1 therapies. The study concludes that active tobacco use induces immunosuppression in HNSCC, suggesting that smoking cessation could improve immune response and treatment efficacy in these patients. [26]

Rochefort J et al. carried out a study to identify immune biomarkers in OSCC based on cigarette smoking and alcohol drinking status, comparing smoker-drinker (SD) patients with non-smoker, non-drinker (NSND) patients. The study analyzed immune cells found in the TME and peripheral blood using flow cytometry and measured the levels of cytokine in tumor supernatants and sera. Results showed that SD patients

had higher levels of monocytes-macrophages, CD4+ T cells and granulocytes expressing CCR6 and CD45RO, while NSND patients exhibited increased CD8+ T cells, correlating with better disease-free survival. Additionally, elevated IL-6 levels in SD patients' sera and tumor supernatants indicated a pro-inflammatory TME associated with poorer prognosis. Surprisingly, Treg cells in SD patients were linked to better outcomes ($p = 0.05$), possibly due to their anti-inflammatory effects. The study suggests that CD45RO+CCR6+CD4+ T cells and granulocytes could serve as negative prognostic markers, while higher CD8+ T cell levels in NSND patients may indicate better prognosis. These findings emphasize the role of immune profiling in OSCC prognosis and highlights the need for personalized immunotherapy strategies targeting distinct immune alterations in SD and NSND patients.[27]

Alexis Desrichard et al. explored tobacco-induced genetic mutations and immune changes affect tumour behaviour and response to immunotherapy in HNSCC and in lung squamous cell carcinoma (LUSC). Using genomic and transcriptomic data from The Cancer Genome Atlas (TCGA) and independent datasets, the study found that a higher tobacco mutational signature correlated with increased tumor mutational burden in both cancer types. However, tobacco's impact on the tumor immune microenvironment varied: in HNSC, it led to immunosuppression (reduced immune infiltration, IFN-γ signaling, and cytolytic activity), while in LUSC, it promoted a pro-inflammatory environment. Smoking-high HNSC tumors were linked with poor survival and lesser response rates to immune checkpoint inhibitors, unlike LUSC tumors, which showed better immunotherapy outcomes. [28]

INTERLINKAGE OF IMMUNE SYSTEM AND TUMOUR CELLS IN THE TUMOUR MICROENVIRONMENT (TME):

Tumor cells in the TME induce nearby cells to release various cytokines and chemokines, creating significant challenges in managing OSCC. Understanding these interactions can improve prognosis assessment and therapy development. Macrophages, particularly tumor-associated macrophages (TAMs), play a MAJOR role in carcinogenesis. TAMs are categorized into M1 (classically activated) and M2 (alternatively activated) subtypes. They secrete cytokines like TGF-β1, IL-8, TNF-α, VEGF, EGF, and MMP, which are crucial for tumorigenesis.[4]

NK cells are cytotoxic effectors in targeting poorly differentiated tumors and cancer stem cells. They use cytokines like IFN-γ and TNF-α to mediate direct cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC). The NK cells are divided into CD56dimCD16+ and CD56brightCD16−/+ subsets, with CD16 receptor activation driving cytokine secretion. Taghavi et al. used CD57 as a marker to evaluate the prognostic value of activated NK cells in OSCC.[31,32]

Dendritic cells (DCs), though rare in tumors, play an essential role in antigen presentation. They out forward the tumor-associated antigens present on MHC molecules and regulate T cell responses through costimulation. DCs are classified into myeloid (mDC) and plasmacytoid (pDC) subtypes. Zhang et al. linked high salivary IL-6 levels in oral cancer patients to DC immunodeficiency, while Xiao et al. associated CD103+ DCs with favorable prognosis. Conversely, Han et al. observed that the TNF-α/NF-κB/CXCR-4 pathway drives OSCC multiplication and its invasion, correlating with increased pDC infiltration.[33-35]

The adaptive immune system is negatively regulated by regulatory T cells (Tregs), which produce TGF-β, retinoic acid, and IL-2. Thymus-derived (tTregs) and periphery-derived (pTregs) subsets of Tregs are distinguished. FOXP3 expression is essential for Treg function. Studies show CCR6+ Tregs are recruited in OSCC via the CCL20/CCR6 axis, contributing to tumor progression. Aggarwal et al. observed altered IL-2, IL-10, and IL-35 expression in CD4+FOXP3+ Tregs in OSCC patients.[36-39]

Additional interactions within the TME include:

- High endothelial venules, associated with increased CD3+ T cells, CD20+ B cells, and chemokine levels ($CXCL^{12}$, CCL21) but reduced CCL20.
- Carcinoma-associated fibroblast (CAF) activation via TGF-β from tumor cells.
- ECM molecules like tenascin-C influencing CD11c+ myeloid cells and lymphatic endothelial cells through integrins.
- Elevated IL-1β, VEGF, and IL-17 secretion by blood neutrophils.

These interactions underscore the complexity of the OSCC TME and highlight targets for therapeutic interventions.[4]

ACTION OF IMMUNE SYSTEM IN CANCER INITIATION AND CARCINOGENESIS:

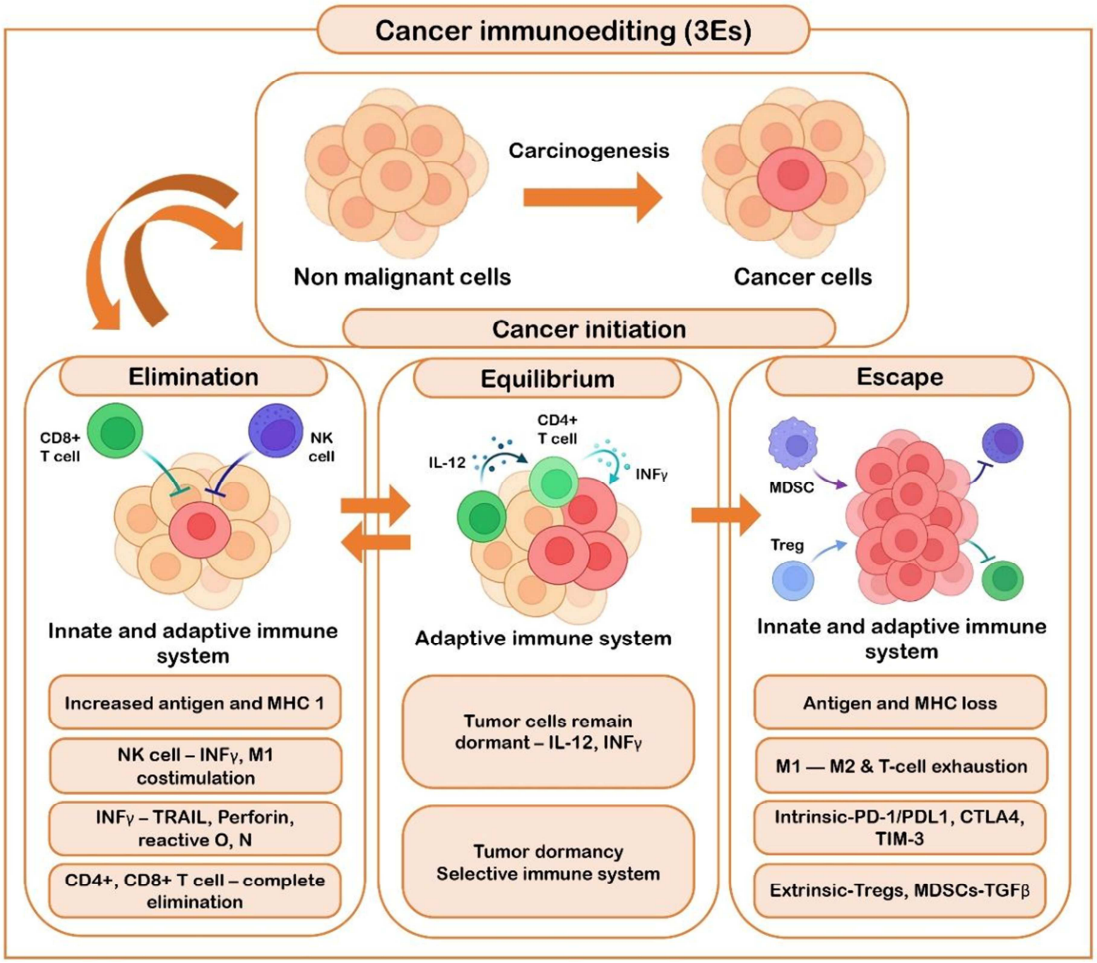


Figure 5: Role of the immune system in carcinogenesis and cancer initiation

Immunoediting in Oral cancer- The components of immune system plays a dual role in cancer, by supporting and restraining tumor progression, making it a two-edged sword in cancer therapy. Cancer immunoediting is a notion that has grown dramatically over the last few decades, with an emphasis on understanding how immune cells aid in cancer formation and how immunotherapy can reverse this process. Cancer immunoediting is often separated into three steps. Cancer immunosurveillance, also known as the elimination phase, is a phase where the immune system targets and eliminates tumour cells. This process begins when tumor-specific and tumor-associated antigens are generated.[4]

IMMUNOPROFILING OF ORAL CANCER:

The American Joint Committee on Cancer (AJCC) established significant updates to oral cancer staging in the "Head and Neck" section of its 8th edition Staging Manual.[25] However, tumors within the same stage often exhibit heterogeneity in their response to therapy and aggressiveness. For forecasting disease progression and overall survival, immunological contexture may be used into staging methods to yield insights that are on par with or even better than traditional TNM staging.[30]

The significance of identifying biological molecules associated with risk assessment, cancer formation, recurrence prediction, invasion, prognosis and metastasis is underscored by molecular and genomic features of oral cancer as well as immune system's infiltration. These factors are also key determinants of immunotherapeutic responses. Immune profiling is particularly significant as patients from different molecular subgroups may show varying responses to treatments.

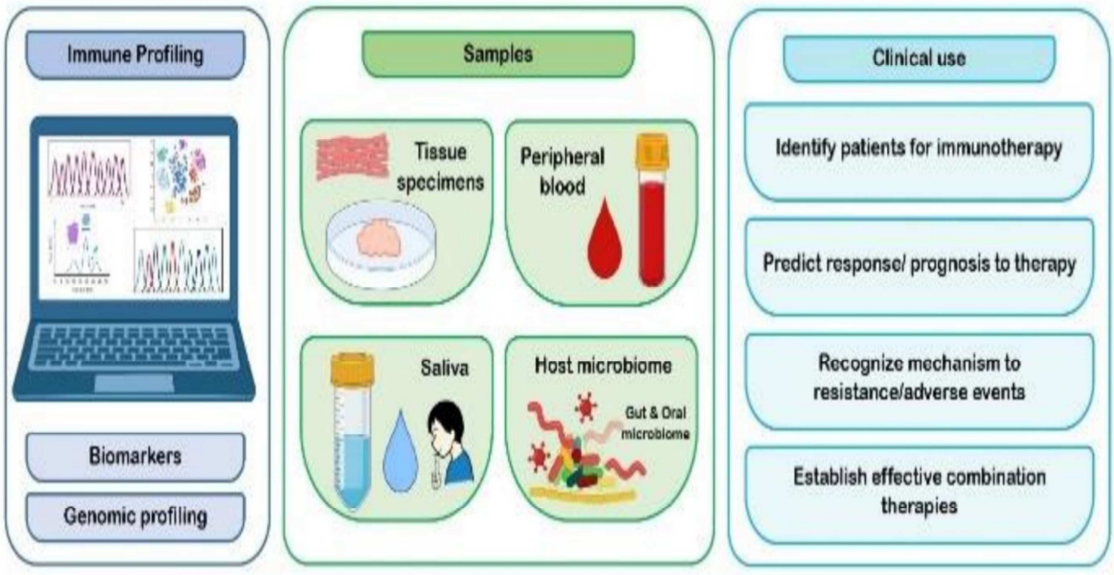


Figure 6: Various samples and clinical use of immune profiling in cancers including OSCC

Technological advancements have transformed immune profiling, moving from single-marker analysis through immunohistochemistry (IHC) to large-scale investigation of immune-related genes via sequencing.

TECHNIQUES EMPLOYED IN IMMUNOPROFILING:

The two main categories of immune profiling technologies are proteomic-based and transcriptomic-based methods. The intricacy of immunological phenotypes and immune heterogeneity were difficult for conventional techniques like immunohistochemistry (IHC) and fluorescent-based flow cytometry to capture. High-dimensional technologies including mass cytometry, multiplex IHC (mIHC), NanoString nCounter, single-cell sequencing, and next-generation sequencing (NGS) have overcome these drawbacks.[30]

NGS encompasses techniques like whole-exome sequencing (WES), whole-genome sequencing (WGS), miRNA profiling, T-cell receptor (TCR) sequencing and RNA sequencing enabling detailed genomic & epigenetic studies. For instance, Goertzen et al. used NGS to identify genes involved in neutrophil recruitment, invadopodia formation, and invasion in TNF-treated OSCC cells. Meanwhile, single-cell genomic techniques**,** though promising for high-resolution cancer analysis at the cellular level, remain in early stages and require further optimization.

mIHC**,** on the other hand, allows simultaneous identification of multiple markers present on a single section of tissue. Qiao et al. leveraged mIHC for PD-L1 expression, tumor-infiltrating lymphocytes (TILs), and immune cells like FOXP3+ Tregs and CD8+ T cells in the TME of diseases like OSCC.[40]

Additionally, **mass cytometry**, along with its integration with flow cytometry (CyTOF), and mass cytometry-based mIHC have proven effective in providing comprehensive immunoprofiling, especially in cancer research.

