MOLECULAR AND BIOCHEMICAL ASPECTS OF THE KALLIKREIN-KININ SYSTEM IN OESOPHAGEAL CARCINOMA.

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AUTHOR'S DECLARATION

This study represents original work by the author and it has not been submitted in any other university. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Experimental and Clinical Pharmacology, Faculty of Medicine, University of Natal, Durban, South Africa under the supervision of Professor K D Bhoola

SIGNED

Z. L. DLAMINI

I hereby certify that the above statement is correct.

SIGNED

PROF. K. D. BHOOLA

DEDICATION

I dedicate this work to:

The sources of my wisdom; my mother, Mrs. Victoria Dlamini, my late father, Mr. Michael Dlamini, the various teachers and lecturers who moulded my academic career and God.

Publications

Dlamini Z L, Raidoo D M and Bhoola K D (1999). Tissue kallikrein and kinin receptors in oesophageal carcinoma. *Immunopharmacology* 43(2-3): 303-310.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

SCC	Squamous cell carcinoma
MMP	Matrix metalloproteinaises
ST3	Stromelysin-3
Gel A	Gelatinase A
Gel B	Gelatinase B
MT1-MMP	Type 1 matrix metalloproteinase
MMP-2	Membrane metalloproteinase-2
MT4-MMP	Membrane type 4 matrix metalloproteinase
MMP-9	Matrix metalloproteinase-9
Cats	Cathepsins
CPs	Cystine proteinases
Cat L	Cathepsin L
Cat B	Cathepsin B
Cat D	Cathepsin D
PAs	Plasminogen activators
UPA	Urinary-type A plasminogen activator
tPA	Tissue-type plasminogen activator
PKC	Protein kinase C
NSCLC	Non-small-cell lung carcinoma

PMA	Phorbol-myristate acetate
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
KKS	Kallikrein-kinin system
TK	Tissue kallikrein
PK	Plasma kallikrein
kDA	Kilodalton
SMG	Submaxillary gland
Leu	Leucine
Met	Methionine
Lys	Lysine
Arg	Arginine
Cys	Cysteine
Gln	Glutamine
Gly	Glycine
Ser	Serine
Phe	Phenylalanine
Val	Valine
Ille	Isoleucine
Pro	Proline
COOH	Carboxyl group (acidic)
NH_2	Amino group
pI	Isoelectric point
KBP	Kallikrein binding protein
HK	High molecular weight kininogen

LK	Low molecular weight kininogen
PPK	Plasma prekallikrein
HF	Hageman factor
PMN	Polymorphonuclear
BK	Bradykinin
K_d	Dissociation constant
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
s	Seconds
nm	Nanometer
KI	Kininase I Carboxypeptidase
KII	Kininase II peptidylpeptidase
KI-CPN	Kininase I-Carboxypeptidase N
KI-CPM	Kininase I-Carboxypeptidase M
KII-ACE	Kininase II-Angiotensin I-Converting Enzyme
KII-NEP	Kininase II-Neutral Endopeptidase
NS	Normal saline
ISH	In situ hybridization
ICC	Immunocytochemistry
°C	Degees Celcius
H&E	Haematoxylin and Eosin Staining
RT	Room temperature
M	Molarity
PBS	Phosphate buffered saline

BSA	Bovine serum albumin
TRIS	(Tris[hydroxymethyl]aminomethane)
HCL	Hydrochloric acid
rpm	Rotors per minute
µ	Micron
SBTI	Soybean trypsin inhibitor
l	Litre
ml	Millilitre
ELISA	Enzyme-linked immunosorbent assay
ID1	Intracellular domain 1
ID2	Intracellular domain 2
EDE4	Extracellular domain 4
%	per cent
PAP	peroxidase antiperoxidase
IgG	Immunoglobulin
tproK	Tissue prokallikrein
Ag	Antigen
Ab	Antibody
pH	-log [Hydrogen ion]
1°	primary
2°	secondary
3°	tertiary
µm	micrometer

MeOH	Methanol
EtOH	Ethanol
DIG	Digoxigenin
DAB	Diaminobenzidine
H_2O	water
mM	millimolar
UV	ultraviolet
DEPC	Diethyl Pyrocarbonate
UTP	Uridine triphosphate
cRNA	copy Ribonucleic acid
cDNA	copy Deoxyribonucleic acid
µg	microgram
mRNA	messenger Ribonucleic acid
LiCl	Lithium chloride
EDTA	Ethylenediamine-tetraacetic acid
DTT	Dithiotreitol
h	hour
NBT	Nitroblue tetrazolium
BCIP	4-bromo-4-Chloro-3-Indolyl Phosphate
ON	overnight
AP	Alkaline Phosphatase
PFA	Paraformaldehyde
ηg	nanogram

HUK	Human urinary kallikrein
pNA	para-nitroaniline
PCR	Polymerase Chain Reaction

ABSTRACT

Introduction:

1. The gene expression of the serine protease, tissue kallikrein, has been determined mainly in epithelial cells derived from the entoderm as evidenced by the induction of this enzyme in pituitary adenomas, and lung, gastric and colonic tumours. Tissue kallikrein is expressed also in prostate and endometrial cancers (Clements & Mukhtar, 1997). Embryologically, the mucosal epithelial cells of the oesophagus are derived also from the entoderm.
2. Tissue kallikrein is a serine protease which in tumourigenesis may convert precursor proteinases and macromolecules into active molecules. In this regard evidence suggests that tissue kallikrein is important in the cascade formation of prostate specific antigen (PSA). Gene expression of tissue kallikrein, and the subsequent formation of the vasoactive kinin peptides could stimulate proliferation of tumour cells, and by increasing vascular permeability, enhance metastasis (Roberts, 1989). Therefore it was considered that tissue kallikrein may be implicated in the process of tumourigenesis both as a serine protease as well as through the cellular actions of bradykinin (Roberts & Gullick, 1989). The observation of increased kinin receptor expression due to oncogenic transformation (Roberts & Gullick, 1989) gives further credence to a mitogenic role for kinins in tumour tissue.

3. Proteinases such as serine proteases are secreted by cancer cells and are responsible for the proteolytic cascade triggered during malignant cell invasion (Bernitez-Brisbiesca *et al.,* 1995). A role for the tissue kallikrein in this regard has still to be researched. Hydrolysis of extracellular matrix is a necessary step for malignant cells to invade functionally normal adjacent tissue. Proteinases that show high activities in malignant tumour homogenates may be related to the degradation of the surrounding cell matrix (Tsuboi *et al.,* 1988).
4. Oesophageal carcinoma is the sixth most common cancer in the world and one of the most lethal tumours (Parkin *et al.,* 1988). Cancer of the oesophagus is the second most important cancer in South African men and the most important cancer in South African Black men, and therefore the current study attempted to correlate histopathological changes with the expression and holistic changes in the kallikrein kinin cascade. This research programme was unique to a primary ethnic group not studied previously in this manner
5. We believed that a comprehensive study involving the cellular localisation and molecular characterisation of the kinin system was necessary to be able to elucidate the role of tissue kallikrein and kinin receptors in oesophageal carcinoma, and provide insights into the role of this enzyme in cancer.

Aim: The aims of this study were:

1. To determine the cellular orientation of tissue kallikrein and the kinin B1 and B2 receptors in oesophageal carcinoma by immunocytochemistry (ICC)
2. To examine the expression of tissue kallikrein by *in situ* hybridisation (ISH) and reverse transcriptase-polymerase chain reaction (RT-PCR)

3. To examine the question whether holistic changes occur in patients with carcinoma by determining the concentration of renal tissue kallikrein (enzyme-linked immunosorbent assay), and its enzymic (amidase) and kininogenase (kinin formation) activities in the urine of three patient groups (oesophageal cancer, non-oesophageal cancer and non-cancer patients).

Methods:

1. Of the 50 oesophageal specimens 33 were biopsies and 17 resections. The control tissue (10 specimens) was tissue adjacent to tumour or normal oesophageal biopsies. Segments from these tissues were prepared for light immunocytochemistry. The antibodies were used to determine the cellular localisation of tissue kallikrein, and the kinin B1 and B2 receptors in normal and oesophageal specimens by standard immunohistochemical techniques. The intensity of immunolabelling was quantified by image analysis.
2. A cRNA probe specific for the KLK1 (tissue kallikrein gene) mRNA was used to determine the expression of tissue kallikrein by *in situ* hybridisation. Also RNA was extracted from fresh oesophageal resection and biopsy specimens, and RT-PCR was performed using gene specific primers for the tissue kallikrein.
3. The functional activity of renal tissue kallikrein was demonstrated by the amidase calorimetric assay in three patient groups and the kininogenase (kinin formation) was demonstrated by competitive ELISA. The total amount of renal tissue kallikrein present in three patient groups was measured in a sandwich enzyme-linked immunosorbent assay (ELISA).

Results and discussion:

1. In oesophageal carcinoma generally the highest intensity for immunoreactive tissue kallikrein, kinin B1 and B2 receptors was in activated mast cells, followed by giant tumour cells. The keratin pearls in oesophageal carcinoma showed the least intensity.
2. The expression of tissue kallikrein mRNA was found in both normal and tumour epithelium of the oesophageal specimens, indicating expression of tissue kallikrein in oesophageal cancer cells.
3. Analysis of renal tissue kallikrein activity: There was significant difference epidemiologically in the number of cases with family history, smokers and drinkers, eyes water and infected maize and beer of oesophageal carcinoma and non-cancer patients subgroups. Also, age distribution was statistically significant between oesophageal carcinoma and non-cancer patients groups Statistically there was no significant difference in the amidase and kininogenase activities, and tissue kallikrein ELISA between the three patient groups.

Conclusions:

1. Holistic changes in the secretion of renal tissue kallikrein was significantly co-related with sub-epidemiological factors.
2. Tissue kallikrein was localised and expressed in the mucosa of the normal and tumour oesophageal epithelium, activated mast cells, giant tumour cells, keratin pearls of the well differentiated Squamous cell carcinoma. The mast cells showed the highest intensity in immunoreactive tissue kallikrein, kinin B1 and B2 receptors.
3. These results are the first that demonstrate the expression of tissue kallikrein, kinin B1 and B2 receptors in oesophageal carcinoma, and this finding may have therapeutic

implications which can lead to possible use of the third generation of kinin receptor antagonists.

CHAPTER 1: INTRODUCTION

1.1 MOLECULAR AND CELLULAR PROCESSES IMPORTANT IN TUMOURIGENESIS

Tumourigenesis is a complex multistep process involving genetic and biological changes. A single normal cell which undergoes an initiating event via exposure to a genotoxic agent, radiation, or a chemical carcinogen, may aquire a heritable form of growth advantage. Following clonal expansion of the population and further mutational events, this leads to the production of a malignant tumour which may undergo metastasis. The malignant phenotype is inherited from cell to cell and is therefore genetically determined. Mutations in the genes which control cell proliferation will increase the rate of cell division and may increase the risk of further genetic errors.

1.1.1 Genetic factors

Genetic alterations can occur sporadically or may be inherited. The types of genetic changes seen in carcinogenesis are gene amplification, gene re-arrangement (e.g. translocation), gene mutations, and deletion of specific genes. These changes can be manifested at the DNA level (i.e. loss, mutation, or amplification of a gene0, at the RNA level (i.e. altered gene expression), or at the protein level (i.e. altered function of the gene product). Alterations in critical genes can confer a survival or growth advantage on the cell and the descendants may accumulate more genetic damage over the next generations.

1.1.2 Nuclear factors

Nuclear proteins are the final site of action for message sent from GFs. Several oncogenes products bind DNA and presumably control the transcription of genes. This is the level at which control of growth and proliferation ultimately operates. The *myc* oncogene encodes a phosphoprotein, p62, that binds DNA, after dimerisation with the Max protein, and is required for cell proliferation (for entry into G_1 from G_0), differentiation, and possible DNA replication. When cells stop proliferating as a result of terminal differentiation, or as part of their life cycle, and revert to a resting state, then *myc* is downregulated. If *myc* is not downregulated, then the cells cannot leave the proliferation cycle. The product of the *jun* oncogene is a transcriptional factor and these control gene expression as they form the initiation complex from which the RNA polymerase starts. Mutation of a transcription factor will alter transcription rates. The *jun* protein binds DNA as a complex with the product of the *fos* proto-oncogene. The *fos/jun* complex binds to a specific DNA sequence, referred to as AP-1 binding site, located in the promoter region of a family of genes, many of which are expressed in transformed, rapidly growing cells.

1.1.3 Growth factors

Growth factors (GFs) cause cells in the resting (G_0) phase to enter and proceed through the cell cycle. The *sis* oncogene codes for a protein homologuus to a subunit of platelet derived growth factor (PDGF). PDGF is normally released by platelets to stimulate fibroblast growth during wound healing. Illegitimate activation of *sis* can induce fibroblasts themselves to produce their own PDGF.

Growth factor receptors: Growth factor receptors (GF_rs) span the cell memrane and most have an extracellular ligand binding domain and an intracellular tyrosine kinase domain that transduces the mitogenic signal. The protein cascade of growth signalling is controlled at the level of phosphorylation by protein kinases. GR receptors encoded by proto-oncogenes can be affected by overexpression or amplification, leading to an increase in the number of receptors; or by mutations resulting in abnormal receptors which do not require GF stimulation. The *erb*B1 product is homologous to the epidermal growth factor receptor (EGFR) and overexpression of *erb*B1 is seen in some breast cancers. The product of *erb*B2/*neu* is EGFR-like and has tyrosine kinase activity. It is highly homologous to, but distinct from *erb*B1. Amplification of *erb*B2/*neu* is common in breast cancer and correlates with poor prognosis. The product of the *fms* oncogene is homolohous to the receptor for colony stimulating factor (CSF) which is the binding site for macrophage CSF, an important regulator of the haemopoietic system.

Signal transducers: Most growth signal transducers are membrane associated and have tyrosine kinase activity. Increased level of signal transmission can occur by overexpression, point mutation, or structural alteration of the tyrosine kinase or GTPase binding proteins. The *src* oncogene has tyrosine kinase activity and activation through strictural change leads to an increase in this activity. The *ras* oncogene (K-*ras*, N-*ras,* H-*ras*) code for a protein, p21 which can bind GTP and exhibits GTPase binding activity. p21 resembles G proteins which act in signal transduction from membrane receptors (e.g. GF receptors) to the cell interior, regulating cell growth and differentiation. Normal *ras* p21 binds GDP in the resting cell and is inactive. When a physiological stimulus is received from another protein e.g. transmembrane receptor, it leads to synthesis of GTP from GDP and causes a conformation change of *ras* p21 into the active GTP-bound state. Mammalian genes have been isolated

which encode proteins called *ras* exchangers which may turn *ras* on in response to GRs by speeding up removal of GDP from *ras* p21 so that it can bind GTP. Active GTP-bound *ras* p21 then transduces signals to the nucleus, after which it normally deactivates immediately by the interaction with the cytoplasmic protein called GTPase stimulating protein (GAP), which speeds up the transition form *ras* p21-GTP to inactive *ras* p21-GDP by stimulating the intrinsic *ras* GTPase activity. Mutant *ras* p21 however has decreased GTPase activity, and also escapes deactivation by GAP, and so does not regulate itself by hydrolysis of GTP to GDT. Mutant *ras* p21 is stabilised in the GTP-bound active state and thus there is a continuous flow of growth stimulatory signals causing unscheduled cell proliferation.

1.1.4 Causes of mutations

As well as environmental carcinogenesis there are endogenous processes known to cause spontaneous mutations. Point mutations can be induced by DNA polymerase infidelity during replication; depurination; oxidative damage; and deamination of methylated cytosine causing C to T transition. Deletions and insertions can be caused by "Slipped mispairing" in DNA replication. Which involves the mispairing of sequences containing direct repeats.

1.1.5 Gene classes involved in cancer

There are several gene classes involved in tumourigenesis which are known to be affected by genetic changes. These classes are oncogenes, tumour suppressor genes, metastasis genes, apoptosis genes and DNA repair genes.

Oncogenes: Oncogenes are important cellular genes which in general act in appositive way in the normal growth regulatory pathways of the cell. Mutations in oncogenes can alter the normal activity of or increase expression of, the gene product, which will have a positive

effect on driving the cell towards malignancy. The oncogenes are involved in basic essential functions of the cell, including growth and differentiation, and thus any alterations in these genes may disrupt normal cell growth. The functions of oncogenes can be separated into four main groups.

Tumour suppressor gene: Tumour suppressor gene products are involved in the negative control of cell proliferation and differentiation. The loss or inactivation of these genes is associated with tumourigenesis by the loss of the normal growth regulation / restraint controls. DNA methylation may play a key role in loss of tumour suppressor gene function by silencing gene transcription or by somehow marking targets for alle loss.

P53: This is a general tumour suppressor gene, located on chromosome 17p, involved in many tumour types. The 53 kD nuclear protein is expressed in most cells of the body. Mutations and LOH of p53 are commonly seen and lead to loss of growth suppression. Normal p53 induces growth arrest of cells in G_1 and is thus a negative regulator of cell cycle progression.

Retinobastoma gene: Fourty per cent of retinoblastoma cases are hereditary with tumours occurring on both eyes. This tumour occurs when both copies of the retinoblastoma (Rb) gene are altered or lost. The Rb product is a nuclear phosphoprotein that is involved in cell cycle regulation and is expressed in all normal tissues. In its unphosphorylated form the Rb protein appears to prevent cells entering S phase and may regulate the cells progress through the cell cycle. When the Rb protein is phosphorylated or the unphosphorylated form is inactivated, by deletion or point mutation, the constraint on cell growth is released. The unphosphorylated Rb binds an unidentified nuclear protein (possible a transcription factor) and this binding may

be critical for growth suppression by Rb since both normal phosphorylated Rb and mutant Rb cannot bind this protein. When the trancription factor is released, then the genes needed for progression through cell cycle (possibly *fos* and *myc*) are expressed. Rb alterations are also seen in other cancers (e.g. breast, lung, colon) which suggest that it may also have a more general role in tumourigenesis.

Other tumour suppressor genes: The MCC gene, mutated in colorectal cancer, is a candidate tumour suppressor gene located close to APC at 5q21. The MCC protein has a short region of homology to the G protein coupled m3 muscarinic acetylcholine receptor and this region of similarity coincides with the part of the receptor that is critical for G protein activation to give signal transduction. The DCC gene, which is deleted in colorectal cancer, is a candidate tumour suppressor gene mapped on 18q. The prohibition gene at 17q21 is homologous to a candidate antiproliferating gene of rat liver cells, and may be a tumour suppressor gene associated with tumour development and / or progression of some cancers. Transforming growth factor B1 (TGF-B1) is a gene product which is a multifunctional regulator of cell growth and differentiation. It is a potent growth inhibitor of lymphid, endothelial and epithelial cells. Many transformed epithelial cell lines are resistant to the inhibitory actions of TGF-B1 and so escape negative growth regulation, which may be a crucial step in cancer development. Cellular oncogenes (e.g. *myc*) may play a role in modulating the inhibitory actions of TGF-B1. Gas-1 is a set of growth arrest specific genes that have been identified that are expressed specifically in cells in the resting stage (G_0) and this expression is linked to growth arrest. If gas is turned on, then cells respond by exciting the cell cycle. Transformed cells cannot stop proliferation as they cannot upregulate gas.

Metastasis genes: Malignant cells which leave the primary tumour and colonise distant sites are the major cause of patients with solid tumours. The complexity of the metastasis process suggests that it is under genetic control. For many patients the metastasis process has probably started by the time of diagnosis / surgery and thus genetic indicators of metastatic potential would be very beneficial

Apoptosis genes: The production of tumours includes disturbances of the mechanisms controlling cell death by apoptosis, since cancer cells escape normal ageing and death. It is thought that TGFBI is involved in the induction of apoptosis in some epithelia. *Myc* acts as a bivalent regulator of both cell proliferation and apoptosis depending on GF availability, and the normal p53 protein appears to be involved in the induction of apoptosis in susceptible cells. Activated *ras* and *bcl*2 fail to undergo apoptosis, leading to a population expansion. Cells with activated *bcl*2 fail to undergo apoptosis in response to a wide variety of stimuli.

DNA repair genes: Mutations in DNA repair genes are known to lead to some cancers. DNA polymerase B, is known to be involved in the repair of chemically induced DNA damage. Mutations of DNA polymerase B, seen in some colorectal tumours, will probably affect the efficiency of DNA repair and may lead to a higher mutation rate.

1.1.6 The metastasis process

Angiogenesis is required for the expansion of the primary tumour and new blood vessels penetrating the tumour are frequent sites for entry of tumour cells into the circulatory system. Cells must first detach from the primary tumour and it has been suggested that malignant cells have a reduced ability to adhere to each other. The detached cells must then attach to, and invade, the basement membrane (BM). Invasion of the BM, which is a continuous dense

matrix, is an active process, involving the secretion of degradative enzymes (e.g. metalloproteinases, to degrade collagen) by the tumour cells. There are cellular inhibitors of metalloproteinases, called tissue inhibitors of metalloproteinases (TIMPs), which suppress invasion and thus these must be deactivated in some way to allow tumour invasion. Once a zone of proteolysis has been created, the cells actively migrate and hence gain access to the vascular system. Throughout the process, the tumour cells must avoid the host immunological response, possibly by the loss of HLA antigens, and finally the cells must adhere to the target organ to colonise the site.

1.2 PROTEINASES AND THEIR IMPLICATION IN THE PROGRESSION OF HUMAN CARCINOMAS

Three groups of proteinases, mainly serine, thiol, and metalloproteinases, are secreted by cancer cells and responsible for the proteolytic cascade triggered during malignant cell invasion (Bernitez-Bribiesca *et al.,* 1995). Proteinases that show high activities in malignant tumour homogenates may be related to the degradation of the surrounding cell matrix in addition to intracellular metabolism (Tsuboi *et al.,* 1988).

1.2.1 Serine proteinases

Proteinases such as serine proteases are secreted by cancer cells and are responsible for the proteolytic cascade triggered during malignant cell invasion (Bernitez-Brisbiesca *et al.,* 1995). Hydrolysis of extracellular matrix is a necessary step for malignant cells to invade functionally normal adjacent tissue. Proteinases that show high activities in malignant tumour homogenates may be related to the degradation of the surrounding cell matrix (Tsuboi *et al.,* 1988). This family includes the kallikreins (tissue and plasma), plasminogen activators (PAs), elastases, thrombin, guanidinobenzoatases and trypsin. Some of these proteases after activation, attack some structural glycoproteins and elastin.

1.2.1.1 The kallikrein- kinin system

Kallikreins are a group of serine proteases that are found in epithelial cells, neutrophils and biological fluids (Bhoola *et al.,* 1992). By means of enzymic action, kallikreins release the vasoactive peptides, kinins from endogenous substrates called kininogens thus they are called kininogenases. In addition to the kallikreins enzymes with kininogenase activity include

trypsin, plasmin and snake vernom proteases. Two types of kallikreins are found in humans: tissue kallikrein (TK) in specific cell types in many organs, and plasma kallikrein (PK) that is synthesised in the liver and secreted into the blood stream. These enzymes can be differentiated on the basis of their molecular weight, isoelectric points, substrate specificity, immunological characteristics, mechanism of activation and type of kinin released. Kinin production is regulated by both its rate of synthesis and its breakdown. Kinins are inactivated via proteolytic cleavage by kininases (Figure 1).

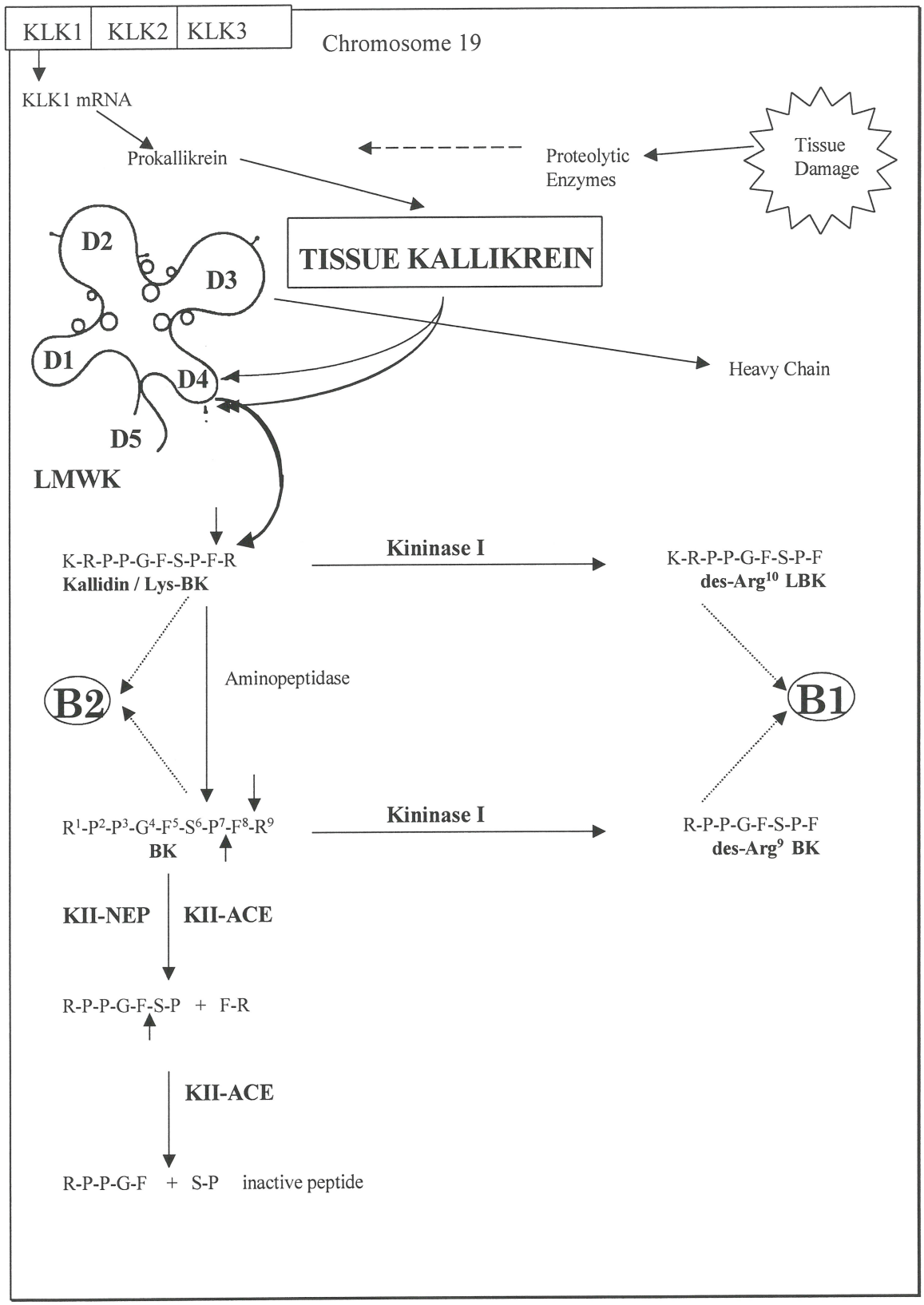


Figure 1 Overview of the Kallikein-Kinin System

1.2.1.1.1 Kallikreins

Tissue kallikrein

Geneology

Tissue kallikrein is a member of a multigene family that shows different patterns of tissue specific gene expression (Mason *et al.,* 1983). The distribution and molecular arrangement of some of the kallikrein genes in the mammalian genome have been studied extensively. They consist of five exons and four introns. Each of the three crucial, active site amino acids are encoded on a separate exon. The gene members are highly conserved, located close to one another, and tightly linked. The intraspecies comparison of the amino acid sequence coded by the kallikrein genes shows a greater homology than a comparison across species. Therefore, whereas the families in the various mammalian species have evolved independently, members within the mouse and rat seem to have co-evolved. Whereas the kallikrein gene members are a distinct subfamily of serine proteases showing between 60 and 75% homologies, a sequence identity of only 35 to 50% is shared with trypsin, chymotrypsin and elastase (Mason *et al,*. 1983). The high degree of homology between the kallikrein-like enzymes suggests that they evolved from a common ancestor through gene duplication.

Gene expression in humans

The gene expression of tissue kallikrein in humans, has been determined mainly in epithelial cells derived from the entoderm as evidenced by the induction of this enzyme in pituitary adenomas, and lung, gastric and colonic tumours. Tissue kallikrein is expressed also in prostate and endometrial cancers (Clements & Mukhtar, 1997). Embryologically, the mucosal epithelial cells of the oesophagus are derived also from the entoderm. Several family members have been characterised in the human: glandular kallikrein (KLK1) and genes

expressed in the prostate, KLK2 and KLK3 (prostate specific antigen, PSA). KLK1/true tissue kallikrein, which is a member of this family of related serine proteases, exhibits a diverse range of enzymic activities. Tissue kallikrein and the kinin B2 receptor are both expressed in prostate and endometrial cancers (Clements & Mukhtar, 1997). This supports the findings that kallikreins have been implicated in the process of tumourogenesis via the action of bradykinin (Roberts & Gullick, 1989). Gene expression of the serine protease, tissue kallikrein and subsequent formation of the vasoactive kinin peptides could stimulate proliferation of tumour cells and by increasing vascular permeability, enhance metastases (Roberts, 1989). The observation of increased kinin receptor expression due to oncogenic transformation (Roberts & Gullick, 1989) gives further importance to a mitogenic role for kinins in tumour tissue. Interestingly, there are suggestions that transformation of cells *in vitro* by the *ras* oncogene, an oncogene found mutated in a large number of human tumours, is associated with increased bradykinin receptor number. Taken with the known release of bradykinin at sites of tissue injury and inflammation (Hargreaves *et al.*, 1988), this implies a role for bradykinin in cell growth control during tumourogenesis. More recent studies performed on the involvement of tissue kallikrein in malignant diseases, shows that TK is secreted by gastric carcinoma cells. It was also recently reported that TK activates *in vitro* matrix degrading metalloproteinases *in vitro* present in carcinoma cells (Hermann *et al.*, 1995). PSA is a kallikrein-like protease mainly expressed in the human prostate and it is reponsible for the proteolysis of the gel-forming proteins in human semen. The concentrations of PSA in serum are normally less than 4 μg/l, but elevated concentrations are found in a majority of patients with prostate cancer (CAP). This is however, not an ideal tumour marker in the sense that there are CAP patients with normal PSA concentrations in serum and patients with benign hyperplasia of the prostate (BPH) with elevated PSA concentrations (Becker & Lilja, 1997). Lately it has been documented that KLK2 is

expressed in prostate cancer, and that the expression is incrementally increased from benign epithelium to high-grade prostatic intraepithelial neoplasia (PIN). Expression of KLK2 indicates that this kallikrein antigen is both prostate localised and tumour associated (Darson, *et al.*, 1997).

Tissue kallikrein in tumourigenesis

In tumourigenesis may convert precursor proteinases and macromolecules into active molecules. In this regard evidence suggests that tissue kallikrein is important in the cascade formation of prostate specific antigen (PSA). Gene expression of tissue kallikrein, and the subsequent formation of the vasoactive kinin peptides could stimulate proliferation of tumour cells, and by increasing vascular permeability, enhance metastasis (Roberts, 1989). Therefore it was considered that tissue kallikrein may be implicated in the process of tumourigenesis both as a serine protease as well as through the cellular actions of bradykinin (Roberts & Gullick, 1989). The observation of increased kinin receptor expression due to oncogenic transformation (Roberts & Gullick, 1989) gives further credence to a mitogenic role for kinins in tumour tissue.

Molecular structure of tissue kallikrein

Tissue kallikreins are acidic glycoproteins of between 24-45 kDa molecular weight and having pI values close to 4.0. Human urinary tissue kallikrein is synthesised bound to a signal peptide of 17 amino acids that is finally cleaved off to produce an inactive precursor (Bhoola *et al.*, 1992). Seven further amino acids are then cleaved to form the active enzyme. In addition to the active enzyme seemingly occurring primarily in the salivary glands, most tissues and body fluids contain in varying proportions the inactive pro form. Although *in vitro* activation of the proenzyme together with the release of the septapeptide has been

formed mainly with trypsin, it can also be achieved with thermolysin. Two endogenously occurring serine proteases, with prokallikrein activation properties have been identified in the rat submaxillary gland (SMG). The presence of such converting enzymes in the SMG raises the possibility of salivary tissue kallikrein being synthesised in a proform but rapidly converted to the active state within the duct cell.

Tissue kallikrein kallikrein is a single chain polypeptide that comprises 238 amino acids with Ile at the NH_2 and Ser at the COOH terminus (Takahashi *et al.,* 1988). The number and position of glycosylation sites seem to vary according to species and synthesising cell. Rat and mouse kallikreins have only one glycosylation site per mole of kallikrein. Porcine pancreatic kallikrein has two or possibly three glycosylation sites. Human urinary kallikrein has three asparigine-linked glycosylation sites (Takahashi *et al.,* 1988), and three additional oxygen glycosylation sites linked to two serine and one threonine residues. Also, the total carbohydrate content of porcine SMG (24% w/w) and human urinary kallikrein (20% w/w) is relatively higher than that of the porcine pancreatic enzyme (12% w/w).

Release of kinins

A characteristic of the true tissue kallikrein of all mammalian species should be its ability to release a kinin (kallidin/Lys-bradykinin) from kininogen. The primary physiological substrate for tissue kallikrein is kininogen from which it forms kinins. Release of kallidin (Lys-badykinin) from the kininogen molecule requires the hydrolysis of Met-Lys in the Leu-Met-Lys (at the NH_2 terminus of kallidin) and Arg-Ser in the Phe-Arg-Ser (at the COOH terminus of kallidin). The inability of tissue kallikrein of most mammalian species to form bradykinin is due to their inability to accommodate the Lys-Arg-Pro sequence for hydrolysis of the Lys-Arg bond in the NH_2 terminus of the peptide.