CLINICAL APPLICATIONS OF IMMUNOPROFILING:

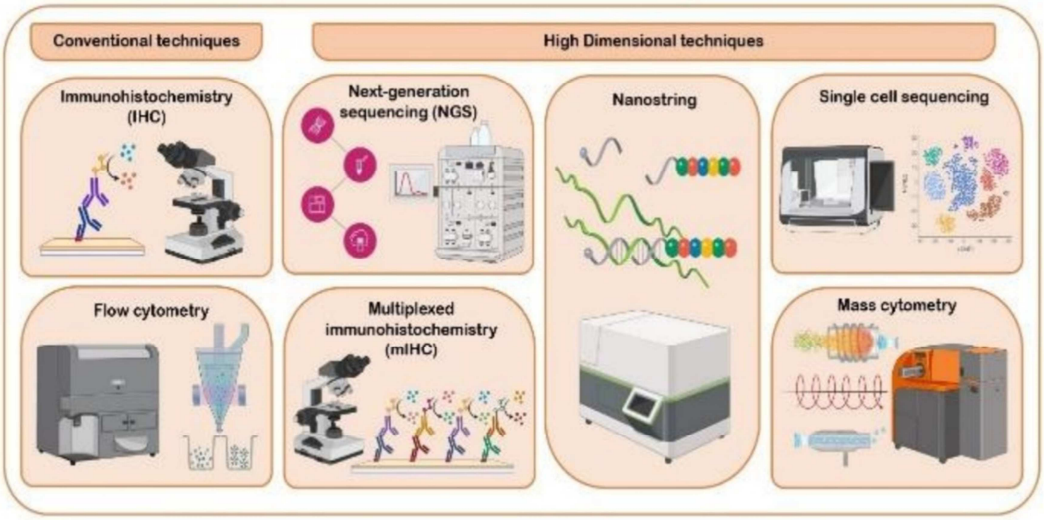


Figure 7: Methods used in Immunoprofiling

Tumor microenvironment (TME) usually determines the immune response to a tumor. Important information on tumor prognosis, especially in regard to immunotherapy, can be obtained by examining these responses using biomarkers, genomic profiling, and cutting-edge technology. In clinical settings, immune profiling makes it possible to choose individuals who are most likely to gain advantages from immunotherapeutic therapies.

For instance, immunotherapies that target PD-1 and its ligand (PD-L1) are essential in the treatment of OSCC and other HNSCC. [41] Research is currently being carried to assess the treatment efficacy of PD-1 and PD-L1 as biomarkers in HNSCC.

In one study, Foy et al. used immunohistochemistry (IHC) for markers such CD4, IDO1, CD3, PD-L1, and CD8, as well as genomic analysis and protein

expression to investigate the immune milieu in HPV-negative OSCC in nonsmokers and nondrinkers. The results validated the use of immunotherapy, specifically pembrolizumab targeting PD-L1 and IDO1.[42]

In a similar vein, Shayan et al. showed that a PD-1 inhibitor in conjunction with the toll-like receptor agonist motolimod (TLR8) and the anti-EGFR antibody cetuximab could improve antitumor responses in HNSCC. The overexpression of T-cell immunoglobulin and mucin domain-3 (Tim-3) was the cause of adaptive resistance to anti-PD-1 therapy, according to a different study conducted by the same group.[43] These results emphasize the significance of identifying and selecting suitable patients for immunotherapy. Immune profiling is integral to predicting therapeutic responses, understanding resistance mechanisms, and designing effective combination therapies tailored to individual patient needs.

MATERIALS AND METHODS

Ethical approval-

Ethical approval for the study was taken from the institutional ethical review committee. Ethical clearance was acquired with clearance number 1661. (Annexure 2)

Sample size estimation-

75 patients aged 18-65 years were included in the study.

Sample size was estimated based on following formula

$$n = \frac{(Z_{1-\alpha/2}+Z_{1-\beta})^2 \times (SD_1^2+SD_2)^2}{(\bar{x}_1 - \bar{x}_2)^2}$$

Where, $\bar{x}_1 = 30.64$, $\bar{x}_2 = 6.47$, $SD_1 = 31.15$, $SD_2 = 3.97$, $Z_{1-\alpha/2} = 1.96$, $Z_{1-\beta} = 1.64$

Sample Size calculated = 22; Final Sample Size rounded = 25 (each group)

So, Total Sample Size = 75 (3 groups)

- 25 Normal (healthy) individuals without any tobacco habit (smoked or chewed form) and systemic illness formed Group 1
- 25 tobacco chewers (15 with clinically visible oral potentially malignant disorders-group 2a and 15 without any visible oral potentially malignant disorder-group 2b) formed Group 2
- 25 oral squamous cell carcinoma patients (clinically and histopathologically confirmed) formed Group 3

All individuals included were as per set inclusion and exclusion criteria. Following were the inclusion and exclusion criterias.

Inclusion criteria:

- Individuals without any tobacco habits who are willing to participate (For group 1)
- Tobacco chewers (For group 2)
- Clinically and histopathologically confirmed oral squamous cell carcinoma patients (For group 3)

Exclusion criteria:

- Those taking medication for any systemic illnesses.
- Congenital/ acquired immunodeficiency states.
- Women during menstrual period or pregnancy and lactation.
- Those under prolonged antibiotics.

Case selection-

After obtaining the institutional ethical clearance the study was conducted on the outpatients reporting to KAHER'S K.L.E V. K Institute of Dental Sciences, Belagavi and KLE Dr. Sampath Kumar Shivanagi Cancer Hospital, Belagavi. Oral screening was performed for all normal healthy individuals, tobacco chewers and OSCC patients.

Written informed consent were obtained from all the individuals included in the control and study groups. Complete case history included- Demographic data for age, sex, habit history, duration and frequency of habit, medical history, duration of the oral potentially malignant lesion and oral squamous cell carcinoma.

Materials:

5 ml syringe	Tryphan blue
Vials containing ethylenediaminetetraacetic acid (EDTA)	coverslip
Tourniquet	Refrigerator
Sterile cotton	Incubator
Surgical gloves, Face masks	DX Flex 2 laser 8 color Beckman coulter flow cytometer
Conical centrifuge tubes	CD3 fluorochrome marker
Micropipette	CD8 fluorochrome marker
Neubaur chamber	CD56 fluorochrome marker

METHODOLOGY

Blood Sample collection-

The venepuncture site was cleaned with 70% alcohol from the centre to periphery and allowed to dry for 30 seconds. Then, 2.5 ml fresh peripheral blood was collected in an anticoagulated tube (EDTA or heparinized).

The samples were immediately shifted to Dr. Prabhakar Kore's Basic Science Research Centre, Belagavi for further processing.

Lymphocyte Isolation-

3–4 mL of HiSep solution was carefully pipetted at the bottom of a 15 mL centrifuge tube, followed by the slow layering of 6–8 mL of diluted blood on top without mixing. The tube was slightly tilted while dispensing to maintain proper layering. The sample was then centrifuged at 400–500 × g for 25–30 minutes at room

temperature without brake, ensuring the formation of distinct layers. The plasma and platelets formed the top layer. The mononuclear cells (PBMCs) appeared as a white ring at the interface, and the bottom layer consisted of RBCs and granulocytes. The PBMC layer was carefully aspirated using a pipette and transferred to a new tube for further processing. To remove residual HiSep, the PBMC suspension was washed with 10 mL of PBS and centrifuged at 300 × g for 10 minutes at room temperature, followed by two additional washes. The final suspension was prepared in PBS for cell counting.

Cell counting and Viability Check-

Cell viability was checked by mixing 10 µL of cell suspension with Trypan Blue and counting with a cell counter. It was ensured that at least 90% of the cells were viable for optimal results and the cells were cryopreserved. The readings of all samples were noted down.

Cryopreservation-

PBMCs were resuspended in freezing medium (90% FBS + 10% DMSO) at 1–10 × 10^6 cells/mL, aliquoted into cryovials, and placed in a -80°C freezer overnight in a controlled-rate freezing container and were later transferred to liquid nitrogen until further analysis.

The cells were further subjected to flowcytometry.

FLOW CYTOMETRY ANALYSIS-

Designing the colour conjugation to the antibodies.

Antibody staining in flow cytometry relies on the use of colour-conjugated fluorochrome-labelled antibodies that bind specifically to cell surface markers. These fluorochromes emit light at distinct wavelengths upon excitation by a laser, enabling the simultaneous identification and analysis of multiple cell populations. Each fluorochrome possesses unique excitation and emission spectra, making it crucial to select fluorochromes compatible with the instrument's lasers and detectors while minimizing spectral overlap.

In this study, using the Beckman Coulter DxFlex flow cytometer equipped with blue and red lasers, three fluorochromes were employed: FITC (Fluorescein isothiocyanate), PE (Phycoerythrin), and APC (Allophycocyanin). These fluorochromes were conjugated to monoclonal antibodies specific to distinct cellular antigens. The selection of fluorochromes was based on key parameters including brightness, photostability, and minimal fluorescence spillover into adjacent detection channels.

To correct for spectral overlap and fluorescence spillover, compensation was applied. Compensation adjusts for emission signal interference between fluorochromes with overlapping spectra. The flow cytometer utilized multiple lasers—blue (488 nm), red (633 nm), and violet (405 nm)—to excite different fluorochromes, allowing for multiparameter analysis of complex cellular populations.

Thawing Cryopreserved PBMCs-

Cryopreserved PBMCs were thawed while maintaining high viability. Pre-warmed RPMI-1640 (or complete medium) was used, and the frozen vial was quickly taken out from liquid nitrogen and placed in a 37°C water bath, gently swirled until only a small ice pellet remained. To minimize osmotic shock, thawed cells were transferred dropwise into a 15 mL tube containing 10 mL of pre-warmed media while swirling.

The cells were then centrifuged at 300 × g for 5–7 minutes at 4°C to remove DMSO, after which the supernatant was aspirated, and the pellet was resuspended in fresh medium. Trypan Blue was used to assess cell viability, and the suspension was adjusted to 1–2 × 10^6 cells/mL in staining buffer, keeping the sample on ice. . Following cell counting, the viable cells were divided into two portions, each containing approximately 1 × 10^6 cells/mL. One portion was reserved as the unstained control, while the other portion was used for staining with secondary antibodies.

Surface staining for flow cytometry-

Approximately 100–200 µL (~1×10^6 cells) were transferred into individual FACS tubes or wells, with an optional Fc receptor blocking step for monocytes to prevent non-specific binding. Followed by adding the 5 µl of FITC anti-human CD3, 5 µl of PE anti-human CD56 and, 5 µl of APC anti -human CD16 secondary antibodies, gently mixed, and incubated for 30 minutes at 4°C in the dark. After incubation, the cells were washed twice with 2 mL PBS and centrifuged at 300 × g for 5 minutes, then resuspended in FACS buffer.

Compensation of antibodies-

In flow cytometry, compensation is essential to exact the spectral overlap between different fluorochromes. Although each fluorochrome is excited by a specific laser and emits light at a characteristic wavelength, some degree of emission can be detected in adjacent channels, leading to fluorescence spill over.

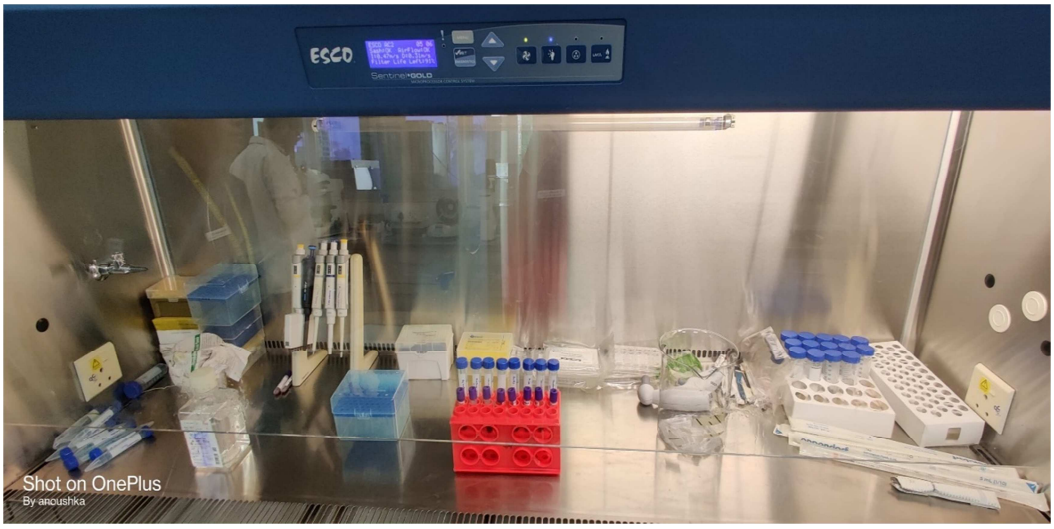
In this study, the fluorochromes used — FITC (for CD3), PE (for CD56), and APC (for CD16) — have overlapping emission spectra. Therefore, compensation was performed to accurately separate the signals and ensure reliable identification of each cell population.

Compensation controls were prepared by staining single-stained samples for each fluorochrome-conjugated antibody individually. These controls allowed the flow cytometer (Beckman Coulter DxFlex) to mathematically subtract the spill over from neighbouring channels and correctly assign fluorescence intensity to the appropriate detector.

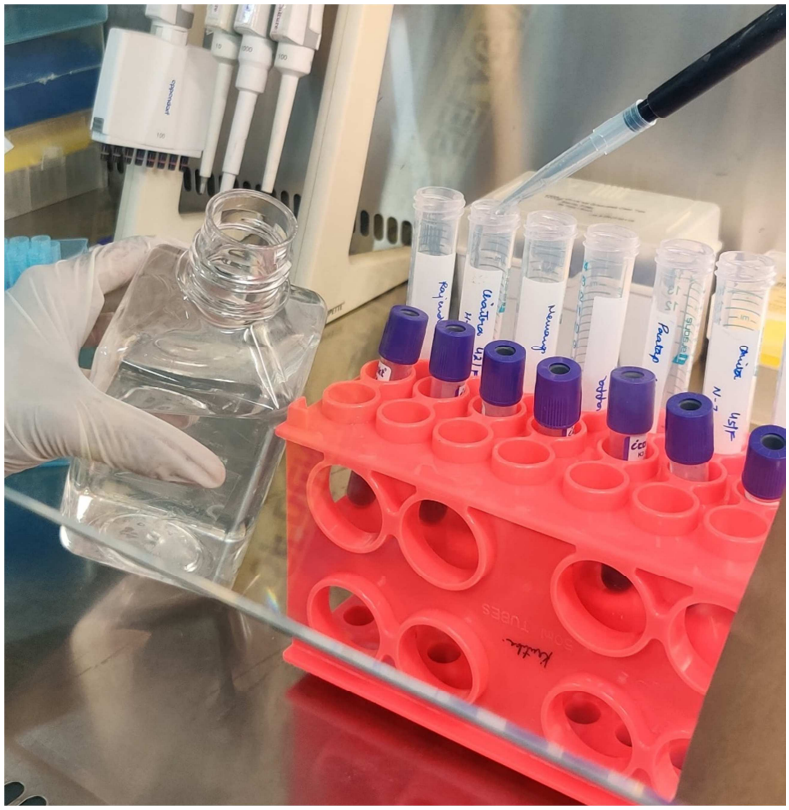
Accurate compensation ensured that the fluorescence measured in each channel truly represented the specific marker expression without interference from other fluorochromes, enabling precise multiparametric analysis of NK cell subsets.