Tissue kallikrein inhibitors

Although inhibitors of tissue kallikrein were first described by Werle and colleagues (Kraut *et al.,* 1930b), another inhibitor of tissue kallikrein (kallikrein binding protein, kallistatin) has been identified in serum and extracts prepared from the liver, lung, and salivary gland of the rat, and this binds specifically to active tissue kallikrein. Additionally it binds to elastase and chymotrypsin, but not to plasma kallikrein, urokinase and, or collagenase. The kallikrein-binding protein (KBP) is an acidic protein (pI 4.2-4.6) with a molecular mass of 60 kDa and five possible glycosylation sites. The enzyme and the inhibitor form a 1:1 stochiometric, heat-stable complex of 92 kDa. The complex is emiocytosed by hepatocytes, a mechanism by which it is cleared from the circulation. The KBP shows a high degree of homology to α_1 - antitrypsin, antithrombin III, plasminogen activator, and α_1 -antichymotrypsin, and therefore, is considered to be a member of the serpin superfamily (Chao *et al.,* 1990a). Another KBP has been isolated from human serum. Like the rat KBP, the human KBP forms a heat-stable 92 kDa complex with the enzyme. The precise functional importance of KBP remains to be elucidated. An *in vitro* inhibitor of the kallikreins is aprotinin (trasylol). Substitution of one of the amino acids essential for the inhibitory action of aprotinin has resulted in a molecule, [Val 15] aprotinin, that selectively inhibits both plasma kallikrein and neutrophil elastase (Wenzel *et al.,* 1986).

Functional role of tissue kallikrein

The exciting new discovery of tissue kallikrein in the neutrophil, and attachment of high molecular weight kininogen (HK), plasma prekallikrein, and low molecular weight kininogen (LK) to the external surface of its membrane has provided new insights for the involvement of kinins in the diapedesis of neutrophils through capillary cell junctions and their

participation in the inflammatory process (Figueroa *et al.,* 1991b). The diverse sites of occurrence of tissue kallikrein has led to the suggestion that the functional role of this enzyme may be specific to cell types. Apart from its kininogenase activity, kallikrein has been implicated in the processing of growth factors and peptide hormones (Mason *et al.,*1983). Tissue kallikrein has been implicated in organ-specific disorders, for example, the extracellular release of glandular (tissue) kallikrein and trypsin into the circulation in acute pancreatitis. Tissue kallikrein plays a major role in homeostatic control of blood pressure. Inhibitors based on the amino acid sequence of kininogen around the cleavage site have been used recently to investigate the role of tissue kallikrein in blood pressure regulation (Burton *et al.,* 1987). The substrate analogue inhibitor KKK7, when infused into rats receiving a diet containing a normal quantity of sodium, produces an increase in blood pressure, prevents the vasodepressor action of exogenous kallikrein, and particularly reverses the hypotensive action of KII-ACE inhibitors (Bhoola *et al.,* 1992).

Plasma kallikrein

Plasma kallikrein (PK) is a serine protease that is synthesised in zymogen form, plasma prekallikrein (PPK) by the hepatocytes. PK is unrelated to tissue kallikrein from which it differs significantly in its biochemical, and immunological characteristics except the capacity to form kinins. PPK is encoded by a single gene, it is synthesised in hepatocytes and secreted into the blood stream where it circulates as a heterodimer complex bound to its substrate, high molecular weight kininogen (HK). The PPK-HK complex, Hageman factor (HF, Factor XII) and Factor XI (plasma thromboplastin antecedent) are the components that initiate the contact activation pathway of the blood clotting cascade. The contact system is activated when blood is exposed to negatively charged surfaces, to which the circulating PPK-HK complex binds through positively charged groups of HK. PPK is converted to PK by active

Hageman factor, HFa, which is itself activated by contact with anionic surfaces. In this way local accumulation of PK occurs. In turn PK activates HF, and through this feedback reaction cycle both Hfa and PK are formed rapidly. As soon as sufficient Hfa is produced the reaction is driven forward to generate activated Factor XI (FXIa). FXIa enhances clotting and activates plasminogen that initiates fibrinolysis. With rising levels, PK reacts with its substrate HK to form the vasoactive peptide bradykinin. Active Hageman factor comprises a heavy chain of 50 kDa which has the binding site for the attachment to the anionic surfaces during activation, and light chain of 28 kDa linked together by a disulphide bond. The light chain of Hfa has the active site for cleaving plasma prekallikrein and, thus, converting the zymogen into the active form. Active enzyme releases bradykinin from HK by hydrolysis of first a Lys-Arg and then Arg-Ser bonds to give the nonapeptide with arginine at both NH_2 and COOH terminals. Although LK is a poor substrate for plasma kallikrein, it will form bradykinin in the presence of neutrophil elastase. It is possible that this reaction occurs *in vivo* for kinin generation from LK, and this activity has important implications because LK has been localised on the external membrane of the neutrophil. PK has a significant effect on PMN leucocytes, and therefore is considered to play an important role in inflammation.

The present view is that, during formation, plasma kallikrein is rapidly inactivated by C1 inhibitor, which alone has the capacity to inhibit with high affinity both plasma kallikrein and HF. Further inhibition could occur with α_2 -macroglobulin, antithrombin III, and mutant α_2-antitrypsins. After the plasma kallikrein inhibitor complexes are formed, they are rapidly cleared from the circulation. It has been proposed that both plasma kallikrein and factor XIa may be protected by HK from inhibition by C1 inhibitor, α_2 -macroglobulin, and α_2-antitrypsin, thereby augmenting the plasma half-life of each enzyme.

1.2.1.1.2 Kinogens

Kininogens are single chain glycoproteins, and are synthesised by hepatocytes. As typical secretory proteins, they undergo posttranslational modifications such as glycosylation prior to secretion into the circulation. Kininogens have also been localised in platelets (Schmaier *et al.,* 1986b), acinar cells of the rat SMG (submaxillary gland) (Chao *et al.,* 1988b), CD (collecting duct) cells of the human kidney (Figueroa *et al.,* 1988), human kidney cortex and medulla (Iwai *et al.,* 1988), endothelial cells (van Iwaarden *et al.,*1988a) and in neutrophils (Figueroa *et al.,* 1990a, 1991b) using specific antibodies. They possess an amino terminal heavy chain, a COOH-terminal light chain with the kinin moiety interleaved between the two polypeptides that are bridged by a single disulphide loop. In association with HF, Factor XI, and plasma prekallikrein, the intact kininogen molecule triggers contact activation of blood clotting, for which it is an essential cofactor (Bhoola *et al.,* 1992). These functions are in addition to the role of kininogens as substrates for kallikreins. Although LK is primarily considered to be a substrate for tissue kallikrein and likewise, HK, for plasma kallikrein, LK can be a substrate for plasma kallikrein, albeit a poor one, with bradykinin released if PMN elastase is present. Additionally, *in vitro*, TK is considered to release kallidin from both LK an HK (Iwanaga *et al.,* 1977). Direct local action by carcinogens (aromatic hydrocarbons, volute nitrosoamines, etc.)

The two kininogens differ in their structure, size and susceptibility to cleavage by plasma and tissue kallikreins. HK, a glycoprotein composed of 626 amino acids, has a molecular mass of 88 to 120 kDa, depending on the species of origin (Yamamoto, 1987). LK consists of 409 amino acids and varies from 50 to 68 kDa in size (Muller-Esterl *et al.,* 1985a). The ability of kininogens to inhibit with high affinity thiol proteinase, such as the cathepsins B, H, and L, ficin, papain, and platelet-derived calpains I and II provides an important bioregulatory

function (Muller-Esterl *et al.*, 1985b). During inflammation, calpains perform a dual function; they release kallidin from kininogens, and thereby dissociate the heavy chain to inhibit the proteolytic activity of calpains coming from damaged tissues. During inhibition of platelet calpain, HK by acting as a substrate for this enzyme, may itself become consumed. T-kininogen forms T-kinins by the action of T-kininogenases in rats and guinea pigs. Although the heavy chain of T-kininogen is structured similar to HK and LK, it does not inhibit calpain. The heavy chain of HK shows a greater inhibition profile against papain than the heavy chain of LK. The inhibitory potency against the different proteases shows species difference.

The difference between the three proteins appears to reside at the amino terminus of the kinin segment. T-kininogen contains the sequence Met-Met-Ile-Ser-bradykinin instead of Met-Lys-bradykinin of HK and LK. The effector peptide, T-kinin (Ile-Ser-bradykinin), is formed by T-kininogenase (a thiol protease), cathepsin D, or trypsin, but not by tissue or plasma kallikrein.

In each of the three kininogens, the light chain distal to the kinin moiety differs in length. The first 12 amino acids of the light chains are identical, but the molecules diverge in subsequent COOH-terminal sequences. Domain 5 of HK is rich in histidine, proline and lysine, and is involved in binding HK to the negative charge on damaged endothelial surfaces, crystals and degraded cartilage products. HK also binds to the platelets and PMNs. After cleavage of the kinin moiety, the downstream light chain enhances blood coagulation through sequential activation of HF, PPK and Factor XI. After formation of active kallikrein, HK continues to act as an essential cofactor, promoting amplification of the cascade. Domain 6

of HK binds PPK and Factor XI. LK has much smaller light chain than HK, lacking contact activation and PPK binding sites, and its function is unknown.

1.2.1.1.3 Kinins

Kinins are potent bioactive peptides formed by the enzymatic action of kininogenases on kininogens (Bhoola *et al.,* 1962). It is generally accepted that kallidin (Lys-bradykinin) is released from LK by tissue kallikrein and bradykinin from HK by the action of plasma kallikrein (Werle *et al.,* 1961). Some conversion of kallidin to bradykinin may occur through removal of the NH_2 -terminal lysine by aminopeptidases (Fig.1). Another analogue, Met-Lys-bradykinin, is formed by pepsin and by uropepsin. In blood, the half-life of bradykinin and kallidin is estimated to be <30 s because of rapid degradation by peptidases called kininases, but may not be so short-lived in other biological fluids and in tissue spaces..

The two mammalian kinins, kallidin and bradykinin, influence the cardinal features of inflammation as well as a number of cellular functions, including blood pressure and local blood flow, electrolyte and glucose transport, and cell proliferation. The cellular actions of kinins are modified by their ability to stimulate the release of many second generation mediators, for example, platelet-activating factor, leukotrienes, prostaglandins, substance P (neurogenic inflammation), acetylcholine, and noradrenaline (sympathetic nerves). Kinins also stimulate the secretion of renin from the kidney, release vasopressin from the neurohypophysis, and secretion of catecholamines from the adrenal medulla (Bhoola *et al.,* 1992). Therefore, the homeostatic regulation of regional blood flow by kinins could be modulated in a manner that has been difficult to evaluate, except with selective antagonists. In addition, it would be necessary to establish the precise role of the secondary autacoids in the control of systemic or local blood flow.

Kinins act as mitogens, stimulating DNA synthesis and thereby promoting cell proliferation. The ability of kinins to induce cell division could enhance the spread of cancerous cells and increase proliferation of epidermal cells in disorders such as psoriasis. Kinins also appear to play an important role in a number of pathological states, namely, reduced sperm motility, allergic and viral rhinitis and asthma, postgastrectomy dumping syndrome, inflammatory bowel diseases, carcinoid, anaphylactic shock, and septick shock.

Most of the actions of bradykinin and kallidin are mediated through the constitutive B2 receptor, which essentially does not respond to desArg^9 -bradykinin or -kallidin, the kinin B1 receptor agonists. On most membranes the K_d of kinins for the B2 receptor is in the range 0.7 to 5 nm. Specific antagonists are essential to understanding the physiological role and cellular actions of endogenously active molecules. Ever since the description of the correct structure of bradykinin in 1960, many analogues have been synthesised. A new class of kinin antagonists (Hoe 140; D-Arg-Arg-Pro-Hyp-Gly-(β-2-thienyl)-Ala-Ser-DTic-Oic-Arg) was developed by introducing several unnatural amino acids into the molecule (Hock *et al.*,1991). Currently, several new generations of kinin antagonists have become available.

1.2.1.1.4 Kinin receptors

The majority of actions of kinins are mediated via an interaction with cell surface bradykinin receptors. Receptors for bradykinin have been reported on such tissues as uterus, intestine, aorta, kidney, heart and spinal cord (Innis *et al.*, 1981; Steranka *et al.*, 1988). These are cell surface, G-protein coupled receptors of the seven-transmembrane domained family. The existence of two subtypes of bradykinin receptor was described over 17 years ago by Regoli and collegues (Regoli and Barabe', 1980). B1 receptors were defined as those where the rank order of potency of kinin analogues on isolated tissues was [desArg^9]-BK>[Tyr(Me)^8]-

BK>BK, and B2 receptors were defined as having the rank order of potency Tyr(Me)^{8}]-BK>BK>[desArg^{9}]-BK.

Kinin B1 receptor

Genes encoding the B1 receptor have been cloned in the mouse (McIntyre *et al.,* 1993), human (Menke *et al* 1994; Webb *et al.,* 1994) and rabbit (MacNeil *et al.,* 1995). The cloned human receptor has a sequence of 353 amino acids and has an overall sequence homology with the B2 receptor of 36% (Menke *et al.,* 1994). The B1 receptors are seven-transmembrane domained and G-protein coupled, and that is consistent with the documented ability of B1 receptors to stimulate the hydrolysis of phosphatidylinositol (Butt *et al.,* 1995).

Kinin B2 receptor

The gene coding for the B2 receptor was cloned, and the receptor protein expressed from the rat (McEachern *et al.,* 1991), and has subsequently been cloned from mice and humans (Hess *et al.,* 1992; Ma *et al.,* 1994). The B2 receptor also belongs to the seven-transmembrane domained G-protein coupled superfamily, and this is consistent with the hydrolysis of phosphatidylinositol in many tissues (Field *et al.,* 1994). The rat and mouse B2 receptor has a sequences of 366 amino acids. The human B2 receptor has a sequence of 364 amino acids, and is about 80% homologous with the mouse and rat receptor.

Biological roles of kinin B1 and B2 Receptors

Bradykinin receptors mediate the majority of the diverse biological actions of kinins, and many of the biological roles attributed to endogenous kinins. The major advances in the area of bradykinin receptor pharmacology have resulted from the use of effective bradykinin

receptor antagonists through molecular cloning, and producing colonies of knock-out mice with deletion of B1 and/or B2 receptors (Borkowski *et al.*, 1995).

1.2.1.1.5 Kininases

The turnover of kinins depends on both the rate of formation and the rate of destruction. After kinins are formed, they are rapidly destroyed by the enzymatic action of peptidases. The sites at which they cleave kinins are indicated in figure 1. This family of enzymes is generally called kininases (Erdos, 1990). There are two families of kininases, KI (Kininase I carboxypeptidases) and KII (KininaseII peptidylpeptidases). The KI family comprises KI-CPN (Kininase I-Carboxypeptidase N) and KI-CPM (Kininase I-carboxypeptidase M) and the KII family includes KII-ACE (Kininase II-Angiotensin I-Converting Enzyme) and KII-NEP (Kininase II-Neutral Endopeptidase). Additional kinin hydrolysing enzymes are prolidase (aminopeptidase P), and two endopeptidases (kininase A and B).

KI-CPN is an arginine carboxypeptidase which is optimally active at pH 7.4 and activated by $CoCl_2$. This enzyme acts on the COOH-terminal end of the bradykinin molecule and by removing the Arg^9 residue produces a kinin B1 receptor agonist. It is synthesised by the liver and secreted into the circulation where it probably accounts for about 90% of the bradykinin-destroying activity in human plasma (Zacest *et al.*, 1974). In addition to kinins CPN hydrolyses the COOH-terminal basic amino acid of several biologically active peptides, namely, enkephalins, anaphylotoxins (C3a, C5a), and fibrinopeptides (Bhoola *et al.*, 1992). This enzyme is a tetrameric complex of 280 to 320 kDa that can be dissociated into two large, identical subunits of 83 to 98 kDa and two small, identical subunits of 42 to 55 kDa. Levels of this enzyme increase during pregnancy (Ito *et al.*, 1990).

KI-CPM (Kininase I carboxypeptidase M) cleaves the COOH-terminal arginine of bradykinin and Lys-bradykinin, resulting also in the formation of des[Arg^9]-bradykinin and des[Arg^9] Lys-bradykinin. The optimum pH for degrading the two kinins is the neutral range. KI-CPM is a 62 kDa metallopeptidase activated by cobalt (more pronounced at pH 5.5). Structurally, KI-CPM is a singe chain cell membrane glycoprotein with 426 amino acids and six asparagine-linkes glycosylation sites. The molecule shows only 41% identity with the active subunit of KI-CPN (Bhoola *et al.,* 1992).