Flow cytometry acquisition and analysis-

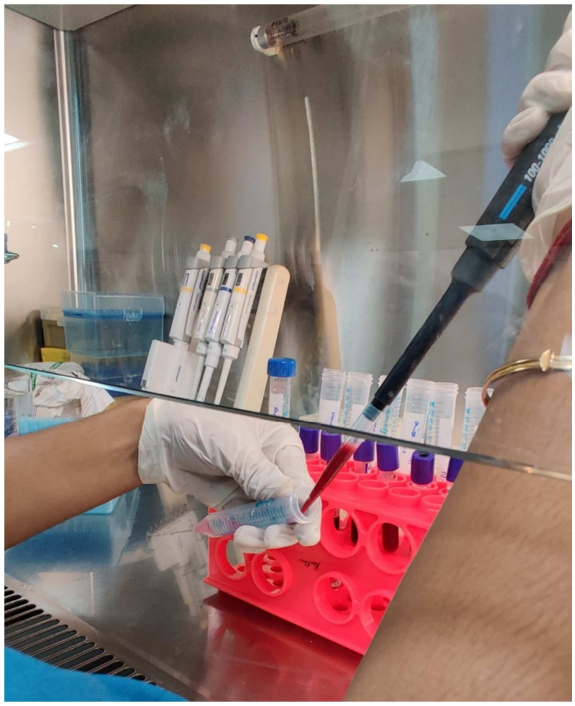
The process involved first running unstained and single-stained controls, followed by singlets gating to exclude doublet cells. The gating strategy consisted of defining lymphocytes based on FSC vs. SSC, excluding dead cells with viability dye, and identifying NK cell populations as $CD3^{-}CD56^{+}$ or $CD3^{-}CD16^{+}$. Subsets included cytotoxic NK cells ($CD56^{+}CD16^{+}$), cytokine producing NK cells ($CD56^{+}CD16^{-}$), and NKT like cells ($CD3^{+}CD56^{+}$) which were excluded from pure NK analysis. The readings were analysed using software.

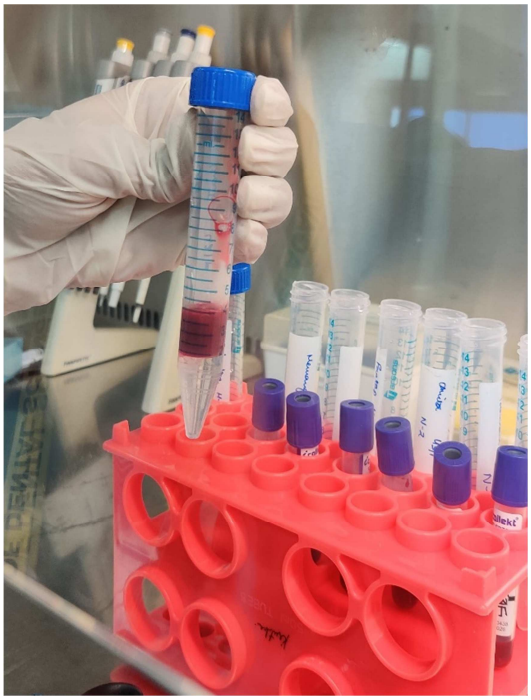


Picture 1: Lymphocyte separation done under sterile condition in laminar flow

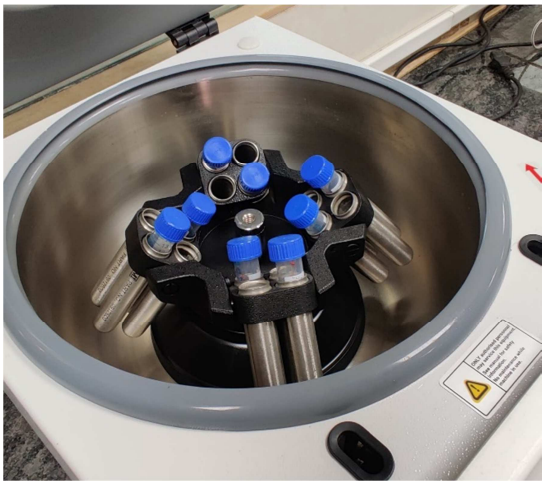


Picture 2: HiSep solution carefully pipetted at the bottom of a 15 mL centrifuge tube

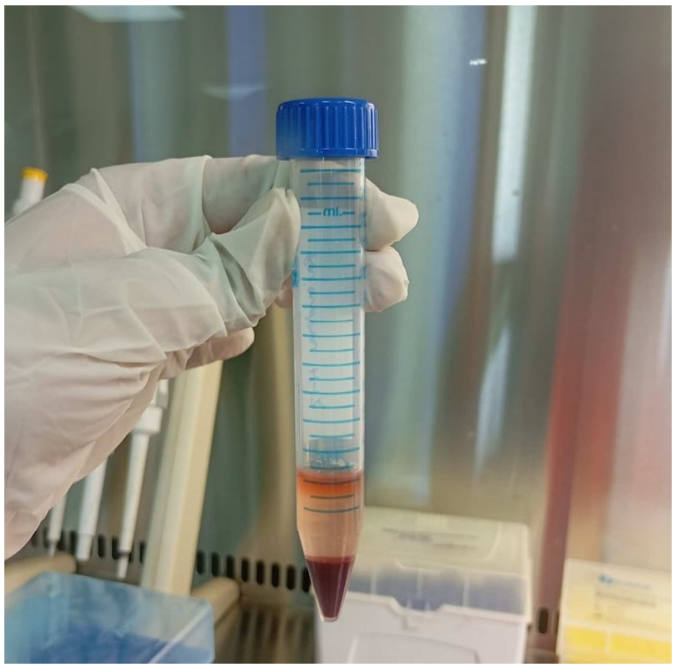




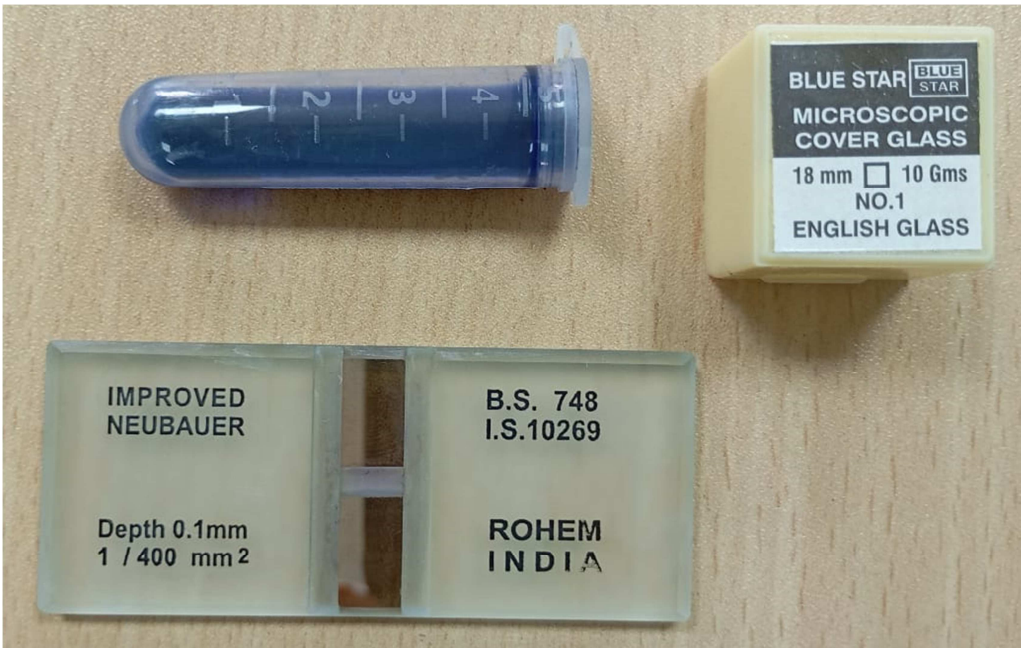
Picture 3a and 3B: The tube was slightly tilted while dispensing to maintain proper layering and slow layering of 6–8 mL of diluted blood was done on top without mixing.



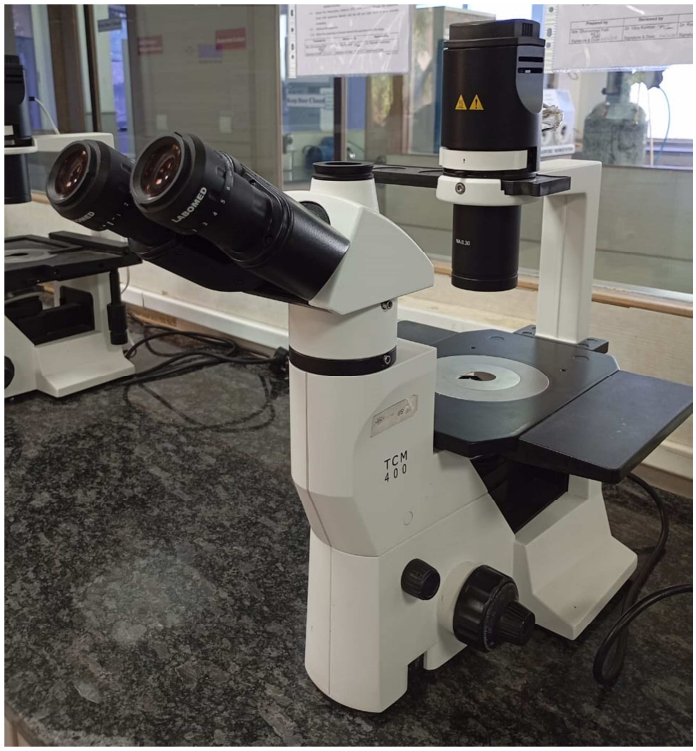
Picture 4a and 4b: Centrifuged at 400–500 × g for 25–30 minutes at room temperature without brake



Picture 5: Plasma and platelets formed the top layer and mononuclear cells (PBMCs) appeared as a white ring at the interface, and the bottom layer consisted of RBCs and granulocytes



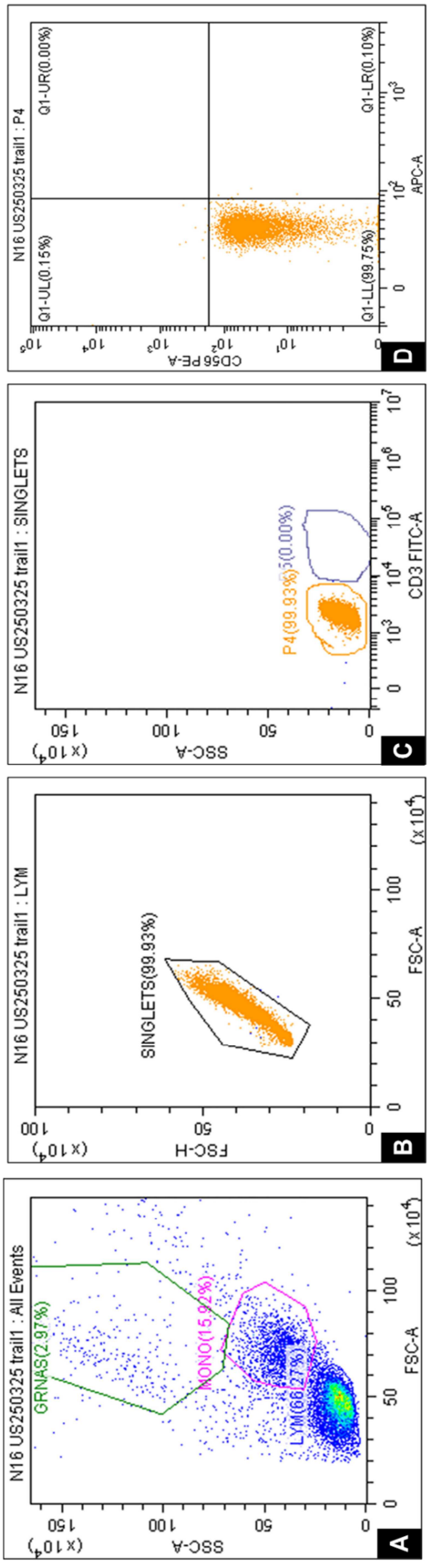
Picture 6: Cell counting materials (Neubauer chamber, tryphan blue, Coverslip)



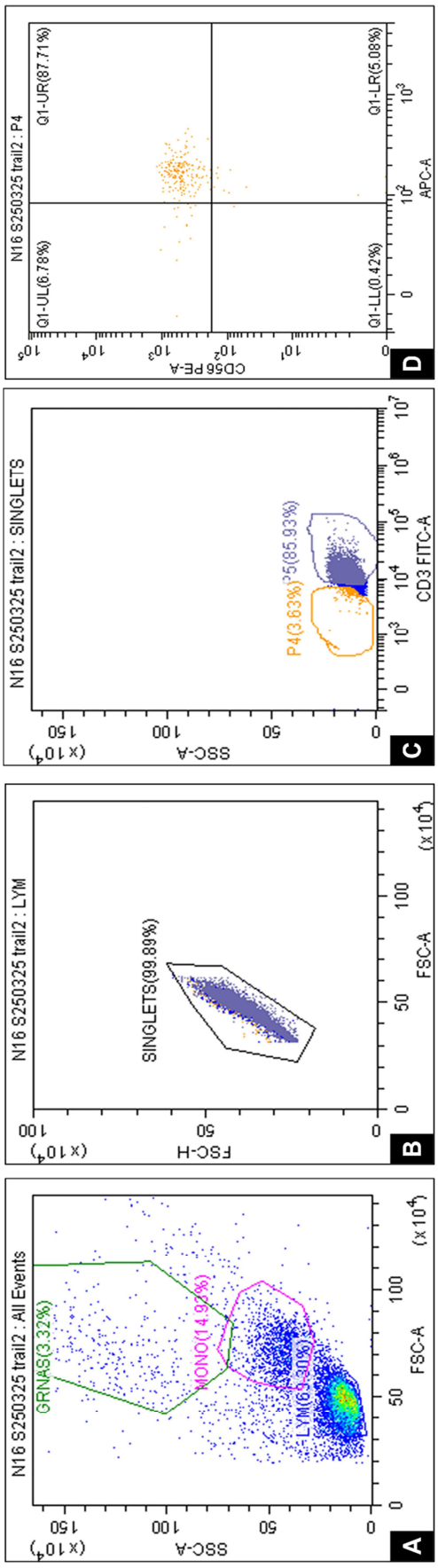
Picture 7: Microscope used for viable cell counting

Picture 8a and 8b : -80^{0}C refrigerator and Liquid nitrogen can

Picture 9 :DX Flex 2 laser 8 color Beckman coulter flow cytometer



Picture 10. Gating strategy applied to an unstained control sample for natural killer (NK) cell analysis by flow cytometry.(A) FSC-A vs SSC-A plot used to identify and gate the lymphocyte population. Cells were classified based on scatter properties into lymphocytes (LYM, 66.27%), monocytes (MONO, 15.92%), and granulocytes (GRNAS, 2.97%). Lymphocytes were gated for further analysis based on their characteristic low SSC-A and moderate FSC-A profile.**(B) FSC-A vs FSC-H plot** of the lymphocyte gate, used to exclude doublets and select singlets. A high proportion of singlet events (99.93%) was retained for further analysis.**(C) CD3 FITC-A vs SSC-A plot** of singlets, used to separate CD3-negative (P4) from CD3-positive (P5) populations. The majority of events fell within the CD3-negative gate (P4, 99.93%).**(D) CD56 PE-A vs CD16 APC-A plot** of CD3-negative events (P4). Nearly all events clustered in the lower-left quadrant (Q1-LL, 99.75%), indicating the absence of CD56 and CD16 staining in the unstained control. This confirms minimal background fluorescence and establishes the negative baseline for NK cell marker expression.This sequential gating strategy ensures accurate identification of NK cells in stained experimental samples by using the unstained control to define population boundaries and background signal.