KII-ACE inactivates circulating bradykinin mainly during its passage through the lung and at the same time converts angiotension I to angiotension II by the removal of the COOH-terminal His-Leu in the pulmonary circulation. This enzyme is a single chain, transmembrane protein bound to cell membranes with COOH-terminal anchor sequence and its molecular mass ranges from 195 to 200 kDa. It hydrolyses two separate bonds on the COOH-terminal end of the bradykinin molecule. First it removes the dipeptide Phe^8 -Arg^9 and next splits the Ser^6 and Pro^7 bond (Erdos, 1979). Several other peptide substrates are hydrolysed by KII-ACE, namely, enkephalins, neurotension, substance P, and luteinising hormone-releasing hormone (Skidgel and Erdos, 1985).

KII-NEP is synthesised as an 88 kDa protein that increases in size to 94 kDa when four to five N-linked oligosaccharides are added. After synthesis, the enzyme migrates to the plasma membrane where it becomes translocated to be anchored on the outer surface of the fibroblast from which it may be secreted into body fluids (Lorkowski *et al.,* 1987). KII-NEP is a 742 amino acid metallopeptidase and it preferentially cleaves bonds in the amino side of hydrophobic amino acid residues. Like KII-ACE, KII-NEP inactivates kinins by removing the COOH-terminal Phe^6 -Arg^9 dipeptide (Gafford *et al.,* 1983). Several of biologically

important peptides cleaved by KII-NEP, described in sequence of affinity for the enzyme, are substance P, kinins, enkephalins, ANF, and neurotension (Gafford *et al.,* 1983). One important action of KII-NEP is the hydrolysis of N-fMLP, a peptide that stimulates chemotaxis. Activation of neutrophil or antigen-antibody reactions triggers the internalisation of KII-NEP, with a rapid loss of its enzymic activity.

Additional kinin peptidases include prolidase that cleaves the Arg^1 Pro^2 bond. The prolidase (aminopeptidase P) has been described in erythrocytes, kidney and lung tissue. Two additional endopeptidases designated as kininase A and B have been purified from rabbit brain. Kininase A cleaves the Phe^5-Ser^6 bond of bradykinin. Kininase B hydrolyses the Pro^7-Phe^8 bond but, unlike ACE, does not split the peptide further, nor does it convert angiotension I to angiotension II.

1.2.2 Serine proteases in neutrophils

The activity of elastases is parallel to the abundance of elastin in the stroma (Clavel & Birembaut, 1988). Polymorphonuclear (PMN) elastase can degrade extracellular matrix components and modulate other proteinases (Bjornland *et al.,* 1998). The presence of PMN elastase in colorectal carcinomas have been demonstrated. An interaction between tumour cells and elastase producing leukocytes is suggested (Bjornland, *et al.,* 1998).

1.2.3 Plasmin family - serine proteases

Plasminogen activators are serine proteinases which catalyse the conversion of the plasma zymogen, plasminogen, to the active proteinase plasmin. Plasmin, a serine proteinase, has a wide rang of substrate activity, can activate latent forms of the other proteolytic enzymes (Sloane & Honn, 1984) and may therefore, have a central role in initiating an enzyme cascade

which results in the degradation of all of the components of the extracellular matrix (Hewin, *et al.,* 1996). There are two types of physiological PA which differ from each other with respect to molecular properties and tissue distribution, urinary-type PA (uPA or urokinase) and tissue-type PA (t-PA) (Dano *et al.,* 1985). Both enzymes efficiently catalyse the specific cleavage of inactive plasminogen at the Arg^{560}-Val^{561} bond. (Stack *et al.,* 1997), resulting in the formation of catalytically active plasmin (Robbins *et al.,* 1967). Production of PAs provides a mechanism by which tumour cell degradation of the extracellular matrix is amplified. Furthermore, co-localisation of cellular receptors for PAs and plasminogen allows for efficient generation of plasmin on the cell surface. Plasmin is a broad spectrum serine proteinase with trypsin-like specificity capable of degrading numerous extracellular matrix and matrix-associated proteins, including fibrin, laminin, fibronectin and vitronectin (Robbins *et al.,* 1967; Dano *et al.,* 1985). The plasminogen activator system is important in the breakdown of extracellular matrix by squamous cell carcinomas and the regulatory mechanisms involved in this proteolytic system may be important targets for chemotherapeutic intervention to limit tumour cell invasion and metastasis (Niedbala & Sartorelli, 1990). Urokinase plasminogen activator (uPA), has a physiological role in invasive processes requiring tissue breakdown, such as inflammatory cell migration, angiogenesis and trophoblast implantation (Stephens *et al.,* 1984; Bacharach , *et al.,* 1992; Lala & Graham, 1990). This protease is up-regulated in breast cancer, and high levels of uPA is strongly associated with poor prognosis (Nielsen *et al.,* 1996). Binding to a specific cell-surface receptor (uPA receptor), allowing the cell a controlled, directional means of initiating pericellular matrix degradation considerably enhances uPA activity. tPA is essential for the intravascular lysis of fibrin clots (de Jong, *et al.,* 1989). The plasminogen activator system is regulated by the activity of specific plasminogen activator inhibitors (PAI-1, PAI-2) which

bind covalently to the attached activator and cause internalisation of the receptor-activator complex (Blasi, 1993).

1.2.4 Serine proteases in cancer

Guanidinobenzoatases (GBs) are cell surface-associated serine proteinases supposed to be involved in cancer metastasis, cell migration, and tissue remodelling. They have a molecular weight of 71 kDa, and are found both free in the epididymal fluids of the mouse and bound to sperm surface (Poustis-Delpont *et al.,* 1992). Invasion and metastasis is aided by the secretion of guanidinobenzoatase, that cleaves the link peptide to fibronectin and urokinase plasminogen activator, which initiates a molecular malignant cell migration through the extracellular martix (Bernstein, *et al.,*1998). The guanidinobenzoatases are associated with human renal carcinoma plasma membrane (Poustis-Delpont *et al.,* 1992).

SP220K, is a newly described high molecular weight tetrameric serine proteinase from human kidney, clear cell carcinoma plasma membranes This enzyme exhibits gelatinase activity which ranges between pH 7.5 to pH 9.0. It also hydrolyses fibronectin and type I collagen (Thaon *et al.,* 1998). This proteinase displays guanidinobenzoatase activity in its inactive form and gelatinolytic activity in its active form (Thaon *et al.,* 1997). There is a potential mechanistic relationship between expression of the matrix proteinase SP220K and invasive phenotype in kidney epithelium proliferative processes and it exhibits a gelatinase activity. Fibronectin and type 1 collagen are hydrolysed by SP220K (Thaon *et al.,* 1998). Trypsin-2, human tumour derived serine proteinase, has the ability to activate latent matrix netalloproteinases. Thus it has been suggested that it plays a role in initiating the proteinase cascade that mediates tumour invasion and metastasis (Sorsa *et al.,* 1997).

1.2.5. Metalloproteinases

Hydrolysis of extracellular matrix is a necessary step for malignant cells to invade functionally normal adjacent tissue. The matrix metalloproteinases (MMPs) are extracellular zinc-enzymes implicated in a number of physiological and pathological tissue remodelling processes, including cancer progression. For a long time they have been thought to be produced by malignant cells and contribute to tumour invasion, through their ability to degrade extracellular matrix components. Recent studies have demonstrated that extracellular proteinases are implicated in the progression of human carcinomas. MMPs are in fact predominantly expressed by stromal and not by cancer cells. In addition to that, membrane receptors, activators and/ or binding sites for some of these proteinases are also predominantly found to be associated with stromal cells (Basset *et al.,* 1997). These findings, together with the observation that the MMPs can cleave some molecules implicated in controlling growth factor activities, suggest that the role of MMPs during cancer progression is not limited to facilitating malignant cell invasion alone but it is also likely to participate in other aspects of the malignant phenotype. MMPs should in fact be regarded as pan-regulators of tissue neoformation characteristic of malignant tumours, which include both epithelial and cell expansion and stroma formation (Basset *et al.,* 1997). The stromal expression is particularly well illustrated in breast carcinomas, where the expression of only two, Matrilysin and Collagenase 3 out of nine MMPs, have been found predominantly in cancer cells (Heppner *et al.,* 1996). Another example of an MMP which is not expressed by stromal cells is that of Stromelysin-2, which has been found to be expressed by cancer cells in carcinomas of the squamous type (Wolf *et al.*, 1993). The cellular expression of matrix metalloproteinases in human breast carcinoma is shown below (Basset *et al.,* 1997).

Table 1. Cellular expression of matrix metalloproteinases in human breast tumours.

Fibroblastic cells	Inflammatory cells	Cancer cells
Stromelysin-3	Gelatinase B	Matrilysin
Gelatinase A	Metalloelastase	Collagenase-3
MT1-MMP		
Collagenase-1		
Stromelysin-1		

MT1-MMP (membrane type 1 matrix metalloproteinase) is expressed on cancer cell membranes and activates the zymogen of MMP-2 (gelatinase A). It is a 72 kDa type IV collagenase. MT1-MMP is an extracellular matrix-degrading enzyme sharing the substrate specificity with interstitial collagenases, and thus it plays a dual role in pathophysiological digestion of extracellular matrix through direct cleavage of the substrate and activation of proMMP-2 (Ohuchi *et al.*, 1997). MT4-MMP (membrane type 4 matrix metalloproteinase) is suggested to have a potential role in tumoural processes. The expression of this proteinase in leucocytes together with its putative membrane localisation suggests that this enzyme could be involved in the activation of membrane-bound precursors of growth factors or inflammatory mediators such as tumour necrosis factor-alpha (Puente *et al.*, 1996). MMP-9 (gelatinase B) is a 92-kd type IV collagenase. Eosinophils have the capacity to synthesise this gelatinase whereas the neutrophils store and probably release the enzyme on demand. Because of its capacity to degrade both basement membrane and interstitial extracellular matrix molecules, the expression, delivery, and secretion of this metalloproteinase by granulocytes may be critical for tumour invasion (Stahle-Backdahl & Parks, 1993). It has

been demonstrated that stromelysin-3 (ST3) and matrilysin act similarly in human carcinomas, these findings further illustrate that MMPs can facilitate tumour growth without contribution to the invasive potential of tumours.

1.2.6 Lysosomal proteinases (Cathepsins-Cats)

Cathepsin B (Cat B), a cysteine proteinase, can degrade basement membrane laminin and thus it is suggested that it may be involved in tumour invasion (Redwood *et al.,* 1992). Abnormal intracellular distribution, increased activities and secretion of cysteine proteinases (CPs) cathepsin B and L (Cat L), a cysteine proteinase, are associated with tumour progression (Lah *et al.,* 1992). The presence of the active mature form of cathepsin D (Cat D), an aspartic proteinase, in metastatic skin lesions of squamous cell carcinoma (SCC) suggests that activated Cat D may be involved in the invasion and metastasis of squamous cell carcinoma (Kawada *et al.,* 1997).

1.2.7 Induction of Protein Kinase C (PKC) in cancer

Activation of protein kinase C and Fos/Jun-dependent signal transduction pathways are thought to be major effects of oncogene action in different tumour systems including human non-small-cell lung carcinoma (NSCLC). It has been shown that the phorbol ester analogue phorbol-myristate acetate (PMA), which is a potent activator of PKC, can induce squamous-type cellular differentiation and expression of proteinases, such as plasminogen activators and pro-cathepsin L, in several NSCLC cell lines (Schuermann *et al.,* 1997).

1.3 CARCINOMA OF THE OESOPHAGUS

Oesophageal carcinoma is the sixth most common cancer in the world and one of the most lethal tumours (Parkin *et al.*, 1988). In the USA and Europe, the incidence of the adenocarcinoma of the oesophagus and gastric cardia including the oesophago-gastric junction, has been increasing rapidly, especially among white men. The squamous cell carcinoma (SCC) is the predominant histological type of oesophageal cancer world-wide (Powell *et al.*, 1990; Blot *et al.*, 1993). Squamous cell papilloma (Squamous papilloma or papillomatosis) of the oesophagus is also believed not to be as rare as thought previously (Shimuzu *et al.*, 1996). Prognosis of this tumour is very poor and is associated with a 5 year survival rate of approximately five percent worldwide. All the stages of oesophageal carcinoma carcinoma requires the action of three kinds of carcinogenic factors: **predisposition** (vitamin deficiencies and oesophagitis); mutagens (tannin from cigarettes, alcoholic beverages and nitroso compounds; **promoters** (phorbol esters present in some beverages) (Correa, 1982).

Due to the wide cultural and geographical variation in incidence it is suggested that environmental exposure is casually related to oesophageal cancer (Schottenfeld, 1984; Sons, 1987). Poor parts of the world whose inhabitants share several dietary characteristics are associated with a high incidence of this tumour (Schottenfeld, 1984; Ghadirian *et al.*, 1992). These are people who subsist on a diet high in starch and low in fresh fruit and vegetables, eat rapidly without sufficient mastication, and consume foods and drinks at very high temperatures (Ghadirian *et al.*, 1992). In the Western world, the incidence of oesophageal carcinoma varies among racial and socio-economic groups. Generally, lower socio-economic groups have a higher risk (De Stefani *et al.*, 1990; Breslow *et al.*, 1974). In the USA, blacks

have a relative risk that is fourfold greater than that of whites (Pottern *et al.*, 1981). World-wide men are more frequently affected than women, although sex distribution shows considerable variation (Yang, 1980; Tuyns, 1970). The causes of the higher risk in males is unclear, but probably reflects sex-related differences in diet, smoking and or alcohol intake.

1.3.1 AETIOLOGY

Not a single environmental factor can account for the pattern of oesophageal carcinoma in all high incidence areas (Schottenfeld, 1984). Oesophageal carcinogenesis has a multifactorial aetiology, and is a complex multistep process. Some factors may act in the promotion and progression of the lesion whereas others may be important in the initiation of the neoplastic state. The development of invasive oesophageal cancer results from the synergistic actions between some or many of these aetiological factors. All the stages of oesophageal carcinogenesis require the action of three kinds of carcinogenic factors: predisposition (vitamin deficiencies, such as those of A, B, C, E, riboflavin and niacin, oesophagitis, with small erosions and ulcerations), mutagens (nitroso compounds, tannin from cigarettes, alcoholic beverages, etc.), and promoters (phorbol esters present in some beverages) (Correa, 1982).

Genetic predisposition.

No genetic factor has so far been identified (Schottenfeld, 1984). Ethnicity is a strong indicator of risk for this disease because ethnic groups tend to share similarities in diet and geography. There are, however, several reports that indicate an increased incidence among blood relatives (Pour *et al.*, 1974; Wu & Ran 1979). A follow-up survey of 622 families with a history of oesophageal carcinoma was carried out in China, and the results showed that

offspring from households where one or both parents died from oesophageal cancer showed a higher mortality from the same disease (Hu, 1990). This points to a strong familial tendency with the possible existence of genetically determined susceptibility.

Environmental factors.

Environmental factors appear to play an important role in oesophageal carcinogenesis. In 1966, Burrel and colleagues proposed the *soil theory* to explain the endemic spread of oesophageal carcinoma in the Transkei (Eastern Cape) region of South Africa. Areas of high incidence of this cancer were distinguishable from those of low incidence by differences in either the plantation productivity on the ground or the content of molybdenum and other trace elements in the soil (Yang, 1980; Burrel *et al.,* 1966). High-risk areas were much lower in both the biological and chemical richness of the soil than low risk areas (Yang, 1980; Burrel *et al.,* 1966; Kodama *et al.,* 1992). Some microelements of the soil may play a role in conditioning the cancer risk (Kodama *et al.,* 1992). There was an inverse correlation between mortality rate from oesophageal cancer and soil content of molybdenum, manganese, zinc, magnesium, silicon, nickel, iron, bromium, iodine, chlorine, potassium. sodium, phosphorus and bicarbonate (Yang, 1980). How these substances affect the oesophageal epithelium is not known but, for example, molybdenum is a co-factor of the enzyme nitrate reductase which affects the nitrite and nitrate content in plants (Yang, 1980; Kodama *et al.,* 1992). Increased incidence of oesophageal cancer has been linked to petroleum contamination of water. Mutagenecity testes on water samples with petroleum contamination indicated the presence of possible carcinogens. Concurrent malnutrition, particularly vitamin A deficiency, may further enhance the carcinogenic risk of such pollutants (Amer *et al.,* 1990). A significant association between oesophageal carcinoma and long term occupational exposure to metal dust (beryllium, chromium, chromates and nickel) have been found (Yu, 1988). These metals

have been shown to be risk factors for respiratory cancers in humans, lending some credibility to the hypothesis that the observed association between oesophageal carcinoma and exposure to metal dust is a causal one. Kaminski (1980) identified that there is a possible occupational hazard in vulcanization workers, plumbers and pipefitters.

Nutritional deficiencies and dietary risks

Much evidence suggests that a chronic low intake of several micronutrients, together with an inadequate protein intake, increases the predisposition of oesophageal epithelium to neoplastic transformation (van Rensburg, 1981). Diets deficient in vitamin A, C, E, niacin, riboflavin and zinc have been suggested as risk factors for oesophageal carcinoma (van Rensburg, 1981; Bjelke, 1975; Nettesheim & Williams, 1976). Food habits, such as long-standing simply starchy diet without vegetables, resulting in nutritional deficiencies, and continual physical and thermal irritation of the oesophagus may play an important role in the aetiology of oesophageal cancer (Schottenfeld, 1984; Ghadirian *et al.*, 1992; Negri *et al.*, 1992; Ghadirian, 1987). Foods that provide large amounts of retinol appear to increase the risk of oesophageal cancer substantially, in a dose-dependent manner (Graham *et al.*, 1990). Also, it is calculated that diets associated with a high risk of oesophageal cancer are deficient in riboflavin, nicotinic acid, magnesium and zinc, whereas diets associated with a low risk of oesophageal carcinoma are rich in these substances (van Rensburg, 1981).

Dietary carcinogens.

Experimental and epidemiological evidence suggests that nitrosoamines are involved in the aetiology of oesophageal cancer (Koga *et al.*, 1988; Magee *et al.*, 1967). Diet with a high content of nitrosoamines is an important risk factor in some African and far East countries (Ghadirian *et al.*, 1992). A clear increase in risk has also been observed for those eating

barbecued food daily compared with those eating it less frequently, even after adjusting for meat consumption. Laboratory investigations showed the presence of animal carcinogens and mutagens in barbecued foods formed by the breakdown of proteins (De Stefani *et al.*, 1990).

Mycotoxins

Fungi have also been implicated as aetiological agents in the cancer of the oesophagus (Chang *et al.*, 1992). Studies in the high-risk areas for oesophageal carcinoma in China showed that some common species of fungus, belonging to the genera *Fusarium, Alternaria, Geotrichum, Aspergillus, Cladosporium* and *Penicillium,* are frequently detected in grain (Yang, 1980; Li *et al.*, 1980; Xia & Zhan, 1978; Luo *et al.*, 1990). Of the various infections of the oesophagus, fungal infections, particularly those of *Candida* species, are by far the most common (Chang *et al.*, 1992; Mathieson & Dutta, 1983). Bacteria are normally present in the upper gastrointestinal tract, including the oesophagus. Deregulation of the normal flora may increase the production of nitrites and nitrates. Salivary nitrates in patients with marked epithelial dysplasia or oesophageal carcinoma were significantly found to be higher than in normal controls (Chang *et al.*, 1992).

Alcohol and tobacco use

The role of alcohol and tobacco abuse in the aetiology of oesophageal carcinoma is well established (Yu *et al.*, 1988; Wynder *et al.*, 1961; Graham *et al.*, 1990; Franceschi *et al.*, 1990; De Stefani *et al.*, 1993). It has been estimated that more than 80 per cent of cases in industrialised countries can be attributed to exposure to these two environmental factors, either singly or jointly (Schottenfeld, 1984; Tuyns, 1970; Wynder *et al.*, 1961; Negri *et al.*, 1992; Tuyns, 1983; La Vecchia & Negri, 1989). Results obtained from well-designed case-controlled studies report an association between cigarette smoking and risk of oesophageal

carcinoma (Pottern *et al.*, 1981; Sankaranarayanan *et al.*, 1991; Negri *et al.*, 1992; Cheng *et al.*, 1992). The tumourigenic activity of tobacco smoke is contained in the particulate matter, or the tar fraction. The primary initiators are believed to be the polynuclear aromatic hydrocarbons, such as benzo(e)pyrene and volatile nitrosoamines (Table 2) (De Stefani *et al.*, 1990; Schottenfeld, 1984). Pipe and cigar smokers have a greater risk of cancer of the oral cavity, the oesophagus than cigarette smokers. It has been proposed that pipe tobacco residues are swallowed into the oesophagus, allowing close contact of the tobacco carcinogens with the oesophagus (De Stefani *et al.,* 1990; Wynder *et a.l,* 1961; De Stefani *et al.,* 1993; Tuyns, 1983; La Vecchia & Negri, 1989). Hand-rolled cigarette smoking is associated also with a higher risk of oesophageal carcinoma, compared with the use of commercial cigarettes (De Stefani *et al.,* 1993). The risk increases with increasing number of cigarettes and duration of the smoking habit (Schottenfeld, 1984; Pottern *et al.,* 1981; Tuyns, 1970; Yu *et al.,* 1988; Tuyns, 1983; De Stefani *et al.,* 1993; La Vecchia & Negri, 1989). Differential risks for oesophageal carcinoma are observed also with different types of alcoholic drink (Breslow *et al.,* 1974; Pottern *et al.,* 1981; Wynder *et al.,* 1961; Yu *et al.,* 1988). For any given level of ethanol intake, the risk from spirits is usually more than twice that from beer; the risk from wine is intermediate between that from spirits and that from beer. Whatever the type of alcohol consumption, however, there is a highly significant correlation with the average daily intake of alcohol (Breslow *et al.,* 1974; Pottern *et al.,* 1981; Wynder *et al.,* 1961; Yu *et al.,* 1988; Chyou *et al.,* 1995; Martinez, 1969). The exact mechanism responsible for the increased risk in alcohol drinkers is not known (Schottenfeld, 1984; van Rensburg, 1981; Palmer, 1978). The independent importance of both tobacco and alcohol as carcinogens of the oesophagus have been established (Tuyns, 1983; La Vecchia & Negri, 1989; Cheng *et al.,* 1995). In addition to their effect as independent risk factors,

alcohol and tobacco exert a synergistic action by potentiating each other (Pottern *et al.,* 1981; Tuyns, 1970; Tuyns, 1983).

Table 2 Summary of the carcinogenic actions of ethanol and smoking

Alcohol	Smoking
1. Direct local action by carcinogens (nitrosoamines, oils, Polycyclic hydrocarbons, etc.)	1. Direct local action by carcinogens (aromatic hydrocarbons, volute nitrosoamines, etc.)
2. Interaction with other agents (tobacco)	2. Exposure to heated smoke
3. Induction of nutritional deficiencies (riboflavin, nicotinic acid, iron, zinc, etc.)	
4. Decreased chemical detoxification and biotransformation due to liver injury.	
5. Impairment of immune system	

Infective factors

The chronic inflammatory processes caused by infectious micro-organisms may result in sustained regenerative proliferation of cells, and increase the risk of cancer development (Chang *et al.,* 1992). An example of a malignant tumour with a possible viral aetiology is shown by the striking geographical distribution of oesophageal carcinoma (Syrjanen *et al.,* 1982; Chang *et al.,* 1990). Human papillomavirus (HPV) infection has been found in chronic oesophageal irritation, papilloma and oesophageal carcinoma (Chang *et al.,* 1992; Syrjanen *et al.,* 1982; Chang *et al.,* 1990; Winkler *et a.l,* 1985; Kulski *et al.,* 1986; Hille *et al.,* 1986; Chang, 1990; Toh *et al.,* 1992; Benamouzig *et al.,* 1992). DNA hybridization studies and polymerase chain reaction techniques disclosed HPV DNA sequences in both benign and malignant oesophageal lesions (Kulski *et al.,* 1986; Chang *et al.,* 1990; Benamouzig *et al.,*

1992). Additionally, *in vitro* and *in vivo* experimental studies have confirmed the oncogenic properties of HPV type 16 and 18 (Benamouzig *et al.,* 1992; Galloway & McDougall, 1989). Protein of HPV types 16 and 18 E6 binds to the tumour-suppressor protein p53 and promotes its degradation (Werness *et al.,* 1990

Gastric surgery

Gastrectomy may cause gastric hypoacidity and favour oesophageal reflux, leading to the development of Barrett's oesophagus and adenocarcinoma of the oesophagus. Increased risk has been reported from several cohort and case-control studies (Caygill *et al.*, 1987; Tersmette, *et al.*, 1990; La Vecchia *et al.*, 1994). These findings have suggested that the gastric stump might produce circulating carcinogens such as nitroso-compounds, which act locally as carcinogens and promote bacterial overgrowth (Tersmette, *et al.*, 1990; La Vecchia *et al.*, 1994).

1.3.2 Associated diseases

1.3.2.1 Schlerodema

Reports of several isolated cases of oesophageal carcinoma associated with schlerodema have raised the possibility of an increased risk of cancer in these patients. The duration of schlerodema before the diagnosis of the oesophageal cancer is generally over 10 years and most of the carcinomas are of squamous cell type (Whitacker & Bishop, 1979). The prognosis is very poor, with an expected survival of less than 6 months after diagnosis (Segel *et al.,* 1984; Cohen, 1979). Cases of adenocarcinoma have also been recently described (Halpert *et al.,* 1983). Gastroesophageal reflux and peptic oesophagitis may result from a weakened lower oesophageal sphincter and delayed gastric emptying (Maddern *et al.,* 1984).

1.3.2.2 Secondary primary tumour.

Oesophageal carcinoma is also frequently associated with other synchronous or metachronous tumours in the upper digestive tract (Slaughter, 1946; Gluckman *et al,* 1980; Shons & McQuarrie, 1985; Blot *et al.,* 1988; Atabek *et al.,* 1990; Ina *et al.,* 1994; Day *et al.;* 1994; Shibuya *et al.,* 1995; Moore, 1971; Schottenfeld *et al.,* 1974; Ribeiro *et al.,* 1996). The risk may be directly proportional to the amount and duration of exposure to carcinogens. About 40 per cent of patients with primary cancers of the oral cavity, pharynx and larynx, who continue to smoke develop a second primary tumour, while only 6 per cent who stopped smoking after onset of the first lesion do so (Moore, 1971). The risk of developing a second primary cancer is enhanced significantly by more intensive, combined exposure to tobacco and alcohol before identification of the primary cancer (Day *et al.,* 1994; Schottenfeld, 1974). When comparing former with current cigarette smokers, prospective studies have generally indicated a 25-50 per cent reduction in the risk of cancer (Day *et al.,* 1994; Schottenfeld, 1974). The field effect theory has been advanced to explain these so-called synchronous and metachronous carcinomas (Slaughter, 1946). This model predicts that tumours will develop from the genotoxic effects of carcinogen shared by many cells in the field. The entire mucosa of the upper gastrointestinal tract, when exposed to such irritants as tobacco smoke and alcohol, is at risk of developing cancer. Because of the clear risk for metachronous cancers, it is recommended that all patients with tumours of the upper gastrointestinal tract undergo close medical follow-up over a long period of time and this should allow early detection of second primary cancers, especially tobacco-related lesions (Ina *et al.,* 1994).

1.3.2.3 Chronic oesophageal irritation.

Chronic physical and or chemical irritation of the oesophageal mucosa is frequently reported by patients who developed oesophageal carcinoma. Ingestion of very hot beverages has been shown to increase the risk of oesophageal cancer (Yang, 1980; Martinez, 1969; De Jong *et al.,* 1974; Cook-Mozaffari *et a.l,* 1979; De Stefani, 1990; Victoria *et al.,* 1990; Khuroo *et al.,* 1992; Chang *et al.,* 1992; Segi, 1975). Irritation of the oesophagus by curry or spicy seasonings has been considered as possible risk factor in oesophageal cancer (Ghadirian *et al.,* 1992). Partially chewed food swallowed rapidly may cause continuous physical irritation to the oesophagus. Epidemiological studies have revealed that most patients with oesophageal carcinoma eat their food very quickly without chewing it well (Ghadirian *et al.,* 1992).

1.3.2.4 Achalasia

Achalasia is a motility disorder caused by degeneration of the intrinsic autonomic nerves in the wall of the oesophagus and lower oesophageal sphincter, and this results in decreased or absent peristalsis in the smooth muscle portion of the oesophagus and impaired relaxation of the lower oesophageal sphincter (Goodman *et al.,* 1990). The tumour usually develops in the dilated portion of the middle and lower thoracic oesophagus (Wychulis *et al.,* 1971; Hankins *et al.,* 1975; Carter *et al.,* 1975). Most oesophageal cancer complicating achalasia is squamous cell carcinoma, although adenocarcinoma associated with Barrett's oesophagus has been reported following surgical myotomy (Goodman *et al.,* 1990; Jaakkola *et al.,* 1994). It is believed that development of the tumour is related to prolonged contact of certain elements of often partially degraded ingested food with the oesophageal mucosa and this results in

increased bacterial growth and chemical irritation that culminates in chronic oesophagitis (Wychulis *et al.,* 1975; Aggestrup *et al.,* 1992).

1.3.2. 5 Chronic lye strictures

Late complications of caustic burns in the oesophagus form scarring srictures and the development of cancer (Schottenfeld, 1984; Gerami *et al.,* 1971; Appelqvist & Salmo, 1980). The risk of oesophageal cancer is approximately 1000 fold greater than for others in the same age group (Appelqvist & Salmo, 1980; Hopkins & Postlethwait, 1981). The average for the development of cancer after caustic injury is about 50 years, compared with approximately 60 years for oesophageal cancer without previous exposure (Appelqvist & Salmo, 1980, Hopkins & Postlethwait, 1981; Bigelow, 1953). The scarring stricture is usually found in the area of the tracheal birfucation and the carcinoma usually develops in the stricture (Sons, 1987; Appelqvist & Salmo, 1980; Hopkins & Postlethwait, 1981; Bigelow, 1953).

1.3.2.6 Oesophageal diverticuli

Malignant transformation in a chronic pharyngo-oesophageal diverticulum is less common (Wychulis *et al.,* 1969; Nanson, 1976). Many authors have speculated that the chronic irritation and inflammation resulting from retained food and secretions in these large pouches are contributing factors in the development of carcinoma (Wychulis *et al.,* 1969; Nanson, 1976).

1.3.2.7 Irradiation and chemotherapy.

Therapeutic irradiation is well known to be associated with an increased risk of neoplasm development in organs within the irradiated field (Goffman *et al.,* 1983; Vanagunas *et al.,* 1990; Brink *et al.,* 1994; Gates & Warren, 1968; Mullen *et al.,* 1979; Sherrill *et al.,* 1984;

Goolden, 1957). The effects of irradiation appear to differ according to the age and time of exposure, dose, duration of radiation and the tissue irradiated. Chemotherapeutic agents have also been known to cause certain tumours (Mullen *et al.,* 1979; Sherrill *et al.,* 1984; Goolden, 1957). Systemic therapy may promote radiation-induced cellular injury or interfere with cellular repair mechanisms.

1.3.2.7 Injection schlerotherapy.

Several isolated cases of oesophageal cancer following endoscopic schlerotherapy have been reported (Kokudo *et al.,* 1990; Wu & Ran, 1979). The middle and lower third of the oesophagus are preferential sites for the development of tumours. Prognosis is generally very poor because of advanced carcinoma and accompanying liver dysfunction (Kokudo *et al.,* 1990). It is also believed that an association with other risk factors such as age, alcohol-tobacco intoxication, or Barrett's oesophagus exists (Guillemot *et al.,* 1988).

1.4.1 HYPOTHESIS

Tissue kallikrein and the kinin B_2 and B_1 receptors are expressed in oesophageal carcinoma.

1.5.1 OBJECTIVE

We believed that a comprehensive study involving the cellular localization and molecular characterization was necessary to be able to elucidate the role of tissue kallikrein and the kinin receptors in oesophageal carcinoma. The experimental findings may suggests a role for the kallikrein-kinin system in malignant disease.

1.6.1 AIMS OF THE PRESENT INVESTIGATION

The aims of this study was to:

1. Examine the question whether a similar expression occurs in patients with oesophageal carcinoma. The concentration and the amidase and kininogenase activities of urinary tissue kallikrein was determined in three patient groups. Evidence suggests holistic changes in tissue kallikrein in patients with carcinoma.
2. Determine enzymic activity (amidase assay), concentration (enzyme-linked immunoassay) and kinin formation (kininogenase assay) by tissue kallikrein of tumour extracts.
3. Examine cellular localisation and molecular properties of tissue kallikrein in patients with oesophageal carcinomas, other carcinomas (non-oesophageal) and no cancer.
4. Determine the cellular orientation of TK, and kinin B1 and B2 receptors in oesophageal carcinoma.
5. Examine the expression of tissue kallikrein and the kinin receptors in oesophageal carcinoma.

CHAPTER 2: MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

2.1.1 Resections

Resection specimens of oesophageal carcinoma were collected on ice from the surgical theatres at King George V Hospital, following oesophagectomy, and processed immediately. Tissue for RNA extraction was snap frozen in liquid nitrogen, and then transferred to an ultralow freezer (NUAIRE) until used.

2.1.2 Biopsies

Biopsy specimens of oesophageal carcinoma were collected at King George V Hospital on ice, and at once fixed in 5% formaldehyde/0.9% saline (v/v). Tissues were then wax embedded and stored at room temperature for future microscopical and immunohistochemical studies.

2.1.3 Urine

Urine samples were collected from oesophageal carcinoma patients, at King George V Hospital and from at King Edward V111 Hospital. The urine from non-oesophageal and non-cancer patients was collected in appropriate specimen bottles, transported on ice and processed quickly.