Picture 11. Gating strategy applied to a stained sample for natural killer (NK) cell identification using flow cytometry. (A) FSC-A vs SSC-A plot of all events used to gate lymphocytes (LYM, 65.30%), monocytes (MONO, 14.93%), and granulocytes (GRNAS, 3.32%) based on light scatter properties. Lymphocytes were gated for further analysis due to their characteristic small size and low granularity.**(B) FSC-A vs FSC-H plot** of gated lymphocytes, used to exclude doublets and retain **singlets (99.89%)** for accurate downstream analysis.**(C) CD3 FITC-A vs SSC-A plot** of singlet lymphocytes, used to separate **CD3-negative (P4, 3.63%)** and **CD3-positive (P5, 85.93%)** populations. The CD3-negative population (P4) was used to identify NK cells.**(D) CD56 PE-A vs CD16 APC-A plot** of CD3-negative events (P4). Most events localized in the upper-right quadrant (Q1-UR, 87.71%), indicating strong co-expression of **CD56 and CD16**, characteristic of mature NK cells. Additional populations were identified in Q1-UL (6.78%, $CD56^{+}CD16^{-}$), Q1-LR (5.08%, $CD56^{-}CD16^{+}$), and Q1-LL (0.42%, double-negative), revealing NK cell heterogeneity.This gating strategy highlights the discrimination of NK cells based on CD3 negativity and dual expression of CD56 and CD16, facilitating phenotypic analysis of NK subsets in stained samples

RESULTS

Table1: Baseline characteristics of study population

	Healthy Individuals (n= 25)	Tobacco Chewers without lesion (n= 12)	Tobacco Chewers with lesion (n= 13)	OSCC patients (n= 25)
AGE (Mean ± SD)	42.36 ± 10.5	45 ± 11.8	45 ± 9.47	53.2 ± 8.03
SEX				
Male	11 (44%)	10 (83%)	8 (61.5%)	20 (80%)
Female	14 (56%)	2 (16%)	5 (38.4%)	5 (20%)
TYPE OF HABIT				
Smokeless Tobacco		8 (66%)	8 (61.5%)	15 (60%)
Pan , Supari		4 (33%)	5 (38.4%)	10 (40%)
FREQUENCY				
1-3 times/day		3 (25%)	3 (23%)	8 (32%)
4-6 times/day		9 (75%)	10 (76.9%)	17 (68%)
DURATION OF HABIT				
< 10 years		1 (8.3%)	2 (15.3 %)	-
10-20 years		4 (33%)	4 (30.7%)	6 (24%)
21-30 years		7 (58%)	5 (38.4%)	10 (40%)
31-40 years		-	1 (7.6%)	7 (28%)
>40 years		-	1 (7.6%)	2 (8%)
TYPE OF LESION				
Leukoplakia			7 (53.8%)	
Erythroplakia			2 (15.3%)	
OSMF			1 (7.6%)	
Tobacco Pouch Keratosis			3 (23%)	
SITE OF LESION				
Buccal Mucosa			10 (76.9%)	14 (56%)
Vestibule			3 (23%)	-
Alveolus			-	4 (16%)
Tongue			-	3 (12%)
Lip			-	2 (8%)
RMT			-	2 (8%)
DURATION OF THE LESION				
<4 years			4 (30%)	20 (80%)
>4 years			8 (61.5%)	5 (20%)

The study included a total of 75 subjects divided into three groups: Healthy individuals (n = 25), tobacco chewers (n = 25), and patients histopathologically diagnosed with OSCC (n = 25). Among the healthy individuals, the mean age of participants were 42.36 ± 10.5 years. Among tobacco chewers the mean age of participants were 45 ± 10.42 and in OSCC patients it was 53.2 ± 8.03 years.

In terms of sex distribution, healthy group had 11 Males and 14 females, tobacco chewers group had 18 males and 7 females, and OSCC group had 20 males and 5 females.

Among the tobacco chewers 13 individuals had visible oral lesions and 12 were without any visible lesion.

66% of the individuals with habit, 61.5% of individuals with habit and lesion, and 60% of OSCC patients had smokeless tobacco chewing habit. The rest used pan and supari along with tobacco.

Among chewers without lesions 75% and chewers with lesions 76.9% , and 68% in the OSCC group, reported to use tobacco 4-6 times/day. A smaller percentage used tobacco 1–3 times per day (25%, 23%, and 32%, respectively).

The habit duration of 21–30 years was observed in 58% of chewers without lesions, 38.4% of chewers with lesions, and 40% of OSCC patients. A duration of 10–20 years was reported by 33% of chewers without lesions, 30.7% of chewers with lesions, and 24% of OSCC patients. Additionally, a duration of 31–40 years was seen in 28% of OSCC patients.

The most common lesion type that the tobacco chewers presented with was leukoplakia (53.8%), followed by tobacco pouch keratosis (23%), erythroplakia (15.3%), and oral submucous fibrosis (OSMF) (7.6%).

The most common lesion site in tobacco chewers group was the buccal mucosa (76.9%), followed by the vestibule (23%). Among OSCC patients, lesions were most frequently located on the buccal mucosa (56%), followed by the alveolus (16%), tongue (12%), lip (8%), and retromolar trigone (RMT) (8%).

Majority of lesions in the OSCC group were reported to have been present for less than 4 years (80%), while in the tobacco chewers with lesions group, most had lesions for more than 4 years (61.5%).(Table1)

Immunoprofiling was carried out in all 75 participants of all groups using flow cytometry. The data were evaluated using SPSS™ .

To evaluate the normality of NK cell distribution among the three groups, Shapiro-Wilk test was applied.

Table 2: Table shows normality of all parameters among three groups (Normal individuals, Tobacco chewers and OSCC cases)

Parameters (% parent)	Groups	Shapiro-Wilk	df	P-value
NK cells	Normal	0.9110	25	0.0330*
	Tobacco	0.8560	25	0.0020*
	OSCC	0.8200	25	0.0010*
CD16+CD56+	Normal	0.9650	25	0.5260
	Tobacco	0.9090	25	0.0280*
	OSCC	0.9410	25	0.1560
CD56+	Normal	0.9630	25	0.4830
	Tobacco	0.9800	25	0.8790
	OSCC	0.7540	25	0.0352*
CD16+	Normal	0.7490	25	0.0001*
	Tobacco	0.8290	25	0.0010*
	OSCC	0.7580	25	0.0001*

*$p<0.05$

NK cells (% parent) percentage showed a non-normal distribution in all groups, with p-values of 0.033 (normal), 0.002 (tobacco), and 0.001 (OSCC), respectively.

$CD16^+$ values were not normally distributed across any group ($p < 0.001$ for all).

For $CD16^+CD56^+$, the normal and OSCC groups showed a normal distribution ($p = 0.526$ and 0.156), while the tobacco group did not ($p = 0.028$).

In the case of $CD56^{+}$, the normal (p = 0.483) and tobacco (p = 0.879) groups displayed normality, but the OSCC group showed a deviation from normal distribution (p = 0.0352). (Table 2)

Based on these outcomes, non-parametric statistical tests were chosen for further analysis.

To assess differences in NK cell levels among the groups, a Kruskal-Wallis test was conducted.

Table 3: Table shows the Comparison of parent NK cells among the Normal individuals, Tobacco chewers and OSCC cases.

Group	Mean	SD	Median	IQR
Normal group	3.85	2.99	2.81	2.08
Tobacco group	3.69	3.34	2.65	1.91
OSCC group	4.57	4.19	2.51	3.03
H-value	0.4012			
P-value	0.8182			

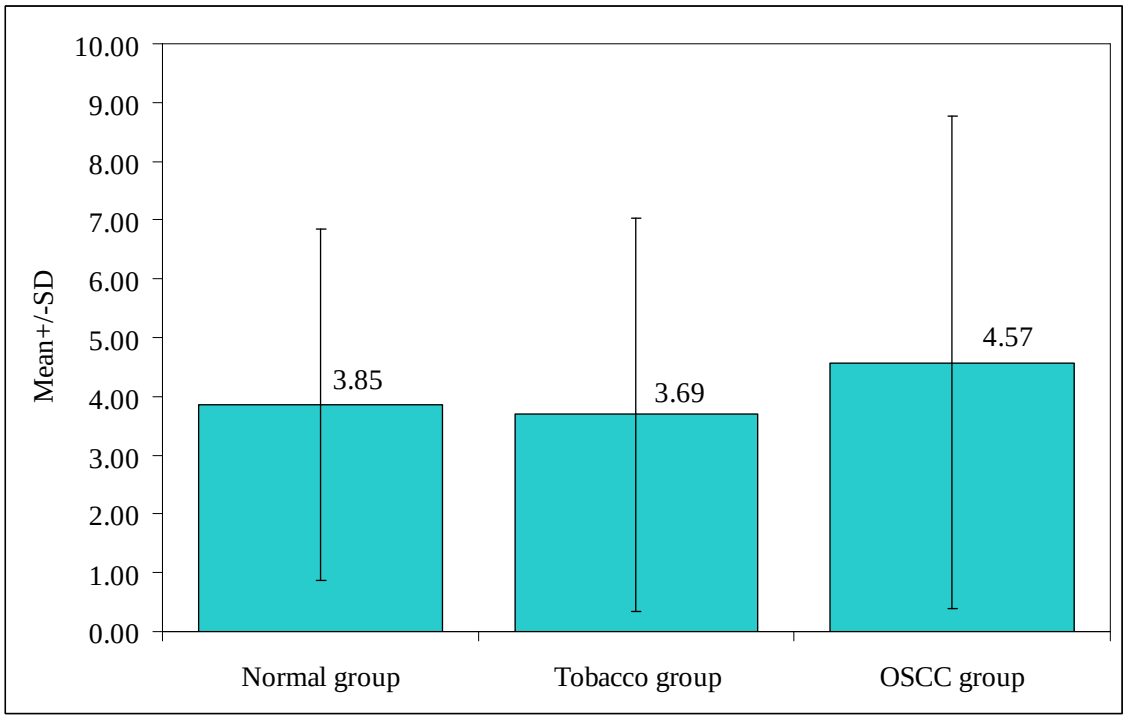
.Descriptive statistics for NK cell percentages are presented in Table 3. The mean NK cell percentages in the normal, tobacco, and OSCC groups were 3.85%, 3.69%, and 4.57%, respectively, while the corresponding median values were 2.81%, 2.65%, and 2.51%. The interquartile ranges (IQR) were 2.08 for the normal group, 1.91 for the tobacco group, and 3.03 for the OSCC group. The test yielded an H-value of 0.4012 and a p-value of 0.8182, indicating no statistically significant difference in NK cell percentages among the three groups.

Pair wise comparison between the groups was done by Mann Whitney U test.

Table 4: Table shows pair wise comparison of three groups (Normal, Tobacco and OSCC) with NK cells (% parent)

Group	Mean	SD	Median	IQR	Mean rank	U-value	Z-value	p-value
Normal group	3.85	2.99	2.81	2.08	26.44			
Tobacco group	3.69	3.34	2.65	1.91	24.56	289.00	0.4463	0.6554
Normal group	3.85	2.99	2.81	2.08	25.46			
OSCC group	4.57	4.19	2.51	3.03	25.54	311.50	-0.0097	0.9923
Tobacco group	3.69	3.34	2.65	1.91	24.20			
OSCC group	4.57	4.19	2.51	3.03	26.80	280.00	-0.6209	0.5347

Bar graph 1: Bar graph shows comparison of three groups (Normal, Tobacco and OSCC) with NK cells (% parent)



When comparing normal individuals and tobacco chewers**,** the mean ranks were 26.44 and 24.56, respectively, with a U-value of 289.00 and a p-value of **0**.6554**,** indicating no statistically significant difference. A comparison between the normal and OSCC groups showed nearly identical mean ranks (25.46 and 25.54), with a U-value of 311.50 and a p-value of 0.9923**,** also not statistically significant. Similarly, the comparison between tobacco chewers and OSCC patients revealed mean ranks of 24.20 and 26.80, respectively, with a U-value of 280.00 and a p-value of 0.5347**,** again showing no significant difference (Table 4 and figure 1). Further, among the total NK cell (% parent) the values were tabulated for CD56+, CD16+ and both CD56+CD16+.

Table 5: Table shows the Comparison of parent CD16+CD56+ NK cells among the Normal individuals, Tobacco chewers and OSCC cases.