2.2 MEASUREMENT OF TK IN TISSUE EXTRACTS AND URINE SAMPLES

2.2.1 Sample processing and storage

2.2.1.1 Urine samples

Urine samples for TK, kinin ELISA and amidase assay, were processed as in schedules 1 and 2. After processing the urine samples were stored at -20°C until they were used.

2.2.2 Assays

2.2.2.1 Amidase assay (Schedule 3).

This is a colorimetric assay for the measurement of enzymatic activity of TK in biological samples, as well as in extracts prepared from oesophageal samples, by an end point microassay using a microtitre plate. The concentration of active TK was measured by assessing the activity of the enzyme on the selective, synthetic substrate, H-D-Val-Leu-Arg-pNA (S2266, Kabivitrum, Sweden) (Amundson, 1979) in the presence of SBTI (Soybean trypsin inhibitor, 0.3 ηg/μl) as modified by Figueroa *et al.*, 1989, and further developed as an end point assay in a microtitre plate by Rahman *et al.*, 1994. For this amidase assay, which involves the release by TK from its selective substrate D-ValLeuArg-pNA, para-nitroaniline (pNA) that has a spectral absorbance at 405 nm, a microassay standard curve was constructed using HUK (Calbiochem, Lucerne). This standard curve was used to calculate in ηg/ml the concentration of TK in the tissue sample extracts. Following the measurement of total protein in an aliquot of the extract, using the Bradford assay (Bradford, 1976) (see schedule17), the enzymic activity of TK was expressed specific activity in ηg TK/ug protein. A control urine sample was included in each run to determine inter-assay coefficient of variation. During each assay, 2 sets of microtiter plates

were processed simultaneously, one being the measurement of zero blank activity of TK, and the other measuring the enzymic activity of TK after a 3 h incubation. All determinations were made in triplicate and the experimental procedure is listed in schedule 16.

2.2.2.2 Enzyme linked immunoassay, ELISA (Schedule 4).

The basic principle of the ELISA is to use an enzyme label to quantify the binding of an analyte, using a solid-phase system for the separation of bound and free portions. First described by Engvall and Perlman (1971) to indirectly measure antibodies using antiglobulins as indicators, it has undergone continuous modification and refinement, including the use of the avidin-biotin binding system (Kendall et al, 1983; Bayer and Wilchik, 1974). Although the ELISA is reported to be almost equal to RIA (radioimmunoassay) and IFA (immunofluorescent assay) it has the advantage of being non-isotopic, safe, flexible, convenient and fast (Voller *et al.*, 1978). The utilisation of an indirect, non-competitive assay 'sandwiches' the analyte between two macromolecules, one of which is linked to an enzyme label (Nilsson, 1990). In this heterogeneous enzyme immuno-assay, the amount of antigen attached is indicated by an enzyme-labelled immunoglobulin and enzyme substrate system whereby the amount of substrate by-product is colorimetrically determined.

An aliquot of the filtrate was used to measure total TK (in triplicate) by a sandwich ELISA using goat anti-human rTK IgG and rabbit anti-human rTK IgG. A third antibody, anti-rabbit IgG (Sigma Chemicals), which interacts with the rabbit anti-human rTK IgG, was conjugated with the enzyme alkaline phosphatase, which acts on the chromogenic substrate disodium p-nitrophenyl phosphate (pNPP) (see schedule 5).

2.2.2.3 Kinin generation assay (Schedule 5).

The kininogen content of the urine samples was determined by initially generating the release of its kinin moiety with endogenous TK followed by the quantitative analysis of the released kinin by ELISA. Activation of the endogenous TK in the sample was achieved by incubating equal volumes of urine sample and generating cocktail for 1 h at 37°C in the presence of kininase inhibitors to limit the degradation of the generated kinin. The released kinin was then extracted by acid ethanol (absolute ethanol/ 0.003% HCl) and measured by a competitive ELISA following overnight incubation with a standard amount of monoclonal mouse anti-BK IgG (SBK1) at 4°C, to bind the kinin released. Known amounts of standard BK (1.25 - 150 ng BK/ml, Sigma Chemicals) were also allowed to react overnight with standard amounts of SBK1. The wells of a Nunc Immulon Maxisorp™ ELISA plate were coated with a standard amount of BK conjugated to cytochrome C using the linker SPDP (the kinin-SBK1 incubates were then added to each well and incubated for 3 h at 37°C so that any remaining free antibody could react with the BK-SPDP(N-succinimidyl 3-[2-pyridyldithio]propionate)-cytochrome C conjugate bound to the ELISA plate. The secondary antibody was an anti-mouse IgG (Sigma Chemicals) labeled with the enzyme alkaline phosphatase, which converts the colourless disodium p-nitrophenyl phosphate (pNPP, Sigma Chemicals) to a yellow chromogen that has a maximal absorbance at 405 nm. The absorbance values obtained for the standard BK was used to plot a standard curve of absorbance versus log concentration from which the basal and generated kinin concentration of the tissue extracts were determined. Similar measurements were performed on control urine samples during each run in order to determine the inter- and intra-assay coefficients of variation.

An important aspect of this assay is the use of two cocktails A and B. Firstly, an aliquot of all samples and control urine are stored in inhibitor cocktail which contained aprotonin (0.8 mg/ml urine), an inhibitor of TK; and SBTI (4 mg/ml), which by inhibiting other trypsin-like proteases

prevents the release of kinin from endogenous kininogen; captopril (2 μg/ml), an inhibitor of the KII family of kininases ensures that the basal kinins present in the samples are not destroyed, and phosphoramidon (0.001 mg/ml urine) another kininase inhibitor. The sample in cocktail A provided the basal kinin value.

A second aliquot of sample and control urine was stored in kinin generating cocktail which was similar to the inhibitor cocktail, except that it lacked the protease inhibitors, aprotonin and SBTI. Their absence ensured the release of kinin from endogenous kininogen in the sample. The presence of captopril (0.43 ng/ml urine) in this cocktail protected the released kinin against degradation by kininases. The sample in the cocktail B provides the generated kinin value.

2.2.2.4 Measurement of protein (Schedule 6)

An aliquot of each sample was used for protein determination (in triplicate) using the Bradford micro assay (Bradford, 1976). The net enzymic activity of TK was expressed as a ratio of the amidolytic activity and the protein concentration (ηg TK/μg protein). To determine the protein concentration a 1 mg/ml protein standard, bovine serum albumin (BSA, fraction V, Boehringer Manheim, Germany) was made up in distilled water. This was serially diluted down to 0.016 mg/ml in distilled water. 30 μl of sample was also added (in triplicate) to wells of a tissue culture plate (Corning Cell Wells™, Corning, USA0. For blanks, 30 μl of distilled water was added (in triplicate) to the plate. 30 μl of sample was also added (in triplicate) to the tissue culture plate. Next, 300 μl of the Bio-rad Protein Assay Reagent (diluted 1:5 in distilled water, Bio-rad, UK) was added to each well containing standard, blank and sample, and the absorbance read at 595 ηm on a Bio-rad Microplate reader 3550 (Bio-rad, UK). The mean blank absorbances were subtracted from each standard and sample absorbance. A standard curve plot

of absorbance versus concentration was set up using the Bio-rad Microplate Manager software and the protein concentrations of each sample were extrapolated from this. The method is simplified in Schedule 7.

2.3 HISTOPATHOLOGY

2.3.1 Haematoxylin and eosin (H&E) staining of wax embedded tissue (Schedule 7).

About 3-5 μm sections of wax embedded tissue were cut on a microtome (Leica Jung), RM2035), and adhered onto poly-L-lysine coated slides. The sections were stained with haematoxylin and eosin (H&E) to determine the histological diagnosis for the resection and biopsy specimens, and to ensure that tissue processing was performed under optimal conditions. Modified Erlich's staining method was performed at RT as listed schedule 8.

2.3.2 Immunocytochemistry

Resection and biopsy specimens and the isolated neutrophils were processed for the cellular localisation of TK and kinin receptors.

2.3.2.1 Tissue fixation and wax embedding for conventional light and confocal microscopy (Schedule 8)

Fresh tissue of about 0.5 cm^3 from resection specimens and biopsy specimens were fixed in 5% formal saline (35% formaldehyde/0.9% NaCl, 1:7 v/v) for 24 h. About 5 mm thick sections from resection and biopsy specimens were orientated and placed in tissue cassettes. These tissue samples were dehydrated and embedded in paraffin wax using absolute ethanol, xylene and wax

under sterile conditions in an automatic tissue processor (Shandon) so that samples could be used for both in situ hybridisation (ISH) and light microscopy. The automated schedule of steps outlining the fixation, dehydration, clearing, infiltration and embedding carried out at the Department of Histopathology, University of Natal.

2.3.2.2 Immunolocalisation-DAB (Schedule 9)

Paraffin waxed sections were dewaxed with xylene, rehydrated through high grade of ethanol to low grade of ethanol and finally in distilled water. The tissue was then boiled in 0.1 M Sodium citrate, pH 6.0 in a microwave oven (Sharp R-4A52) at high watt for 3 min and at low watt for 5 min for antigen retrieval and allowed to cool at RT for 20 min. Endogenous peroxidase was inactivated by incubating with 5% H_2O_2 in absolute methanol for 30 min. Then sections were incubated with DIG-blocking agent (DIG wash and blocker set, Boehringer, Mannheim/Germany) for 30 min at RT to prevent non-specific binding. Following this sections were probed for the presence of immunoreactive TK, TproK and kinin B1 and B2 kinin receptors using specific antibodies. After overnight incubation at 4^0C, the sections were thoroughly washed and incubated with biotinylated secondary antibody followed by Streptavidin-peroxidase complex (Dako LSAB Kit Peroxidase, Dako Corporation, USA). The enzyme-substrate reaction was detected by the chromogen, diaminobenzidine. After immunostaining, tissue sections were lightly counterstained with Harris Haematoxylin, dehydrated and mounted in Entellan (Merck, Germany). For control Primary antibody was pre-absorbed with an excess of antigen.

2.3.2.3. Immunolocalisation-FITC (Schedule 10)

After blocking non-specific binding with DIG blocker for 30 min at RT, tissue was probed with primary specific antibodies against TK, TproK and kinin B1 and B2 and incubated at 4^0C overnight. After jet washing, tissue was incubated with an FITC conjugated secondary antibody diluted 1:32 for 1hr at RT. Slides were mounted with fluorescent mounting medium and viewed under Leica TCS TD-4-true confocal microscope and counted number of pixels versus fluorescence staining intensity.

2.3.3 Immunocytochemistry

2.3.3.1 Antibody profiles

2.3.3.1.1.Anti-rTK antibody.

Recombinant TK was obtained from DR M Kemme (Institute for Biochemistry, Technical University of Darmstadt, Germany). The antibody was produced in a goat host using 100 μg of recombinant tissue kallikrein conjugated to 125 μl TiterMaxTM adjuvent injected intra-muscularly at four week intervals over a four month period. Isolation and characterisation of IgG was performed according to the method described by Johnstone and Thorpe (1982). Aliquots (30 μl) were stored at 20°C and reconstituted when required. On immunoblotting, single band reaction with recombinant TK and no reaction was shown against plasma kallikrein (Naidoo *et al.,* 1999).

2.3.3.1.2 Anti-tProk antibody

The antibody to recombinant TK (rTK) detects both the active and pro-forms of the enzyme. To generate a specific antibody that discriminated between these two forms, the peptide Cys-

Ala1-Pro-Pro-Ile-Gln-Ser-Arg^{7}-Ile-Val-Gly^{10} (CAP 11) covering the aminoterminal residues of prokallikrein, namely the first 7 residues of the peptide (Ala^{1} to Arg^{7}), followed by the first residues (Ile^{8} to Val^{10}) of mature enzyme was synthesised (Angermann *et al.*, 1992). An additional Cys residue was introduced at the aminoterminus of the synthesised peptide for coupling of the peptide to the carrier protein, key hole limpet haemocyanin (KLH). This conjugate was used for the production of antisera to CAP 11 in rabbits. The antibody to CAP 11 specifically reacted with TproK.

2.3.3.1.3 Anti-B1 receptor antibody

Fred Hess (Merck Research Laboratories, R80M-213, Rahway, NJ 07065), kindly supplied an antibody directed at the C-terminus peptide of kinin B1 receptor (I-S-S-S-H-R-K-E-I-[F-Q-L]-F-W-R-N) which was characterised for specificity.

2.3.3.1.4 Anti-B2 receptor antibody

Eight polyclonal antipeptide antibodies to the transmembrane loop of the receptor (fully characterised for specificity) raised to synthetic peptides of the aminoterminal and loop regions encoded by the rat B2 receptor cDNA, and was based on the homology between these regions with the human receptor. However, only four were shown to react strongly with human epithelial cells and neutrophils (Haasemann, *et al*, 1994). The match between the peptide sequences of intracellular domains one (ID1, LHK) and two (ID2, DRY) and the fourth extracellular domain (ED4, DTL) in the rat and human receptors was 80%, 100% and 75% respectively. It was therefore decided to perform experiments with a combination of antibodies directed to these regions. The antibodies were kindly donated by Werner Muller-Esterl (Department of Pathobiochemistry, Johannes Gutenberg-University of Mainz, Duesberg

6, D-6500 Mainz, Germany). The antibodies were fully characterised for specificity (Henderson *et al.,* 1994).

2.3.4 Immunolocalisation of TK, TProK, kinin B1 and B2 receptors by conventional light confocal (laser scanning) microscopy

Thin sections (3 μm) of the wax embedded tissue were adhered onto adhesive coated (poly-L-lysine, Sigma Chemicals) slides and used for the detection of immunoreactive TK, TProK, kinin B1 and B2 receptors. In the immunolabelling techniques, polyclonal goat anti-human rTK IgG, rabbit anti-human CAP 11 (TProK), rabbit anti-human kinin B1 and B2 receptor were used as primary antibodies, and secondary antibodies were conjugates of either the peroxidase-antiperoxidase (PAP) immunoenzyme complex with diaminobenzidine immunoprecipitation as the chromogen visualised by light microscopy (schedule 9), or linked to a fluorescent probe visualised by confocal microscopy (schedule 10).

2.3.4.1 Immunocytochemistry controls

2.3.4.1.1 TK positive control

Since TK is abundant in the duct cells of human salivary glands, samples of fresh normal human salivary gland were collected at post mortem, fixed in 5% formaldehyde/0.9% NaCl (v/v) and embedded in paraffin wax. During each labelling run, this appropriate positive control tissue, demonstrated the presence of TK in the ducts of the human salivary gland (Schachter, 1978; Simson, 1988).

2.3.4.1.2 Tissue prokallikrein (TProK) positive control

Since TProK is abundant also in the human salivary gland, samples of fresh normal human salivary gland were collected at post mortem, fixed in 5% formaldehyde/0.9% NaCl and embedded in paraffin wax. During each labelling run, this appropriate positive control tissue, demonstrated the presence of TProK in the tubules of the human salivary gland.

2.3.4.1.3 B1 receptor positive control

The B1 receptor is abundant in neurons of the human spinal cord and therefore fresh normal spinal cord samples were collected at post mortem, fixed in 5% formadehyde/0.9% NaCl and embedded in paraffin wax. During each labelling run, this appropriate positive control demonstrated the presence of the B1 receptor in neurons of the human spinal cord.

2.3.4.1.4 B2 receptor positive control

Sine the B2 receptor is abundant in human salivary glands, samples of fresh normal human salivary glands were collected at post mortem, fixed in 5% formaldehyde/0.9% NaCl and embedded in paraffin wax. During each labelling run, this appropriate positive control tissue, demonstrated the presence of B2 receptor in the ducts of the human salivary gland.

2.3.4.2 Negative control

The loss of immunolabelling following preabsorbtion of the primary antibody with an excess of human urinary kallikrein (HUK) demonstrated the specificity of the antibody utilised. The goat anti-human rTK antibody was diluted 1:2500 with 1 mM phosphate buffered saline, pH 7.2 (PBS), 25µl of that was added to 200 ul HUK 5 µg/ml and 175 ul of PBS to yield a final

concentration of 2.5 ηg antibody (anti-human rTK) per ml and 2.5 ug antigen (HUK) per ml. This was vortexed and incubated over a week-end or for 36 h at 4°C to allow formation of antigen antibody complexes. Following centrifugation at 10 000 rpm for 15 min at 4°C (Heraeus, Biofuge 13R) 100 ul of the supernatant replaced the primary antibody in the immunolocalisation procedure. For the negative controls of the kinin receptor antibodies, primary antibodies were replaced with non-immune serum or omission and replacement by PBS (Henderson *et al.,* 1994).

2.3.5 Photomicrography

Sections were examined and areas of interest photographed with a Nikon binocular Optiphot photomicroscope with objective magnifications ranging from x 10 to x 100.