Group	Mean	SD	Median	IQR
Normal group	28.26	15.00	26.83	9.82
Tobacco group	23.14	18.50	18.37	13.51
OSCC group	21.12	16.06	18.18	10.73
H-value	2.6444			
P-value	0.2665			

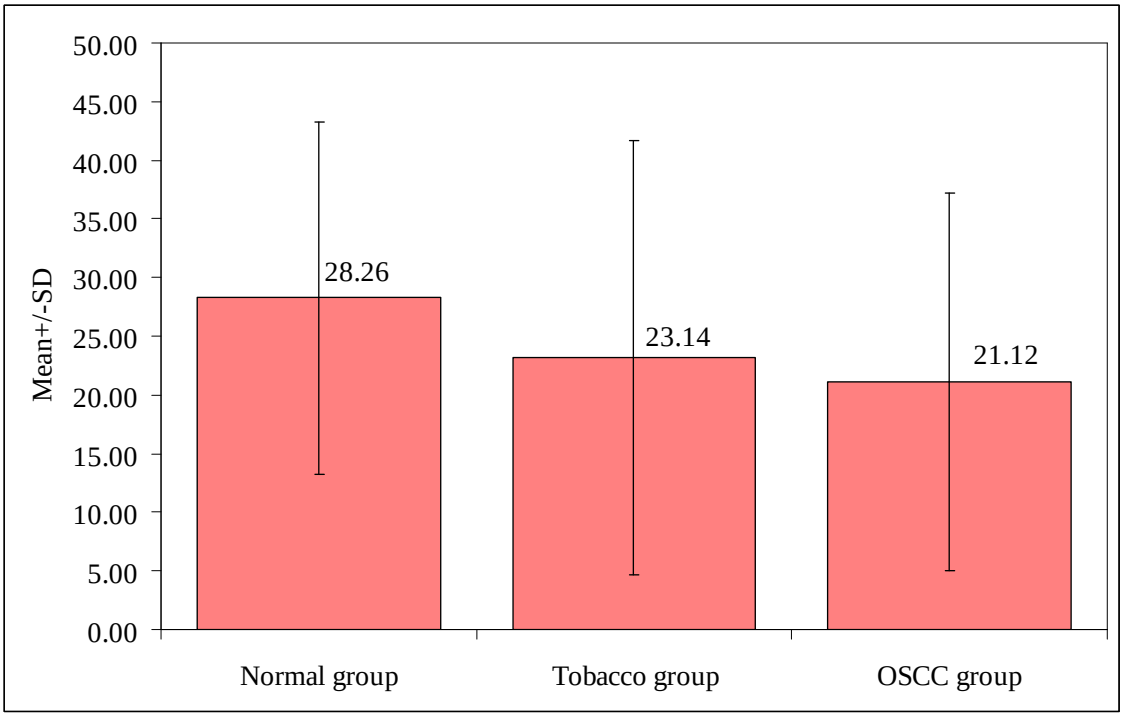
The distribution of $CD16^{+}CD56^{+}$ (% parent) NK cells was analyzed among normal individuals, tobacco chewers, and patients with oral squamous cell carcinoma (OSCC). The mean percentage of $CD16^{+}CD56^{+}$ cells was highest in the normal group (28.26 ± 15.00), followed by the tobacco group (23.14 ± 18.50), and lowest in the OSCC group (21.12 ± 16.06). Median values mirrored this trend: 26.83% in normal

individuals, 18.37% in tobacco users, and 18.18% in OSCC patients. The interquartile ranges (IQR) were 9.82, 13.51, and 10.73 for the normal, tobacco, and OSCC groups, respectively, indicating higher variability in tobacco users. The test yielded an H-value of 2.6444 with a p-value of 0.2665, suggesting that the differences observed were not statistically significant.

Table 6: Table shows pair wise comparison of three groups (Normal, Tobacco and OSCC) with CD16+CD56+ (% Parent) cells

Group	Mean	SD	Median	IQR	Mean rank	U-value	Z-value	p-value
Normal group	28.26	15.00	26.83	9.82	27.92			
Tobacco group	23.14	18.50	18.37	13.51	23.08	252.00	1.1642	0.2444
Normal group	28.26	15.00	26.83	9.82	28.76			
OSCC group	21.12	16.06	18.18	10.73	22.24	231.00	1.5716	0.1160
Tobacco group	23.14	18.50	18.37	13.51	26.04			
OSCC group	21.12	16.06	18.18	10.73	24.96	299.00	0.2522	0.8009

Bar graph 2: Bar graph shows comparison of three groups (Normal, Tobacco and OSCC) with CD16+CD56+ (% Parent) cells



Further pairwise comparisons using the Mann-Whitney U test revealed similar non-significant findings:

- Between normal and tobacco groups, the mean ranks were 27.92 and 23.08, with a U-value of 252.00, Z = 1.1642, and p = 0.2444.
- Between normal and OSCC groups, the mean ranks were 28.76 and 22.24, with a U-value of 231.00, Z = 1.5716, and p = 0.1160.
- Between tobacco and OSCC groups, the mean ranks were 26.04 and 24.96, with a U-value of 299.00, Z = 0.2522, and p = 0.8009.

Although numerically the percentage of $CD16^{+}CD56^{+}$ NK cells appears reduced in tobacco users and OSCC patients compared to healthy individuals, these differences were not statistically significant in this study. This trend, however, may indicate a gradual decline in functional NK cell populations in association with tobacco exposure and malignant transformation.

Table 7: Table shows the Comparison of parent CD56+ NK cells among the Normal individuals, Tobacco chewers and OSCC cases.

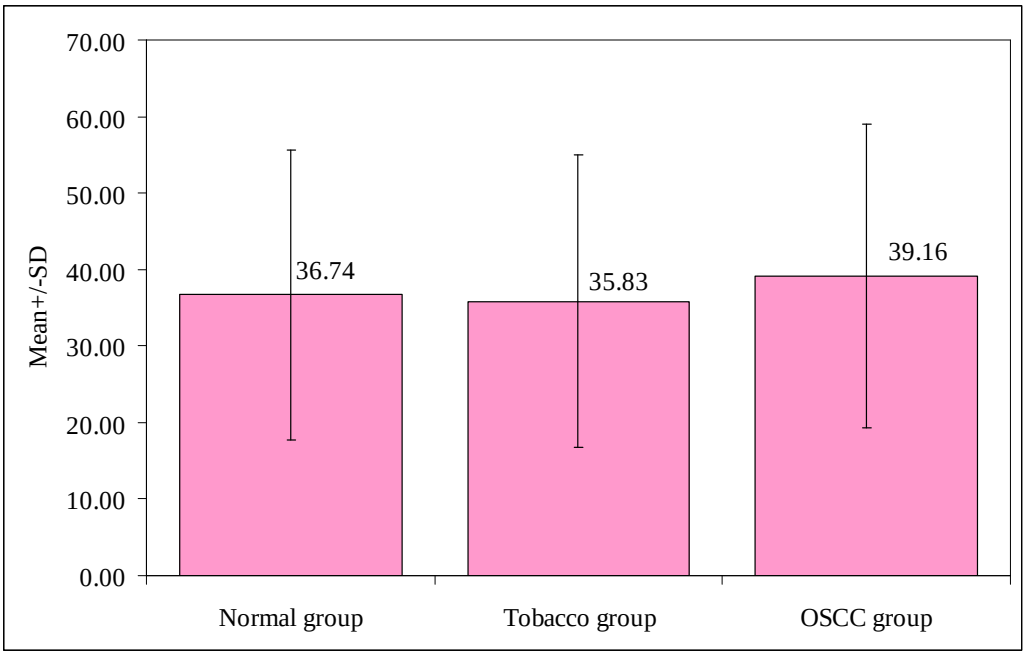
Group	Mean	SD	Median	IQR
Normal group	36.74	18.95	33.81	12.22
Tobacco group	35.83	19.10	33.77	14.21
OSCC group	39.16	19.81	38.18	8.83
H-value	0.5041			
P-value	0.7772			

The percentage of $CD56^+$ (% parent) NK cells was evaluated across the normal, tobacco, and OSCC groups using both descriptive and inferential statistics. The mean values were 36.74% for the normal group, 35.83% for tobacco users, and 39.16% for OSCC patients. The median values were similar, at 33.81%, 33.77%, and 38.18%, respectively. The interquartile range (IQR) was highest in the tobacco group (14.21), followed by the normal group (12.22), and lowest in the OSCC group (8.83), indicating relatively tighter data spread in OSCC cases. The test yielded an H-value of 0.5041 and a p-value of 0.7772**,** indicating no statistically significant difference in $CD56^+$ NK cell percentages across the three groups.

Table 8: Table shows pair wise comparison of three groups (Normal, Tobacco and OSCC) with CD56+ (% Parent) cells

Group	Mean	SD	Median	IQR	Mean rank	U-value	Z-value	p-value
Normal group	36.74	18.95	33.81	12.22	25.70			
Tobacco group	35.83	19.10	33.77	14.21	25.30	307.50	0.0873	0.9304
Normal group	36.74	18.95	33.81	12.22	24.20			
OSCC group	39.16	19.81	38.18	8.83	26.80	280.00	-0.6209	0.5347
Tobacco group	35.83	19.10	33.77	14.21	24.28			
OSCC group	39.16	19.81	38.18	8.83	26.72	282.00	-0.5821	0.5605

Bar graph 3: Bar graph shows comparison of three groups (Normal, Tobacco and OSCC) with CD56+ (% Parent) cells



Further pairwise comparisons using the Mann-Whitney U test also showed no significant differences:

- Between normal and tobacco groups**,** the mean ranks were 25.70 and 25.30, with a U-value of 307.50, Z = 0.0873, and p = 0.9304.
- Between normal and OSCC groups, the mean ranks were 24.20 and 26.80, with a U-value of 280.00, Z = -0.6209, and p = 0.5347.
- Between tobacco and OSCC groups, the mean ranks were 24.28 and 26.72, with a U-value of 282.00**,** Z **=** -0.5821, and p = 0.5605.

Although the OSCC group showed a slightly higher mean and median $CD56^+$ expression compared to the other groups, these differences were not statistically significant**.** The findings suggest that the percentage of $CD56^+$ NK cells remains relatively consistent among healthy individuals, tobacco chewers, and OSCC patients within the context of this study.

Table 9: Table shows the Comparison of parent CD16+ NK cells among the Normal individuals, Tobacco chewers and OSCC cases.

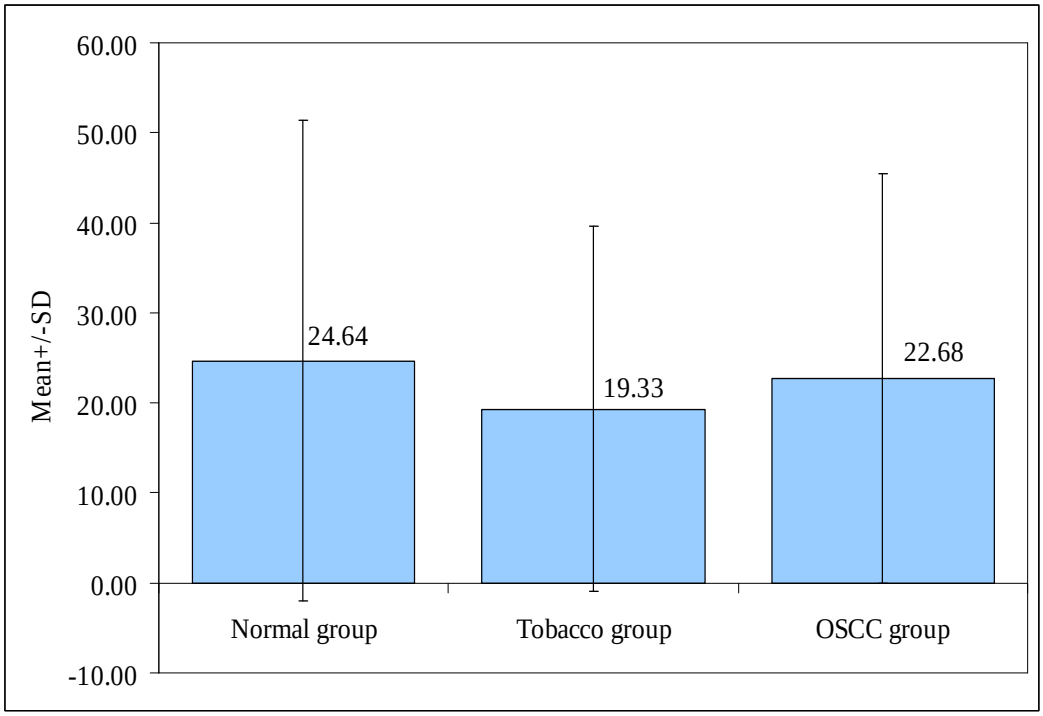
Group	Mean	SD	Median	IQR
Normal group	24.64	26.68	13.82	10.43
Tobacco group	19.33	20.30	13.51	7.75
OSCC group	22.68	22.71	13.21	10.34
H-value	0.6658			
P-value	0.7169			

The distribution of CD16+ % parent cells was compared among three groups: Normal, Tobacco users, and patients with Oral Squamous Cell Carcinoma (OSCC). Using the Kruskal-Wallis ANOVA test, the mean percentage of CD16+ cells was observed to be highest in the Normal group (Mean = 24.64%, SD = 26.68), followed by the OSCC group (Mean = 22.68%, SD = 22.71), and lowest in the Tobacco group (Mean = 19.33%, SD = 20.30). The median values were relatively similar across groups: 13.82% in the Normal group, 13.51% in the Tobacco group, and 13.21% in the OSCC group. The interquartile range (IQR) was 10.43 for the Normal group, 7.75 for the Tobacco group, and 10.34 for the OSCC group. The Kruskal-Wallis test yielded an H-value of 0.6658 and a corresponding p-value of 0.7169, indicating that there was no statistically significant difference in CD16+ % parent cell distribution among the three groups.

Table 10: Table shows pair wise comparison of three groups (Normal, Tobacco and OSCC) with CD16+ (% Parent) cells

Group	Mean	SD	Median	IQR	Mean rank	U-value	Z-value	p-value
Normal group	24.64	26.68	13.82	10.43	27.04			
Tobacco group	19.33	20.30	13.51	7.75	23.96	274.00	0.7373	0.4609
Normal group	24.64	26.68	13.82	10.43	25.08			
OSCC group	22.68	22.71	13.21	10.34	25.92	302.00	-0.1940	0.8462
Tobacco group	19.33	20.30	13.51	7.75	24.16			
OSCC group	22.68	22.71	13.21	10.34	26.84	279.00	-0.6403	0.5220

Bar graph 4: Bar graph shows comparison of three groups (Normal, Tobacco and OSCC) with CD16+ (% Parent) cells

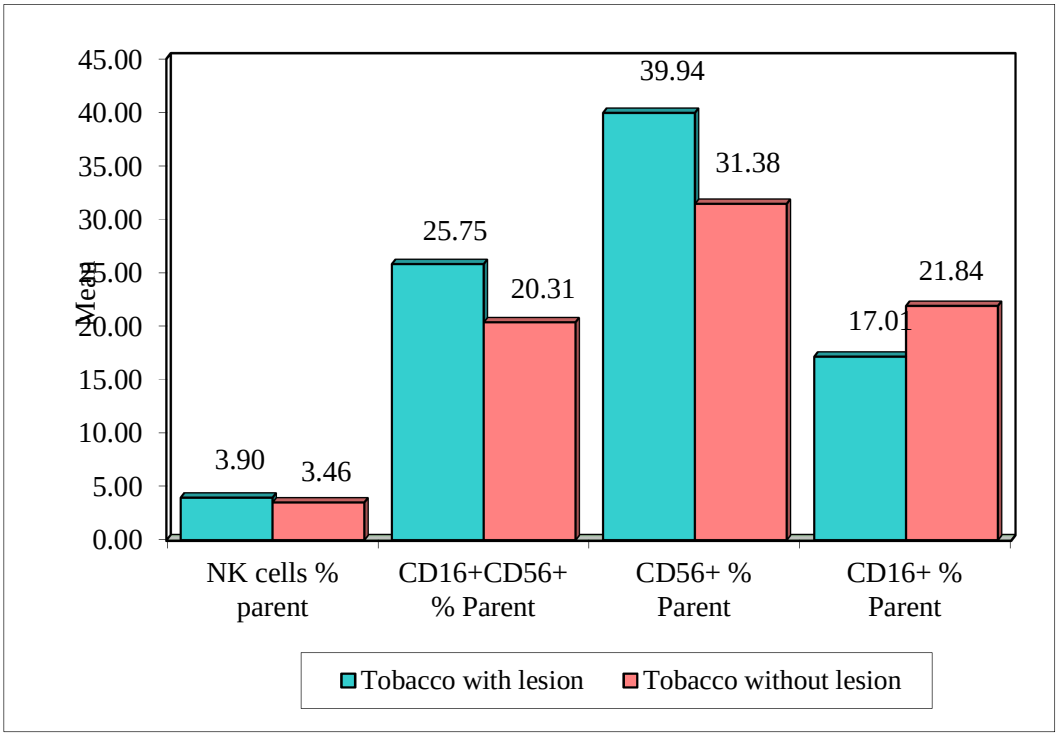


Further, pairwise comparisons using the Mann-Whitney U test were conducted. Between the Normal and Tobacco groups, the U-value was 274.00 with a Z-value of 0.7373 and a p-value of 0.4609. The mean rank was 27.04 for the Normal group and 23.96 for the Tobacco group. Comparing the Normal and OSCC groups, the U-value was 302.00, Z-value was -0.1940, and p-value was 0.8462; with mean ranks of 25.08 and 25.92, respectively. Between the Tobacco and OSCC groups, the U-value was 279.00, Z-value was -0.6403, and p-value was 0.5220. The mean ranks were 24.16 for the Tobacco group and 26.84 for the OSCC group. These results indicate that none of the pairwise comparisons showed statistically significant differences in CD16+ % parent cells across the groups.

Table 11: Table shows comparison of Tobacco with lesion group, Tobacco without lesion group among tobacco chewers with all parameters

Variable	Tobacco	n	Mean	SD	SE	t-value	P-value
NK cells (% parent)	Tobacco with lesion	13	3.90	3.80	1.05	0.3199	0.7520
	Tobacco without lesion	12	3.46	2.92	0.84		
CD16+CD56+ (% Parent)	Tobacco with lesion	13	25.75	16.09	4.46	0.7272	0.4745
	Tobacco without lesion	12	20.31	21.16	6.11		
CD56+ (% Parent)	Tobacco with lesion	13	39.94	16.50	4.575	1.1260	0.2718
	Tobacco without lesion	12	31.38	21.40	6.177		
CD16+ (% Parent)	Tobacco with lesion	13	17.01	17.12	4.75	-0.5857	0.5638
	Tobacco without lesion	12	21.84	23.80	6.87		

Bar graph 5: Bar graph shows comparison of Tobacco with lesion group, Tobacco without lesion group among tobacco chewers with all parameters



When compared the NK cells (% parent) between tobacco users with or without lesion, the mean was slightly higher in tobacco users with lesions (3.90 ± 3.80) compared to those without lesions (3.46 ± 2.92), though this difference was not statistically significant ($t = 0.3199$, $p = 0.7520$).

In terms of $CD16^{+}CD56^{+}$ (% parent) cells, individuals with lesions also showed a higher mean value (25.75 ± 16.09) than those without lesions (20.31 ± 21.16), but again, this difference did not reach statistical significance ($t = 0.7272$, $p = 0.4745$).

The mean percentage of $CD56^{+}$ cells was 39.94 ± 16.50 in the lesion group and 31.38 ± 21.40 in the non-lesion group. Although the lesion group had a noticeably higher mean, the result was not statistically significant ($t = 1.1260$, $p = 0.2718$).

Interestingly, the mean value of $CD16^+$ cells was slightly lower in tobacco users with lesions (17.01 ± 17.12) compared to those without lesions (21.84 ± 23.80), but this trend was also not significant (t = -0.5857, p = 0.5638).

Overall, although individuals with lesions showed numerical differences in NK cell markers — with trends toward higher $CD56^+$ and $CD16^+CD56^+$ percentages — none of these findings were statistically significant.

DISCUSSION

During tumor immunosurveillance, the immune system can either suppress or stimulate immune responses. By identifying tumor-specific antigens, the host's immune system is capable of identifying and eradicating the developing tumour cells. Similar to homograft rejection, immunosurveillance helps in maintaining tissue equilibrium in complex organisms. But the tumor cells often adopt various mechanisms to evade detection, including the release of immune-modulating substances. The key players in this defense are the cytotoxic T lymphocytes and NK cells, which directly destroy cancer cells and are vital in both preventing and eradicating tumor development.[60]

NK cells are the bone marrow derived, cytotoxic innate lymphocytes classified as large granular lymphocytes (LGLs). They are the part of innate lymphoid cell (ILC) family, known for their ability to recognize and destroy virus-infected and tumor cells without prior sensitization, comprising about 5–20% of human circulating lymphocytes.[5] Natural Killer (NK) cells detect abnormal cells by integrating signals like IL-12, IL-15, IL-18 and balancing activating vs. inhibitory receptors, especially those interacting with MHC-I. [63]During infection and/or inflammation, NK cells are activated rapidly, proliferated quickly, and it contributes significantly to innate and adaptive immunity.[8,66] Despite their innate nature and the lack of precise target recognition, NK cells depends on supporting cells like dendritic cells (DCs) for activation (e.g., IL-15 trans-presentation) and monocytes expressing MICA, which enhances their anti-tumor activity through Fc receptors.[61] Also, NK cells can also develop immunological memory, known as trained immunity — a feature once thought exclusive to adaptive immune cells.

Repeated inflammatory exposure in humans can induce the change of conventional NK cells into memory-like NK (NKm) cells, which exhibit boosted responses, including increased production of IFN-γ, perforin, and granzymes, particularly within tumor microenvironments. However, chronic stimulation through tumor-expressed ligands such as NKG2D can lead to NK cell dysfunction and will contribute to tumor immune evasion and metastasis (Cerwenka & Lanier, 2016).[69]

Overall, NK cell action and function is maintained via interaction of activating and inhibitory signals. While tumor cells may evade CTLs by lowering MHC-I levels, this simultaneously diminishes inhibitory signaling to NK cells, resulting in enhanced activation and cytotoxicity. By identifying and destroying tumor cells that evade T cell-mediated immune responses, mature NK cells play a critical role in tumor immune surveillance and act as an essential defense.(Wu et al., 2020; Anfossi et al., 2006)[64,65]

Smokeless tobacco**,** one of the major risk factors for oral cancer, has been found to impair NK cell-mediated tumor immune surveillance**.** Chronic exposure to tobacco-specific nitrosamines and other immunosuppressive agents present in tobacco may reduce NK cell cytotoxic activity, thereby compromising anti-tumor immunity and contributing to a higher tumor burden.[10]

Given the critical role of NK Cells in tumor immunosurveillance and antitumor responses, smokeless tobacco use may alter their immunoprofile, potentially disrupting their functional balance and leading to abnormal activation, migration, or cytotoxic activity—ultimately allowing tumor cells to evade immune detection. Due to the paucity of research in this area, the present study aims to assess the peripheral NK cell immunoprofile among healthy individuals, tobacco chewers, and patients with oral squamous cell carcinoma using flow cytometry.

To assess NK cell phenotypes in peripheral blood, immunophenotyping was performed using flow cytometry**.** PBMCs were isolated from the freshly obtained blood sample and was stained with fluorochrome-conjugated monoclonal antibodies targeting specific surface markers—CD3 (FITC) to exclude T cells, CD56 (PE) to identify NK cells, and CD16 (APC) to evaluate cytotoxic activity. A Beckman Coulter DxFlex flow cytometer equipped with multiple lasers and compensation controls was used for accurate multiparametric analysis. The gating strategy involved excluding doublets and dead cells, followed by identifying NK cells as $CD3^{-}CD56^{+}$ and $CD3^{-}CD16^{+}$**,** and further subclassifying them into $CD56^{+}CD16^{-}$**,** $CD56^{+}CD16^{+}$**,** and $CD16^{+}$ only subsets. This allowed for a comprehensive evaluation of both the regulatory (cytokine-producing) and cytotoxic compartments of NK cells across study groups. The data acquired provided the basis for assessing potential shifts in NK cell distribution due to tobacco exposure and malignant transformation.

The present study included 75 participants divided into three groups with 25 healthy individuals, 25 tobacco chewers and 25 OSCC patients, these three groups were matched for age and sex. All the individuals were in 4^{th} decade of life, with slight increase in age of OSCC patents. This age-related shift line up with existing literature suggesting that increasing age is a risk factor for immune dysregulation and cancer susceptibility, particularly among long-term tobacco users (Gupta et al., 2017)[84]. Also, a male predominance was noted in the tobacco-exposed groups and OSCC patients accounting for 80% in the OSCC group and over 60% in chewers simulating findings from other Indian cohorts where sociocultural patterns often associate with higher tobacco use by males (Kumar et al., 2016; Ranganathan et al., 2019).[85,86]

More than 60% of chewers and OSCC patients reported the use of tobacco product in chewed form. This is in line with reports from Southeast Asia, where smokeless tobacco remains a major etiological factor for oral lesions (Asthana et al., 2019).[87]

The type and duration of oral lesions emerged as relevant factors potentially linked to immune alterations, particularly in NK cell dynamics. In the present study, among tobacco chewers with lesions, leukoplakia was the most prevalent type (53.8%), followed by tobacco pouch keratosis (23%), erythroplakia (15.3%), and oral submucous fibrosis (OSMF) (7.6%). These patterns mimic with epidemiological data from South Asia, where leukoplakia remains the most common potentially malignant disorder associated with smokeless tobacco use (Warnakulasuriya et al., 2020). Majority of individuals in the present study with such lesions had a lesion duration of less than 4 years (30%), compared to 80% in OSCC patients, whose lesions had persisted for over four years. This increase in lesion chronicity could be a sign of both protracted immunological dysregulation, including potential NK cell exhaustion over time, and delayed clinical presentation. Long-standing oral lesions are frequently linked to decreased NK cell cytotoxicity and changed cytokine profiles, which may facilitate immune escape pathways during malignant transformation, according to studies done by Rivera et al. (2017) and Gupta et al. (2019).[82,83] These findings emphasizes the necessity of taking chronicity and lesion type into account as extra immunological risk factors in oral cancer surveillance.

As we see the lesion distribution, the buccal mucosa emerged as the most common site for lesions in both precancer and cancer groups (76.9% and 56%, respectively), which could be due to direct and prolonged contact with the tobacco quid. The results of Mohit Sharma et al. (2020)[89], who have highlighted the buccal mucosa as a main location of early dysplastic alterations in SLT users, are consistent

with this site-specific trend. These demographic findings highlight the significance of exposure site and habit intensity in influencing both local and systemic immune responses, in addition to reflecting the typical high-risk group impacted by oral potentially malignant disorders and OSCC.

In our study, the percentage of NK cells among healthy individuals ranged from 0.19% to 12.24%, with the average tending slightly lower than some previously published studies. The broad range—especially the lower limit—may reflect the underlying population-specific factors, such as genetic background, environmental exposures, or lifestyle habits.

Study by, Apoil et al. (2017) [75]documented NK cell percentages ranging from 1.1% to 17.7%, with a mean value of 6.4%, while Angelo et al. (2015)[76] reported a range between 0.61% and 16.87%, with a mean of 6.47% in healthy adults. The lower end observed in our study may also be explained by natural temporal fluctuations in immune status, as highlighted by Angelo et al., who found that even within the same individuals, NK cell percentages could vary between 2% and 11% across several months.

Similar observations were seen in, major laboratory databases such as ARUP Laboratories have set the NK cell reference range at 4% to 26% of total lymphocytes, with absolute counts falling between 78 and 470 cells/μL. Labcorp similarly lists a reference percentage range of 1% to 19%. In a separate clinical study, Dendle et al. (2019)[77]found NK cell percentages in healthy individuals between 7% and 28%, also including functional assessments such as cytotoxic activity, which serve as an important benchmark for evaluating immune competence. Moreover, findings from Wolfgang Merkt et al. (2021)[90] demonstrated a wide spectrum in NK cell counts, ranging from 82 to 594 cells/μL, with an average of 253 cells/μL, emphasizing that even within healthy populations, NK cell distributions can vary considerably.

These comparative insights underline the intrinsic variability of NK cell levels among healthy individuals. They also highlight the value of establishing local, population-specific baseline values, which are critical for detecting subtle immune deviations in clinical settings—particularly in conditions such as chronic tobacco use or oral squamous cell carcinoma, where immune surveillance by NK cells plays a key role.

In the current study, the NK cells percentage in individuals who used smokeless tobacco ranged from 0.35% to 12.88%, with a mean of 3.69%. This average is slightly lower than that observed in the normal healthy group of our study, which had a mean NK cell percentage of 3.85%. Although the difference appears minimal, it may hold biological significance and reflects a trend reported in previous studies indicating immunomodulatory effects of smokeless tobacco products.

Tobacco, particularly in its smokeless form, contains several bioactive compounds, including nicotine and tobacco-specific nitrosamines, which are known to interfere with immune function. Bagaitkar et al. (2013)[73] showed that the exposure to smokeless tobacco extracts suppresses the expression of key cytokines such as TNF-α and IFN-γ in NK cells and impairs their cytolytic activity. These impairments may not necessarily reflect sudden reductions in NK cell percentages but do also indicate a suppression of immune surveillance process in tobacco users. In parallel, Malovichko et al. (2019)[74] reported that prolonged exposure to smokeless tobacco in murine models led to a significant reduction in circulating immune cells, including NK cells, and a decrease in plasma IFN-γ levels, suggesting a state of chronic immune suppression.