2.3.6 Image analysis and statistics

Semi-quantitative estimates of immunolabelled of TK, Tprok, B1 and B2 receptors, as well as their respective mRNAs expressed immunolabelled proteins were determined by image analysis. The mean high intensity immunolabelling in (n) number of cells was quantified in pixels/μm^2 using the Analysis 2.1 Pro-System (Soft-Imaging Software GmbH, 1996, Münster, Germany).

2.4 DETECTION OF TK MESSENGER RNA BY IN SITU HYBRIDISATION (ISH) (SCHEDULE 12)

2.4.1 Cleaning and sterilisation of ISH apparatus

All glassware and plastic-ware for ISH use were treated as follows to obtain clean and RNAse free working conditions:

2.4.1.1 Coating of glass slides

Before tissue sectioning, the microtome, water bath and all other apparatus were cleaned with ethanol and sprayed with RNAase away (RNAses and DNAses decontaminant). The water bath was filled with 0.1% DEPC (diethyl pyrocarbonate, Sigma Chemicals) water and maintained at 37°C. About 4 μm thick sections were cut, picked up on the silane (3-aminpropyltriethoxysilane, Sigma Chemicals) pre-treated glass slides and dried at 55°C for 2 h followed by 37°C overnight. These were stored in a clean dust-free slide box until used for ISH.

2.4.2 Probe preparation

cDNAs which had been subcloned into the Not 1/Cla 1 site of the plasmid, Bluescript (Stratagene), linearized with the appropriate restriction enzymes were received from Dr. J. Clements (Prince Henry's Institute of Medical Research, Australia) in an aqueous salt/ethanol suspension. They were centrifuged at top speed in a microfuge (Biofuge 13R, Heraeus) for five min to precipitate the DNA. The supernatant was discarded and the walls of the microfuge tube rinsed down with 70% ethanol (to ensure minimal losses after transit) and recentrifuged. The DNA pellet was air dried (tube upside down on bench for an hour) and the DNA was reconstituted in 20 μL (~1 μg/μl concentration) deionized, distilled and filtered water. The probes were then ready to label with non-radioactive UTP (uridine triphosphate), from

Boehringer, to give antisense (positive detection of mRNA) or sense (negative control) cRNA probes.

2.4.2.1 Generation of Digoxigenin (DIG) labelled cRNA sense and anti-sense transcripts by in vitro transcription using hKLK1 (TK) cDNA

To generate DIG labelled cRNA sense and anti-sense transcripts to hKLK1 mRNA, approximately 1 μg of linearised purified bluescript plasmid containing the hKLK1 gene was used as the template incubated with a mixture of NTPs (nucleotides) containing DIG-UTP (uridine triphosphate) for 2 h at 37°C with the following transcription enzymes:

T7 RNA polymerase (Boehringer Manheim) for human AS probe

T3 RNA polymerase (Boehringer Manheim) for human S probe

To determine the specificity of the labelling reaction, a DIG labelled rat antisense cRNA probe was also generated under the same conditions using SP6 RNA polymerase (Boehringer Manheim) and control DNA PSPT 18-Neo/Pvu II supplied in the DIG labelling kit (Boehringer Mannheim).

To prepare the reaction mixture for DIG labelling of the antisense, sense and control cRNA probes, the following reagents were added in order into 3 autoclaved 0.5 ml eppendorfs according to the procedure described by Boehringer Mannheim, Cat No. 1 175 025 and tabulated in schedule 12.

2.4.2.2 Estimation of minimal probe concentration that can be detected

To determine the minimal dilution of DIG labelled cRNA sense and anti-sense transcripts, generated in section 2.5.2.1, that can be detected with the DIG detection system (sheep anti-

DIG IgG conjugated to alkaline phosphatase and NBT/BCIP(nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate) we used the method described in the DIG users guide (Boehringer Mannheim). 10 fold serial dilutions of these transcripts and DIG labelled control RNA (10 µg/100 µl, provided in the DIG RNA Labelling Kit, Boehringer Mannheim) were prepared with DEPC treated dH_2O as indicated in schedule 14.

2.4.2.3 In situ hybridisation

In order to establish tissue specific expression of TK in oesophageal carcinoma samples, we had to first establish tissue specific expression of TK in human salivary gland, a tissue known to contain a large amount of TK, we attempted to detect and localise the mRNA encoding TK by *in situ* hybridisation.

Pre-hybridisation of tissue samples

A fresh sample of 4% PFA (paraformaldehyde) was prepared at the beginning of each run by dissolving 4g PFA in 100 ml 0.1 M phosphate buffer by gentle stirring and heating on a magnetic stirrer hotplate (SM26, Stuart Scientific) placed in the fume hood. The heating plate was set not to exceed 60°C. When the temperature of the solution reached 60°C all the PFA dissolved and the solution became totally clear, it was covered with parafilm, transferred to the refrigerator and maintained at 4°C until used in step 7. While the PFA was being prepared we commenced with the dewaxing, rehydration and pre-hybridisation treatment of the tissue sections as stated in the schedule.

Hybridisation

From section 2.4.2.2 the minimal dilution of both antisense and sense cRNA hKLK1 probe that could be detected was found to be 1 ng/µl and 100 pg/µl respectively. The probes, stored at -70°C, were thawed slowly in ice and 1 µl (200 ηg) of each probe was added to 150 µl hybridisation buffer. The diluted probes were denatured by heating in a boiling water bath for 5 min followed by rapid cooling on ice. 75 µl of this, containing 100 ηg probe, was spread over each section limited by an adhesive square and covered with plastic. To further limit evaporation of the hybridisation mixture a solution of 5XSSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0) buffer and 50% formamide was poured into the hybridisation chamber. Using the Hybaid Omnislide Flat Block Humidity Chamber (Hybaid Ltd.,UK) the optimal hybridisation temperature for all tissues was found to be 55°C while the optimal hybridisation time for salivary gland was 20 h and for oesophageal tissue 44 h.

Post-hybridisation washes and detection of bound probes

After the appropriate hybridisation time, the slides were taken off the Hybaid Omnislide Flat Block Humidity Chamber and the plastic covers were removed. Excess probe was washed off with various concentrations of SSC and after slides were mounted with aqueous glycerol jelly and viewed under a Leica DMLB microscope and images captured with a Sony Power HAD 3CCD colour video camera (Model DXC-950P, Sony corporation, Japan). were analysed for intensity of labelling using the Analysis 2.1 Pro system (Soft-Imaging Software GmbH, 1996, Germany). The post hybridisation procedure was as tabulated in schedule 16.

2.5 DETECTION OF THE TK (HKLK1) GENE EXPRESSION BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

To study the differential expression of genes in tissues we study the RNA by PCR. To apply the PCR methodology to the study of RNA, the RNA sample is first transcribed to cDNA, thereby providing the necessary DNA template for PCR. This process is known as Reverse Transcription Polymerase Chain Reaction (RT-PCR). The PCR products are then southern blotted and probed for the presence of a specific gene (TK gene)

The primers used are either random primers, oligo (dT) primers or sequence specific primers. RNA template can be total RNA or poly $(A)^+$ RNA but must be intact and free of contaminating genomic DNA.

2.5.1 Toatal mRNA extraction

Total mRNA was extracted using the Boehringer Tripure RNA isolation (schedule 12).

2.5.1 1 Determination of RNA purity

The purity of RNA was determined using a standard assay (schedule 13).

2.5.1.2 Electrophoresis of RNA

Agarose gel was prepared as in schedule 14 and RNA samples were loaded with a loading gel buffer in schedule 14 and run at a constant voltage of 200V for 1hr and then viewed and photographed under uv light.

2.5.2 Reverse transcription (RT)

The reverse transcription was performed using KLK1 specific primers and the method is as shown in schedule 15.

APPENDIX A

Experiment Schedules

2.2.1.1 Processing of urine samples

Schedule 1

1. An amount of 200µl SBTI (0.08 mg/ml)/Bacitracin (0.029 mg/ml)/TRIS/HCl (pH 8.2) buffer was added to urine samples (10 ml) at 4°C.
2. This was centrifuged at 2300 rpm at 4°C for 10 min.
3. About 200 µl 0.2M TRIS-HCl pH (8.2) was added in each 1 ml aliquot of supernatant.
4. One set of plain urine with no SBTI/Bacitracin/TRIS/HCl buffer (10 ml) was spun at 2300 rpm at 4°C for 10 min.
5. The supernatant of plain urine was stored as 1 ml for total protein determination.

Schedule 2 for ELISA

1. An amount of 200µl SBTI/Bacitracin/TRIS/HCl (pH 8.2) buffer was added to urine samples (10 ml) at 4°C.
2. This was centrifuged at 2300 rpm at 4°C for 10 min.
3. In one set of supernatant (1 ml) about 200µl kinin generating cocktail was added and

4. In another set of supernatant (1 ml) about 200 µl Kinin inhibitor cocktail (see appendix B was added).

Schedule 3-TK amidase microassay

1. – Prepare HUK standards-600 ul of 400 ng/ ml HUK from a 1200 ηg/ml aliquot stored at -20°C using amidase buffer (0.2 M Tris-HCl, pH 8.2).
2. Use 300 ul of this and double dilute to 6.25 ng/ml in amidase buffer
3. Add 50 ul of each dilution in triplicate to two 96-well tissue culture plates (Corning Cell Wells™, 25860, Corning, New York).
4. Blank-add 50 ul amidase buffer (triplicate) to the two microtitre plates
5. Controls-add 50 ul control urine, double diluted to 1:64 (with amidase buffer), in triplicate to both plates
6. Samples-add 50 ul oesophageal tissue extract in triplicate to the remaining wells
7. Assay buffer-add 50 ul Assay buffer (60 mg/ml SBTI, 75 mg/ml EDTA in amidase buffer) to each well of both plates
8. Incubate at 37°C for 30 min
9. Zero activity-add 50 ul dH_2O to each of the wells of one plate and read absorbance at 405 nm on the Biorad Microplate Reader 3550.
10. Enzymic activity-add 50 ul S-2266 (1.5 mM in deionised dH_2O) to each well of the second plate and incubate at 37°C for 3 h
11. Read absorbance at 405 nm after 3 h
12. Calculation - for each plate subtract the absorbance of the blanks from the absorbance of the standards, controls and samples. Then subtract the zero activity absorbance

from the enzymic activity and use this value to plot absorbance versus concentration of HUK standards. Read concentration of samples from this graph.

During the 3 h incubation period, an aliquot of the filtrate was used for protein determination (in triplicate) using Bradford microassay (Bradford, 1976).

The enzymic activity of TK was thus expressed as ηg/μg protein.

Schedule 4 Enzyme linked immunoassay

1. Primary antibody-Coat the ELISA plate (Corning) with 100 ul of 30 ng/ml Goat α-human rTK IgG in coating buffer at 4^0C overnight
2. Wash the plate with 0.01 M PBS/0.5%Tween (pH 7.4) for 9 min (3 changes of 3 min each) at room temperature
3. Block 1-add 200 ul of 5% milk blocker (see appendix B) to each well at room temperature for 30min
4. Wash the plate with 0.01 M PBS/0.5%Tween for 9 min (3 changes of 3 min each) at room temperature
5. Block 2 - add 200 ul of 5% milk blocker to each well for 30 min at room temperature
6. Wash the plate with 0.01 M PBS/0.5%Tween for 9 min (3 changes of 3 min each) at room temperature
7. Standards-a 1200 ng/ml aliquot of HUK standard (Calbiotech, California) stored at -20°C was double diluted 40 ng/ml to 0.625ng/ml, and 100 ul of each dilution was added in triplicate

8. Blanks-100 ul 0.01 M PBS pH (7.4) was added to 3 wells
9. Controls-Control urine from section 2.4.3.5 was double-diluted from 1/8 to 1/512 in 0.01 M PBS (pH 7.4), and 100 ul of each dilution added in triplicate. Also, known samples are rerun to test for interassay variation
10. Sample-100 ul of sample was loaded onto the remaining wells of the plate in triplicate
11. Incubate for 1 h at 37^{0C}
12. Wash the plate with 0.01 M PBS/0.5%Tween for 9 min (3 changes of 3 min each) at room temperature
13. Secondary antibody-add 100 ul of 25 ng/ml rabbit α- human rTK to each well and incubate for 1 h at 37^0C
14. Wash the plate with 0.01 M PBS/0.5%Tween for 9 min (3 changes of 3 min each) at room temperature
15. 100 ul of sheep anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1:250 with 0.01M PBS was added to each well and incubated for 1 h at 37^0C
16. Wash the plate with 0.01 M PBS/0.5%Tween for 9 min (3 changes of 3 min each) at room temperature
17. Chromogen-100 ul of 1 mg/ml pNPP (p-Nitrophenyl phosphate) substrate was added to each well (1 tablet in 5 ml of substrate buffer) and allow colour to develop for 1 h at 37^0C
Read absorbance at 405 nm.
Calculation - subtract the absorbance of the blanks from the absorbance of the standards, controls and samples.

Plot absorbance versus concentration of HUK standards. Read TK concentration of samples and controls from this graph.

Schedule 5-Kinin Generation assay

1) Bradykinin-Spdp (N-succinimidyl 3-[2-pyridyldithio]propionate)-Cytochrome C Conjugate

1. Cytochrome C-SPDP conjugate-Mix 1 volume 13 Um SPDP with 1 volume 10 uM Cytochrome C (Sigma Chemicals) for 1 h at 22^0C
2. Remove unconjugated SPDP and centrifuge solution in Millipore 10 000 nominal weight cut off filters at 13200 rpm for 2-3 h at 22^0C
3. Resuspend retained solution in two volumes of 0.3 M phosphate buffer (pH 6) to give original volume
4. In Cytochrome C-SPDP-BK-add 1 volume of 1 mM BK for 1 h at 22^0C
5. Measure protein concentration (Bradford Method)
6. Determine optimal protein concentration that can be detected by ELISA-usually between 3-4 ug protein/ml or approximately 1:160 dilution
7. Aliquot and store at -20^oC

2) Extraction of basal kinin (endogenous kinins)

8. Precipitate proteins-thaw urine samples and centrifuge at 6600 rpm for 10 min at 4°C (stored with inhibitor cocktail without SBTI and Aprotinin)
9. Remove 500 µl and add to 500 ul acid-alcohol (absolute alcohol/ 0.003% HCl pH 3.0) to each for 90 min at 20°C
10. Centrifuge at 6600 rpm, aspirate and keep supernatants for 10 min at 4°C
11. Wash pellets with 500 ul acid-alcohol diluted 1:2 in dH_20 and centrifuge at 6600 rpm 10 min at 4°C
12. Pool supernatants and place in 24 well cell culture plate (Corning) and evaporate to dryness overnight at 55°C
13. Reconstitute dry residue in 500 ul assay buffer (original volume), centrifuge (6600 rpm) for 10 min at 4°C and use clear supernatants for ELISA

3) Generation and extraction of kinin in control urine and tissue extracts

1. Kinin generation-, thaw urine samples and centrifuge at 6600 rpm for 10 min at 4°C (stored with kinin inhibitor cocktail with SBTI (4 mg/ml) and Aprotinin (4 mg/ml)
2. Mix 500 µl of urine with 500 ul kinin generating cocktail to each and incubate for 60 min at 37°C
3. Remove 500 µl and add to 500 ul acid-alcohol (absolute alcohol/ 0.003% HCl pH 3.0) to each for 90 min at 20°C

4. Centrifuge at 6600 rpm, aspirate and keep supernatants for 10 min at 4°C
5. Wash pellets with 500 ul acid-alcohol diluted 1:2 in dH_20 and centrifuge at 6600 rpm 10 min at 4°C
6. Pool supernatants and place in 24 well cell culture plate (Corning) and evaporate to dryness overnight at 55°C
7. Reconstitute dry residue in 500 ul assay buffer (original volume), centrifuge (6600 rpm) for 10 min at 4°C and use clear supernatants for ELISA

4) Measurement of basal and extracted kinin by ELISA

1. Coat microtitre plate (Nunc Immulon Maxisorp™) overnight with 100 ul of BK-SPDP-cytochrome C conjugate (optimal concentration determined for each batch: ±3-4 μg/ml) in sodium carbonate/coating buffer, (pH 9.6) at 4°C
2. Blank - add 100 ul coating buffer only to 3 wells at 4^0C overnight
3. BK Standards (Sigma) – double dilute one aliquot of 1 mg/ml BK, stored at –20°C, down to 1.25 ng/ml in PBS
4. Also overnight, incubate sample or standard with equal volume of SBK1 α-BK antibody (1:1000 in PBS, pH 7.4)
5. Wash plate - 0.01 M PBS/0.5% Tween 200 ul/well for 9 min (2 changes of 3 minutes each) at room temperature
6. Block 1 - add 200 ul of 5% Milk blocker (see appendix B) to each well for 30 min

at room temperature
7. Wash plate - 0.01 M PBS/0.5% Tween 200 ul/well for 9 min (2 changes of 3 min each) at room temperature
8. Block 2 - add 200 ul of 5% Milk blocker to each well for 30 min at room temperature
9. Wash plate - 0.01 M PBS/0.5% Tween 200 ul/well for 9 min (2 changes of 3 min each) at room temperature
10. Add SBK1 kinin-incubate from D6-100 ul/well in triplicate for 1 h at 37^0C-
11. Wash plate - 0.01 M PBS/0.5% Tween 200 ul/well for 9 min (2 changes of 3 min each) at room temperature
12. Alkaline phosphatase conjugated anti-mouse IgG (Sigma Chemicals)-diluted 1:250 in PBS (pH 7.4) add 100 ul/well for 2 h at 37^0C
13. Chromogen (pNPP, 5 mg tablet, Sigma Chemicals)-add 100 ul of 1 mg/ml pNPP (p-Nitrophenyl phosphate), diluted in coating buffer) for 1 h at room temperature
14. Read absorbance (Biorad microplate reader 3550) at 405 nm

BLANKS:

Non-specific binding to the plate was determined by pre incubating SBK1, diluted 1/800 in PBS (pH 7.4), with an equal volume of PBS; and adding 100 ul to the wells coated with coating buffer only, and no conjugate.