More recent evidence has proposed that nicotine can directly impair NK cell function by binding to nicotinic acetylcholine receptors (nAChRs)—specifically the

β2 subunit—on NK cells. This interaction has been shown to disrupt cytotoxic functions and enhance tumor progression in experimental models (Zhang et al., 2013)[80]. Although the precise mechanism in humans remains to be fully defined, it raises the possibility that long term nicotine exposure from smokeless tobacco may downregulate NK cell activity through receptor-mediated pathways.

The duration and frequency of tobacco chewing appear to have a crucial role in modulating NK cell dynamics**.** Prolonged and repeated exposure to smokeless tobacco constituents—like nicotine**,** tobacco-specific nitrosamines**,** and reactive oxygen species—can lead to the gradual suppression of NK cell-mediated immune surveillance. In the present study, individuals with longer chewing histories (21–30 years and beyond) and higher usage frequency (four to six times daily) demonstrated a trend toward reduced NK cell percentages, particularly within the cytotoxic $CD56^{+}CD16^{+}$ subset**.** This is in consistent with findings that chronic antigenic stimulation from tobacco use habit may induce NK cell exhaustion**,** functional impairment**, or** downregulation of receptor, thereby weakening early immune responses aiding to dysplastic transformation and tumorigenesis. Jung et al. (2020)[91] reported significantly reduced NK cell activity among chronic smokers, with greater suppression correlating with longer duration and higher quantity of tobacco use. Similarly, our demographic analysis revealed that most tobacco users in the study reported high-frequency chewing (four to six times daily), with 58% of chewers without lesions and 40% of OSCC patients falling into the 21–30 year habit duration group. These findings line up with the results of Jain et al. (2018)[88]**,** who demonstrated that prolonged, high-frequency tobacco exposure was associated with both mucosal alterations and systemic immune modulation**,** further confirming

tobacco's function as a chronic immunological stressor that could lead to oral cancer and immune suppression.

These results provide validity to the theory that, even in the absence of obvious disease, smokeless tobacco may have immunosuppressive effects on NK cells. Although our study's decrease in NK cell percentage might seem lesser, the functional deficiencies that go along with it, as indicated by previous research, may lower tumor immunosurveillance and make chronic users more vulnerable to infections or precancerous alterations. Further research is warranted to explore not only the quantitative shifts but also the functional competency of NK cells, particularly their cytokine production, degranulation capacity, and receptor expression profiles, in the context of SLT exposure.

In the current study, the percentage of NK cells in OSCC patients ranged from 0.71% to 20.6%**,** with a mean value of 4.57%**.** This average was notably higher than the NK cell mean percentages observed in both healthy individuals (mean: 3.85%) and smokeless tobacco chewers (mean: 3.69%), suggesting a trend of increased NK cell representation in the peripheral immune profile of OSCC patients**.** This increase can be the result of a complicated immunological response to immune surveillance dynamics, tumor-associated inflammation, and malignant transformation.

The increase in NK cell count may be attributed to a reactive immune surveillance response, where immune system attempts to recognize and eliminate tumour cells. NK cells, as innate cytotoxic lymphocytes, play an important role in early tumor immunosurveillance by releasing interferon-gamma (IFN-γ) and directly killing target cells through perforin-dependent cytotoxicity (Agarwal et al., 2016).[94]

As tumor progress, the tumor microenvironment (TME) becomes a crucial modulator of immune cell recruitment and function. John et al. (2024)[93] showed a significantly higher expression of $CD57^+$ NK cells in OSCC tissues compared to oral epithelial dysplasia (OED) and normal mucosa. The mean index of $CD57^+$ cells increased progressively from 3.21 in normal mucosa**,** to 8.40 in OED, and increased at 12.04 in OSCC, with this difference being statistically significant ($p = 0.01$). This supports the notion that NK cell infiltration increases with disease severity and could serve as a surrogate marker for malignant transformation.

However, while increased NK cell counts may suggest an activated immune state, their functional integrity is often compromised in the TME. According to Devillier et al. (2021)[92], NK cells frequently encounter metabolic and phenotypic remodeling in cancer patients, which results in functional exhaustion. Tumor-derived immunosuppressive agents such as TGF-β, IL-10, and PGE2 downregulate activating receptors like NKG2D, NKp30, NKp46, and impair NK cell-mediated cytotoxicity.

From a prognostic standpoint, higher NK cell infiltration in OSCC has been linked with improved survival outcomes. Agarwal et al. (2016)[94] reported that patients with higher $CD57^+$ NK cell labeling index had better three year survival as compared to those with lower indices. Specifically, the mean CD57 labeling index was 10.67 in surviving patients versus 3.67 in those who had died of the disease. This significant correlation ($p < 0.0001$) underscores the prognostic value of NK cells in OSCC and supports their use as potential biomarkers.

Similarly, John et al. (2024)[93] concluded that both CD57 (NK cell) and CD8 (cytotoxic T-cell) expression increased progressively from normal mucosa to OED to OSCC, and suggested that these markers could aid in assessing the malignant potential of oral lesions.

Higher percentage of NK cells seen in OSCC patients in this study, most likely reflects two realities either a tumor environment that is working to suppress or neutralize the immune response, and an immune system that is actively trying to control tumor growth. Even if there may be an increase in NK cells count, their functional integrity is frequently damaged, which emphasizes the significance of a thorough and perceptive assessment that takes into account both quantitative abundance and qualitative efficacy.

These results support the theory that NK cell enumeration, in conjunction with functional analysis, might provide important insights into the immunological landscape of OSCC and its progression. Future work should aim to combine phenotypic, functional, and spatial data to improve therapeutic targeting of NK cells in oral cancer

In the present study, natural killer (NK) cells were immunophenotypically categorized according to their surface marker expression—$CD56^+$, $CD16^+$ and $CD56^+CD16^+$—to understand their functional distribution among healthy individuals, tobacco chewers (with and without lesions), and oral squamous cell carcinoma (OSCC) patients. These markers are essential in identifying distinct NK cell subsets with different immunological roles.

$CD56^+$ NK cells, particularly those in the $CD56^+CD16^-$ subset, are known to secrete cytokines such as IFN-γ ,TNF-α which are predominantly immunoregulatory rather than cytotoxic. In our study, $CD56^+$ NK cells in healthy individuals ranged from 15.38% to 75%, with a mean value of 36.74%. This subset showed a mean of 35.83% in tobacco chewers and 39.16% in OSCC patients. Although the increase in OSCC cases was not statistically significant, the trend indicates a possible enrichment of $CD56^+$ cells in response to chronic inflammation or tumor presence. Literature by Cooper et al. (2001)[45] and Romee et al. (2013)[67] supports this pattern, where $CD56^+$

NK cells are often more prevalent in inflammatory environments and function through cytokine-mediated immune regulation.

$CD16^+$ NK cells, characterized by their expression of FcγRIIIa, play a pivotal role in antibody-dependent cellular cytotoxicity (ADCC). In our analysis, the mean percentage of $CD16^+$ NK cells was 24.64% in healthy individuals, 19.33% in tobacco users, and 22.68% in OSCC patients. The reduced CD16 expression among tobacco chewers may suggest early immune modulation or downregulation of cytotoxic NK function due to chronic tobacco exposure. This observation is in line with findings by Bagaitkar et al. (2013)[73], who reported suppressed NK cell cytotoxicity and IFN-γ expression in individuals exposed to tobacco, likely mediated by oxidative stress and immunosuppressive signaling pathways. Interestingly, although $CD16^+$ NK cells appeared to recover slightly in OSCC patients, their functional activity may be impaired within the immunosuppressive tumor microenvironment, as evidenced in studies by Devillier et al. (2021)[92].

The $CD56^+CD16^+$ phenotype predominantly represents the CD56+CD16+ NK cell subset, which constitutes the majority of peripheral blood NK cells and exhibits potent cytotoxic capabilities. In our study, the mean percentage of $CD56^+CD16^+$ NK cells was 28.26% in the normal group, 23.14% among tobacco chewers, and 21.12% in OSCC patients. This downward trend may indicate a gradual depletion or dysfunction of the cytotoxic NK cell population with increasing disease burden. Although these differences were not statistically significant, they suggest a shift from functional cytotoxic subsets toward either exhausted or suppressed phenotypes in the context of chronic tobacco exposure and malignancy. This observation resonates with the findings of Zhou et al. (2022), who reported altered NK cell phenotype and reduced expression of cytotoxic receptors such as NKG2D and NKp30 in OSCC

patients. Additional research by Agarwal et al. (2016) [94]and John et al. (2024)[93] demonstrated that although NK cell infiltration or peripheral levels may increase in OSCC, their tumor-suppressive capacity is often compromised due to immune escape mechanisms employed by tumor cells.

Furthermore, when comparing tobacco chewers with and without lesions, the mean levels of $CD56^+$, $CD16^+$, and $CD56^+CD16^+$ NK cells were higher in tobacco chewers with lesion group, although not statistically significant. This trend might reflect an early immune response to premalignant transformation, which, over time, becomes increasingly dysfunctional or suppressed as lesions progress toward malignancy. Despite the lack of statistical significance across groups, the biological implications of these trends are noteworthy. A gradual loss of cytotoxic NK subsets and possible enrichment of immunoregulatory or exhausted NK cells could contribute to impaired immune surveillance in OSCC pathogenesis.

In summary, the study reveals subtle but potentially meaningful differences in NK cell functional phenotypes among healthy individuals, tobacco chewers, and OSCC patients. While $CD56^+$ cells tend to increase in OSCC, the $CD16^+$ and $CD56^+CD16^+$ cytotoxic subsets show a declining trend. These patterns reflect an evolving immune response from regulation to dysfunction during carcinogenesis. The findings are consistent with existing literature and underscore the importance of evaluating both quantitative and functional aspects of NK cells in disease progression. A mere increase in NK cell counts does not necessarily indicate effective immune function; rather, a comprehensive understanding of their activation status, receptor expression, and cytotoxic potential is essential. Future scope of this study will be to integrate these immunophenotypic profiles with functional assays to better elucidate the title role of NK cells in oral cancer immunity.

SUMMARY AND CONCLUSION

Present study provides valuable insights into the immunoprofiling of NKCs and their phenotypes in health, tobacco exposure, and oral squamous cell carcinoma. While the total percentage of NK cells do not show statistical significance among the study groups, subtle variations in phenotypic subsets were observed. The $CD56^+$ subset showed a mild increase in OSCC patients, possibly reflecting a regulatory immune response to tumor development. In contrast, the cytotoxic $CD16^+$ and $CD56^+CD16^+$ populations showed a decreasing trend from healthy individuals to tobacco chewers and further to OSCC patients, indicating a gradual impairment in cytotoxic immune surveillance. Although statistically non-significant, these trends align with established mechanisms of immune exhaustion and suppression in tumor microenvironments. Tobacco chewers, especially those with lesions, showed intermediate alterations, suggesting early immunomodulatory effects of tobacco even before malignant transformation. Overall, the study underscores the importance of not only quantifying NK cells but also examining their functional phenotypes to understand their evolving roles in oral carcinogenesis. These findings support the potential of NK cell immunoprofiling as a biomarker in oral precancer and cancer, and emphasize the need for region-specific reference values in immune evaluation.

LIMITATIONS

- The sample size for each group, particularly tobacco chewers with and without lesions, was relatively small, which may have limited the statistical power to detect subtle but biologically important differences.
- The study focused exclusively on surface marker expression and did not assess NK cell functionality through assays such as degranulation, cytotoxicity, or cytokine production. This limits the interpretation of whether observed shifts in phenotype translated into functional impairment or activation.
- External variables such as nutritional status, systemic inflammation, infections, and circadian rhythm—factors known to influence immune parameters—were not controlled in this study.
- Additionally, the cross-sectional nature of the study precludes causal interpretations of the changes.

FUTURE SCOPE

- To include larger and more diverse sample populations to validate and expand upon these findings.
- Incorporating functional assays, such as CD107a degranulation, intracellular IFN-γ staining, and target cell lysis assays, would provide a more comprehensive understanding of NK cell activity across the disease spectrum.
- Longitudinal studies tracking immune changes from healthy tobacco users to lesion development and malignant transformation would offer invaluable insights into the temporal dynamics of immune escape.
- Integrating NK cell profiling with additional immune markers, such as T-cell subsets, dendritic cells, and checkpoint inhibitors (e.g., PD-1, TIGIT), could yield a broader perspective on the immune landscape in oral carcinogenesis.
- Use of tissue-based techniques, such as immunohistochemistry or immunofluorescence, to correlate peripheral immune changes with local tumor microenvironmental alterations.
- Such integrative approaches would enhance the clinical utility of NK cell profiling in early diagnosis, prognosis, and potentially, therapeutic targeting in OSCC.

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ANNEXURE 1

BIOSTATISTICS CERTIFICATE

K L E
VISHWANATH KATTI
INSTITUTE OF DENTAL SCIENCES
A Constituent college of
K.L.E. Academy of Higher Education and Research
J.N.M.C. Campus, Nehru Nagar Belagavi -590010 Karnataka, India.
Department of Oral & Maxillofacial Pathology and Oral Microbiology

BIOSTATISTICS CLEARANCE CERTIFICATE

This is to certify that the Biostatistics art of Dissertation/ Research work of **IH0222002** **Postgraduate student** under the guidance of **Professor, Department of Oral & Maxillofacial Pathology and Oral Microbiology** entitled "Immunoprofiling of Natural Killer cells in Normal individuals, Tobacco chewers and patients with Oral Squamous Cell Carcinoma- A flow cytometry study" has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 15.4.2025

Name and signature of Biostatistician
Dr. S. B. Javali. Ph.D.
Professor In Statistics
Department of Community Medicine
USM KLE International Medical Programmes,
BELAGAVI-590010.

ANNEXURE 2

ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
KLE VK INSTITUTE OF DENTAL SCIENCES
A Constituent Unit of KLE Academy of Higher Education & Research
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362 Web: http://www.kledental-bgm.edu.in
FAX: 0831-2470640 E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1661

CERTIFICATE

This is to Certify that the synopsis titled

Immunoprofiling of Natural Killer cell in normal individuals, tobacco chewers and patients with oral squamous cell carcinoma - A flow cytometry study

Submitted by

Dr. **IH0222002** *P. G. Student /*

Staff, Guided by *from Department of*

Oral Pathology and Oral Microbiology *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 16/11/85

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belgaum

ANNEXURE -3

CONSENT FORM

KAHER's KLE VISHWANATH KATTI INSTITUTE OF DENTAL SCIENCES, BELAGAVI

DEPARTMENT OF ORAL AND MAXILLOFACIAL PATHOLOGY AND ORAL MICROBIOLOGY

TITLE: IMMUNOPROFILING OF NATURAL KILLER CELLS IN NORMAL INDIVIDUALS, TOBACCO CHEWERS, AND PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA: A FLOW CYTOMETRY STUDY

I, _____ have been informed about the involvement in the study, aged _____ *(in years)* in the study in the language that I can understand.