Schedule 6-Measurement of total protein (Bradford, 1976)

1 Protein standard-100 ml of 1 mg/ml Bovine Serurm Albumin (BSA, Fraction V, Boehringer) was prepared and stored in 1 ml aliquots at -20C.
1 ml aliquot of this standard was double diluted with dH_2O from 1000 to 16 ug/ml.
30 ul of each dilution was added to a microassay plate in triplicate.
3 Blank-30 ul dH_2O was added to 3 wells of the plate
Samples were added in triplicate to the plate
4 Biorad Protein Assay Reagent (Biorad) was diluted 1/5 in dH_2O and 300 ul was and added to each well of the plate
5 Absorbance was read at 595 nm.
6 CALCULATIONS-Subtract the absorbance of the blanks from the absorbance of the standards and samples. Plot absorbance vs concentration of BSA standards. Read protein concentration of samples from this graph. TK enzymic activity is expressed as ng TK/ug protein

Schedule 7-H & E staining method

1. Slides were dewaxed in xylene for 10 min (2 changes of 5 min each
2. Rehydrated in absolute ethanol for 2 min (2 changes of 1 min each)
3. Rehydrated in 90% ethanol for 1 min.
4. Rehydrated in 70% ethanol for min.
5. Rehydrated in water for 1 min
6. Slides were immersed in Mayer's Haematoxylin (Sigma Chemicals) for 5 min

7. Slides were rinsed in running tap water (blued) for 5 min.
8. Immersed in Eosin (Sigma Chemicals) for 2 min
9. Quickly immersed in 95% ethanol for 30 sec.
10. Dehydrated in absolute ethanol for 2 min (2 changes of 1 min each).
11. Dehydrated in xylene for 1 min.
12. Maintained in xylene until mounted in Entellen (Merck Chemicals).

Schedule 8-Fixation and embedding for conventional light and confocal microscopy

1. Fixation in 5% formaldehyde/0.9% NaCl (v/v) at 24°C for 1 h
2. Fixation in 5% formaldehyde/0.9% NaCl (v/v) at 24°C for 1 h
3. Dehydration in absolute ethanol at 24°C for 1 h
4. Dehydration in absolute ethanol at 24°C for 1 h
5. Dehydration in absolute ethanol at 24°C for 1 h
6. Dehydration in absolute ethanol at 35°C for 1 h
7. Dehydration in absolute ethanol at 35°C for 1 h
8. Dehydration in absolute ethanol at 35°C for 1 h
9. Dehydration in absolute ethanol at 35°C for 1 h
10. Clearing in Xylene at 35°C for 1 h
11. Clearing in Xylene at 35°C for 1 h
12. Vacuum infiltration 1 in paraffin wax at 60°C for 1 h
13. Vacuum infiltration 2 in paraffin wax at 60°C for 1 h
14. Embedding in paraffin wax at 20°C for 20 min.

Schedule 9-Immunocytochemistry: [Peroxidase-antiperoxidase (PAP)-Diaminobenzidine(DAB) method

1. Sections were dewaxed in xylene for 20 min (2 changes of 10 min each) at room temperature
2. Rehydration was done in – 100% ethanol for 10 min (2 changes of 5 min each) at room temperatute
3. Endogenous peroxidase was quenched by 100% methanol for 20 min at room temperature.
4. Rehydration of sections was done in 90% ethanol for 8 min (2 changes of 4 min each) at room temperature
5. Rehydration in 70% ethanol for 3 min at room temperature
6. Antigen retrieval was done by boiling sections at 80^0C for 2 min high and 5 min low in 0.1 M Sodium-Citrate (pH 6.0) in microwave (Sharp, Carousel).
7. Allow to cool to room temperature for ± 20min
8. Wash in 0.01 M PBS (pH 7.4) for 2 min at room temperature
9. Block endogenous peroxidase in 10% H_2O_2/ 90% MeOH for 20 min at room temperature
10. Wash - 0.01M PBS (pH 7.4) for 2 min at room temperature
11. Incubate with DIG blocking agent [Maleic acid (1X), Boehringer Manheim, 10% blocking reagent) for 30 min at room temperature
12. Incubate with 1°antibody (diluted in Maleic acid (Boehringer manheim/milk blocker) overnight at 4°C under humid conditions.

13. Wash in 0.01 M PBS (pH 7.4) for 2 min at room temperature
14. Incubate with anti-species IgG Biotin link (DAKO K0690) for 20 min at room temperature
15. Wash in 0.01M PBS (pH 7.4) for 2 min in room temperature.
16. Incubate with 2^0 antibody - Strepavidin-peroxidase (DAKO K0690) for 20 min at room temperature.
17. Wash in 0.01M PBS (pH 7.4) for two min at room temperature
18. Incubate with liquid DAB-Chromogen (Dako, K3465) for 1 min at room temperature in darkness.
19. Counterstain in Mayer's Heamotoxylin (Sigma) for 5 min at room temperature
20. Blue in tap H_2O for 5 min at room temperature
21. Dehydrate in 70% ethanol for 3 min at room temperature
22. Dehydrate in 90% ethanol for 8 min (2changes of 4 min each) at room temperature.
23. Dehydrate in 100% ethanol for 10 min (2 changes of 5 min each) at room temperature
24. Dehydrate in xylene for 20 min (2 changes of 10 min each) at room temperature
25. Mount – Entellen (Merck Chemicals)

NOTES

- Controls for these procedures involved replacing the primary antibody with goat/rabbit non-immune serum diluted in 1 mM Phosphate buffer (pH 7.4)/ 1%BSA.
- Method controls are 1^0 antibody replaced with buffer and pre-absorbed antibody
- All dilutions made up in 0.1 M PBS (pH 7.4)/1%BSA
- Tissue sections were not allowed to dry out.

- Labelled slides are stored in the dark.

Schedule 10-Immunocytochemistry: Fluorescent probe (FITC-fluorescein isothiocyanate)-confocal microscopy

1. Sections were dewaxed in xylene for 20 min (2 changes of 10 min each) at room temperature
2. Rehydration was done in – 100% ethanol for 10 min (2 changes of 5 min each) at room temperature
3. Endogenous peroxidase was quenched by 100% methanol for 20 min at room temperature.
4. Rehydration of sections was done in 90% ethanol for 8 min (2 changes of 4 min each) at room temperature
5. Rehydration in 70% ethanol for 3 min at room temperature
6. Antigen retrieval was done by boiling sections at 80^0C for 2 min high and 5 min low in 0.1 M Sodium-Citrate (pH 6.0) in microwave (Sharp, Carousel).
7. Allow to cool to room temperature for ± 20 min
8. Wash in 0.01M PBS (pH 7.4) for 2 min at room temperature
9. Block endogenous peroxidase in 10% H_2O_2/ 90% MeOH for 20 min at room temperature
10. Wash – 0.01M PBS (pH 7.4) for 2 min at room temperature
11. Incubate with DIG blocking agent (1X Maleic acid, 10% blocking reagent) for 30 min at room temperature
12. Incubate with 1°C antibody (diluted in Maleic acid/milk blocker, Boehringer

Manheim) overnight at 4°C under humid conditions.
13. Wash in 0.01M PBS (pH 7.4) for 2 min at room temperature
14. Incubate with anti-species fluorescent conjugate (antibody cojugated to fluorescein isothiocyanate-FITC) for 30 min at room temperature
15. Wash in 0.01M PBS (pH 7.4) for 2 min in room temperature.
16. Mount with 10% PBS (pH 7.4)/90% Glycerol.

NOTES

- Fluorescent emission was analysed using the Leica TD4 confocal microscopy system (Leica, Germany).
- Method controls are 1^0 C antibody replaced with PBS (pH 7.4)/preabsorbed antibody
- All dilutions made up in 0.1M PBS (pH 7.4)/1%BSA
- Tissue sections were not allowed to dry out.
- Labelled slides were stored in the dark at 4°C

Schedule 11- In situ hybridisation (ISH)

1) Cleaning and sterilisation

1. Apparatus were thoroughly cleaned and soaked in detergent overnight
2. Extensive rinsing was done under tap water twice
3. They were then soaked in millipore water for 1 h
4. Soaking was done in 2% H_2O_2 to kill infectious agents for 15 min
5. Soaked in 0.1M HCl to denature proteins for 15 min
6. Rinsed with autoclaved millipore water
7. Sprayed with RNAse Away (Molecular Bio-products) to destroy RNAses

8. Rinsed with 100% ethanol to cleanse and facilitate drying
9. Kept under UV light in fume hood to sterilise and dry overnight
10. All glassware was then covered in aluminium foil and autoclaved for 20 min

2) Digoxigenin labelling

Reagents / Procedure	Antisense cRNA to hKLK1	Sense cRNA to hKLK1	Control cRNA to PSPT 18-Neo/Pvu II
HKLK 1 cDNA digested with NOT I	-	1 µl (≈ 1µg)	
HKLK 1 cDNA linearised with ACC I	1 µl (≈ 1 µg)	-	
Control DNA PSPT 18-Neo/Pvu II	-	-	4 µl (≈ 1 µg)
10 x NTP labelling mix	2 µl	2 µl	2 µl
10 X Transcription buffer	2 µl	2 µl	2 µl
DEPC H_2O	13 µl	13 µl	10 µl
Mix and Quick Spin			
SP6 RNA polymerase (2 U/µl)			2 µl
T3 Polymerase (2 U/µl)	_	2 µl	
T7 Polymerase (2 U/µl)	2 µl	_	_
TOTAL	20 µl	20 µl	20 µl
PCR – Incubate in thermocycler for 2h @ 37^{O}C.			
Stop reaction - 0.2 M EDTA, (pH 8.0)	2 µl	2 µl	2 µl
Mix and quick spin			

4 M LiCl	2.2 µl	2.2 µl	2.2 µl
Precipitate – Cold absolute EtOH	75 µl	75 µl	75 µl
Precipitate – Mix, quick spin and incubate at -20°C for 2 h			
Precipitate pellet – Centrifuge (13 000 g, 15 min, 4 °C) and decant supernatant			
Wash pellet – cold 70% EtOH	100 µl	100 µl	100 µl
Precipitate washed pellet – Centrifuge (13 000 g, 15 min, 4°C) and remove supernatant with pipette			
Dry pellet - 2h in air in laminar flow			
Dissolve pellet -DEPC treated dH_2O	50 µl	50 µl	50 µl
Dissolve pellet - Leave at 4 °C for approximately 1h			
Store DIG labelled cRNA in 2 x 20 µl and 1 x 10 µl alliquots @ -70°C in screw top eppendorf cups, with a rubber seal			

NOTES

- Although the labelling procedure recommended that 1 µl RNAase inhibitor be added before aliquoting and storage, we did not add RNAase inhibitor because RNAase inhibitor is usually isolated from placenta and require high concentrations of DTT (dithiotreitol) for activity. The DTT affects T3 and T7 polymerase.
- We did not add DNAase 1, because the amount of DNA template left would be very small and also the DNAase 1 may not be RNAase free.
- For the initial precipitation the sample can be left at -20 °C for 2 h or ON or in the ultra-freezer at -70°C for 30 - 60 min.
- The concentration of RNA in each sample should be 10 µg/50 µl (200 ng/µl).
- By scaling up the reaction 1µg DNA plasmid can yield up to 40 µg RNA transcript.

- The aliquots can be thawed and frozen a few times without loss of activity.

3) Estimation of minimal probe concentration

DILUTION / CONC.	PROBE		
	DIG Labelled Control RNA (BM)	DIG labelled cRNA to control DNA	Sample probe (AS and S)
Initial Concentration	10 µg/100 µl	250 ng/50 µl	10 µg/50 µl
Dilution 1 Conc. 20 ng/ul	(1:5) 1 µl + 4 µl dH_2O		(1:10) 1 µl + 9 µl dH_2O
Dilution 2 Conc. 1ng/µl	(1:20) 2 µl + 38 µl dH_2O	(1:5) 2 µl + 8 µl dH_2O	(1:20) 2 µl + 38 µl dH_2O
Dilution 3 (1:10) Conc. 100 pg/µl	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O
Dilution 4 (1:10) Conc. 10 pg/µl	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O
Dilution 5 (1:10) Conc. 1 pg/µl	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O
Dilution 6 Conc. 0.1 pg/µl	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O
Dilution 7 (1:10) Conc. 0.01 pg/µl	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O	5 µl + 45 µl dH_2O

The optimal concentration of antisense and sense probes that can be detected was determined by comparing spot intensities with that of DIG labelled control RNA and the method used is tabulated bellow:

4) Concentration estimation for the probe

1. Mark spot points lightly with pencil on a Nylon membrane (Hybond)
2. Spot 1µl of each dilution onto membrane and air dry for 10 min
3. Fix the probe to membrane by placing under UV light for 5 min
4. Wash – 1x washing buffer (BM) to cover membrane while shaking 5 min
5. Block with 1x blocking buffer (BM) to cover membrane with shaking for 30 min
6. Incubate in anti-DIG AP (BM) - diluted 1:10 000 with 1x blocking buffer-prepare sufficient volume to cover the membrane for 30 min
7. Wash with 1x washing buffer (BM) to cover membrane while shaking for 15 min
8. Wash in 1x washing buffer (BM) to cover membrane while shaking for 15 min
9. Immerse in1x Detection buffer (BM) - to cover membrane with shaking for 2 min
10. Incubate in NBT/BCIP (BM) diluted 1:50 with detection buffer-enough to cover membrane and allow colour to develop in the dark overnight
11. Terminate colour reaction in 50 ml of TE buffer for 5 min
12. Air dry and estimate minimal dilution of probe that can be detected against that of DIG labelled control RNA

NOTES

- Fixing could also be performed by baking the membrane at 120°C for 30 min. These (UV & baking) induce radicals in the nylon membrane to cross-link with the DIG labelled nucleotide samples.
- All incubations and washes were at RT.
- The detection buffer has $MgCl_2$ which activates the alkaline phosphatase conjugated to the anti- DIG IgG by increasing the pH to alkaline for the colour reaction.
- BM-Boehringer Mannheim

5) Pre-hybridisation treatment of sections

1. Dewax in clean fresh xylene for 30 min (3 changes of 10 min each at room temperature
2. Rehydrate in clean fresh absolute Ethanol for 6 min (2 changes of 3 min each) at room temperature
3. Rehydrate in 90% ethanol for 3 min at room temperature
4. Rehydrate in 70% ethanol for 3 min at room temperature
5. Rehydrate in 50% ethanol for 3 min at room temperature
6. Rehydrate in DEPC H_2O for 3 min at room temperature
7. Fixation - freshly prepared 4% PFA (prepared above) for 20 min at room temperature
8. Rinse in fresh TBS for 3 min (3 changes of 1 min each) at room temperature RT
9. Denature proteins in 0.1M HCl for 10 min at room temperature
10. Rinse in fresh TBS for 3 min (3 changes of 1 min each) at room temperature
11. Limit non-specific labelling with freshly prepared 0.5% Acetic anhydride in 100 mM Tris, (pH 8) for 10 min at room temperature
12. Rinse in fresh TBS for 3 min (3 changes of 1 min each) at room temperature
13. Permeabilise cell membrane with Proteinase K for 20 min at 37 ^{o}C
14. Rinse in fresh TBS for 3 min (3 changes of 1 min each) at room temperature
15. Terminate Proteinase K activity in TBS for 5 min at 4^{o}C
16. Dehydrate in 50% ethanol for 1 min at room temperature
17. Dehydrate in 70% ethanol for 1 min at room temperature
18. Dehydrate in 90% ethanol for 1 min at room temperature

Dehydrate in absolute ethanol for 2 min (2 changes of 1 min each) at room temperature
19. Dry in Chloroform for 10 min in the fume hood
20. Store in dust free environment for days at room temperature

NOTES

- All dilutions of ethanol were made with DEPC treated millipore water
- The concentration of Proteinase K required was optimised for each tissue by observing morphological changes in H&E stained sections