I agree to give my personal details like name, age, sex, address, previous dental history, and the details required for the study to the best of my knowledge.

STUDY PROCEDURE INCLUDES :5 ml of venous blood will be collected by using a routine venipuncture method and stored in vials containing EDTA. The blood samples will be processed in a flow cytometer and the immunoprofile evaluation of peripheral natural killer cells will be done.

I will cooperate with the investigator for intra-oral and extra-oral examination. I will follow the instructions given by the investigator during the study.

I have understood the nature of the study and permitting the investigator to collect blood samples and store the blood samples for further investigation.

I permit the investigator to utilize the information given by me and the results obtained from this study for presentation and publication.

If for any reason I am unable to participate in the study, for reasons unknown, I can withdraw from the study.

I have read, gone through and understood the above information given by the investigator about the study.

I have agreed and signed this application.

Participants name :

Address :

Phone no. :

Participant Signature **Witness Signature** **Investigator Signature**

ಕಾಹೆರ್ಸ್ ಕೆಎಲ್ಇ ವಿಶ್ವನಾಥ ಕಟ್ಟಿ ದಂತ ವಿಜ್ಞಾನ ಸಂಸ್ಥೆ, ಬೆಳಗಾವಿ

ಮೌಖಿಕ ಮತ್ತು ಮ್ಯಾಕ್ಸಿಲೊಫೇಶಿಯಲ್ ರೋಗಶಾಸ್ತ್ರ ಮತ್ತು ಮೌಖಿಕ ಸೂಕ್ಷ್ಮ ಜೀವವಿಜ್ಞಾನ ವಿಭಾಗ

ಒಪ್ಪಿಗೆ ಪತ್ರ

ಶೀರ್ಷಿಕೆ: ಸಾಮಾನ್ಯ ವ್ಯಕ್ತಿಗಳು , ತಂಬಾಕು ಜಗಿಯುವವರು ಮತ್ತು ಬಾಯಿಯ ಸ್ಕ್ಯಾಮಸ್ ಸೆಲ್ ಕಾರ್ಸಿನೋಮಾ ಹೊಂದಿರುವ ರೋಗಿಗಳಲ್ಲಿ ನೈಸರ್ಗಿಕ ಕೊಲೆಗಾರ ಕೋಶಗಳ ಇಮ್ಯುನೊಫೆನೊಟೈಪಿಂಗ್: ಒಂದು ಫ್ಲೋ ಸೈಟೋಮೆಟ್ರಿ ಅಧ್ಯಯನ

ನಾನು _____ ಅರ್ಥಮಾಡಿಕೊಳ್ಳಬಹುದಾದ ಭಾಷೆಯಲ್ಲಿ ಅಧ್ಯಯನದಲ್ಲಿ ವಯಸ್ಸಾದ *(ವರ್ಷಗಳಲ್ಲಿ)*_____ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯ ಬಗ್ಗೆ ನನಗೆ ತಿಳಿಸಲಾಗಿದೆ.

ಹೆಸರು, ವಯಸ್ಸು, ಲಿಂಗ, ವಿಳಾಸ, ಹಿಂದಿನ ದಂತ ಇತಿಹಾಸ, ಮತ್ತು ಅಧ್ಯಯನಕ್ಕೆ ಅಗತ್ಯವಾದ ವಿವರಗಳಂತಹ ನನ್ನ ವೈಯಕ್ತಿಕ ವಿವರಗಳನ್ನು ನನಗೆ ತಿಳಿದ ಮಟ್ಟಿಗೆ ನೀಡಲು ನಾನು ಒಪ್ಪುತ್ತೇನೆ.

ಅಧ್ಯಯನ ಕಾರ್ಯವಿಧಾನವು ಒಳಗೊಂಡಿದೆ: ವಾಡಿಕೆಯ ವೆನಿಪಂಕ್ಚರ್ ವಿಧಾನವನ್ನು ಬಳಸಿಕೊಂಡು 5 ಮಿಲಿ ರಕ್ತನಾಳದ ರಕ್ತವನ್ನು ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ ಮತ್ತು ಇಡಿಟಿಎ ಹೊಂದಿರುವ ಬಾಟಲಿಗಳಲ್ಲಿ ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ. ರಕ್ತದ ಮಾದರಿಗಳನ್ನು ಫ್ಲೋ ಸೈಟೋಮೀಟರ್ ನಲ್ಲಿ ಸಂಸ್ಕರಿಸಲಾಗುವುದು ಮತ್ತು ಬಾಹ್ಯ ನೈಸರ್ಗಿಕ ಕೊಲೆಗಾರ ಕೋಶಗಳ ರೋಗನಿರೋಧಕ *ಫಿನೊಟೈಪ್ ಮೌಲ್ಯಮಾಪನವನ್ನು ಮಾಡಲಾಗುತ್ತದೆ.*

ಅಂತರ್-ಮೌಖಿಕ ಮತ್ತು ಬಾಹ್ಯ-ಮೌಖಿಕ ಪರೀಕ್ಷೆಗೆ ನಾನು ಪರಿಶೋಧಕರೊಂದಿಗೆ ಸಹಕರಿಸುತ್ತೇನೆ. ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ಪರಿಶೋಧಕರು ನೀಡುವ ಸೂಚನೆಗಳನ್ನು ನಾನು ಅನುಸರಿಸುತ್ತೇನೆ.

ನಾನು ಅಧ್ಯಯನದ ಸ್ವರೂಪವನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ ಮತ್ತು ರಕ್ತದ ಮಾದರಿಗಳನ್ನು ಸಂಗ್ರಹಿಸಲು ಮತ್ತು ಹೆಚ್ಚಿನ ತನಿಖೆಗಾಗಿ ರಕ್ತದ ಮಾದರಿಗಳನ್ನು ಸಂಗ್ರಹಿಸಲು ಪರಿಶೋಧಕರಿಗೆ ಅನುಮತಿ ನೀಡಿದ್ದೇನೆ.

ನಾನು ನೀಡಿದ ಮಾಹಿತಿ ಮತ್ತು ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ಫಲಿತಾಂಶಗಳನ್ನು ಪ್ರಸ್ತುತಿ ಮತ್ತು ಪ್ರಕಟಣೆಗಾಗಿ ಬಳಸಿಕೊಳ್ಳಲು ನಾನು ಪರಿಶೋಧಕರಿಗೆ ಅನುಮತಿಸುತ್ತೇನೆ.

ಯಾವುದೇ ಕಾರಣದಿಂದ ನನಗೆ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಸಾಧ್ಯವಾಗದಿದ್ದರೆ, ಅಜ್ಞಾತ ಕಾರಣಗಳಿಂದಾಗಿ, ನಾನು ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯಬಹುದು.

ಅಧ್ಯಯನದ ಬಗ್ಗೆ ಪರಿಶೋಧಕರು ನೀಡಿದ ಮೇಲಿನ ಮಾಹಿತಿಯನ್ನು ನಾನು ಓದಿದ್ದೇನೆ, ಓದಿದ್ದೇನೆ ಮತ್ತು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

ನಾನು ಈ ಅರ್ಜಿಯನ್ನು ಒಪ್ಪಿದ್ದೇನೆ ಮತ್ತು ಸಹಿ ಹಾಕಿದ್ದೇನೆ.

ಭಾಗವಹಿಸುವವರ ಹೆಸರು:

ವಿಳಾಸ:

ಫೋನ್ ಸಂಖ್ಯೆ.:

ಭಾಗವಹಿಸುವವರ ಸಹಿ **ಸಾಕ್ಷಿ ಸಹಿ** **ವೈದ್ಯರ ಸಹಿ**

कहार के क्ले विश्वनाथ कट्टी दंत चिकित्सा विज्ञान संस्थान, बे□गावी

ओर□ और मक्सिि□ोफेशिय□ पथो□ॉजी और ओर□ माइक्रोबायो□ॉजी विभाग

सहमति पत्र

शीर्षक: तम्बाकू चबाने वा□ों, गु-चबाने वा□ों और मौखिक स्क्मस से□ कार्सिनोमा वा□ रोगियों में प्राकृतिक हत्यारे कोशिकाओं की इम्यूनोप्रोफि□िंग: एक प्रवाह साइटोमेट्री □ ध्यन

मुझे,_____ अध्ययन में शामिल होने के बारे में सूचित किया गया है, मेरी उम्र_____ (वर्षों में) उस भाषा में है जिसे मैं समझ सकता हूँ।

मैं अपने व्यक्तिगत विवरण जैसे नाम, आयु, लि□, पता, पिछला दं इतिहास और अध्ययन के लिए आवश्यक विवरण अपनी जानकारी के अनुसार देने के लिए सहमत हू□

□ ध्ययन प्रक्रिया में शामि□ हैं: नियमित वेनिपं चर विधि का उपयोग करके 5 मिलीलीटर शिरापरक रक्त एकत्र किया जाएगा और ईडीटीए युक्त शीशियो□ें सं हित किया जाएगा। रक्त के नमूनो□ को एक फ्लो साइटोमीटर में सं ाधित किया जाएगा और परिधीय प्राकृतिक किलर कोशिकाओ□ का इम्यूनोप्रोफाइल मूल्यां न किया जाएगा।

मैं इ ा-ओरल और एक्स्ट्रा-ओरल परीक्षा के लिए अन्वेषक के साथ सहयोग कर गा। मैं अध्ययन के दौरान अन्वेषक द्वारा दिए गए निर्देशो□ का पालन करूँगा।

मैंने अध्ययन की प्रकृति को समझ लिया है और अन्वेषक को आगे की जां के लिए रक्त के नमूने एकत्र करने और रक्त के नमूनो□ को सं हीत करने की अनुमति दे दी है।

मैं अन्वेषक को मेरे द्वारा दी गई जानकारी और इस अध्ययन से प्राप्त परिणामो□ को प्रस्तुति और प्रकाशन के लिए उपयोग करने की अनुमति देता हूँ।

यदि किसी कारण से मैं अध्ययन में भाग लेने में असमर्थ हूँ, अज्ञात कारणो□ से, मैं अध्ययन से हट सकता हूँ।

मैंने अध्ययन के बारे में अन्वेषक द्वारा दी गई उपरोक्त जानकारी को पढ़ लिया है, पढ़ लिया है और समझ लिया है।

मैंने इस आवेदन पर सहमति व्यक्त की है और हस्ताक्षर किए हैं।

प्रतिभागियों का नाम :

पता :

फोन नंबर:

प्रतिभागी हस्ताक्षर गवाह हस्ताक्षर □ न्वेषक हस्ताक्षर

काहेरची के विश्वनाथ कट्टी इन्स्टिट्यूट ऑफ डेंट सायन्सेस, बे गावी

ओर आणि मक्सिोफेशिय पथॉॅजी आणि ओर मायक्रोबायो ॅजी विभाग

संमती पत्र

शीर्षक: सामान्य व्यक्ती, तखाखू चेव्हर्स आणि तोडी स्क्रॅमस सेल कार्सिनोमा असलेल्या रूग्णामध्ये नैसर्गिक किलर पेशीचे इम्युनोप्रोफाइलिंग: एक फ्लो सायटोमेट्री अभ्यास

मी, _____ अभ्यासातील सहभागाबद्दल माहिती देण्यात आली आहे, युग _____ मला समजेल अशा भाषेतील अभ्यासात

नाव, वय, लिंग, पत्ता, पूर्वीचा दंत इतिहास आणि अभ्यासासाठी आवश्यक तपशील यासारखे माझे वैयक्तिक तपशील माझ्या माहितीप्रमाणे देण्यास मी सहमत आहे

अभ्यास प्रक्रियेचा समावेश : नियमित व्हेनिपंक्चर पद्धतीचा वापर करून 5 मिली शिरासंबंधी रक्त गोळा केले जाईल आणि ईडीटीए असलेल्या कुपीमध्ये संग्रहित केले जाईल. रक्ताच्या नमुन्यावर फ्लो सायटोमीटरमध्ये प्रक्रिया केली जाईल आणि *परिघीय नैसर्गिक किलर पेशीचे इम्मुनओप्रोफाइल* मूल्यांकन केले जाईल

- आंतर-तोडी आणि अतिरिक्त-तोडी तपासणीसाठी मी अन्वेषकाला सहकार्य करीन. अभ्यासादरम्यान अन्वेषकाने दिलेल्या सूचनांचे मी पालन करेन
- मी अभ्यासाचे स्वरूप समजून घेतले आहे आणि अन्वेषकाला रक्ताचे नमुने गोळा करण्याची आणि पुढील तपासणीसाठी रक्ताचे नमुने साठवण्याची परवानगी दिली आहे
- मी अन्वेषकाला मी दिलेली माहिती आणि या अभ्यासातून मिळालेले परिणाम सादरीकरण आणि प्रकाशनासाठी वापरण्याची परवानगी देतो
- जर कोणत्याही कारणास्तव मी अभ्यासात भाग घेऊ शकलो नाही, अज्ञात कारणास्तव, तर मी अभ्यासातून माघार घेऊ शकतो
- या अभ्यासाविषयी अन्वेषकाने दिलेली वरील माहिती मी वाचली आहे, पाहिली आहे आणि समजून घेतली आहे
- मी या अर्जावर सहमती दर्शविली आहे आणि स्वाक्षरी केली आहे

सहभागींची नावे :

पत्ता :

फोन नं. :

सहभागी स्वाक्षरी साक्षीदार स्वाक्षरी न्वेषक स्वाक

ANNEXURE 4

CASE HISTORY PERFORMA

KLE VISHWANATH KATTI INSTITUTE OF DENTAL SCIENCES,

BELAGAVI

DEMOGRAPHIC DETAILS:

Name: Case No.:

Age: OPD No.:

Sex:

Occupation:

Address & Contact No.:

HISTORY:

Chief Complaint:

History of present illness:

Family History:

Past medical/ drug history:

Past dental history:

Habit history:

Type (specify)	Smokeless	Smoked	Drinking Alcohol
Frequency / day			
Age of initiation			
Duration of Use			

CLINICAL EXAMINATION:

Extra-oral examination:

Asymmetry:

Swelling:

Ulcer:

Sinus opening:

Lymph node:

Mouth opening:

Intra-oral examination:

Buccal mucosa:

Palate:

Tongue:

Floor of mouth:

Lips:

DIAGNOSIS:

Description of the lesion/ tumor:

Differential diagnosis:

Provisional Diagnosis:

Biopsy no.:

Type of biopsy: Incisional/ Excisional

Histopathological Diagnosis: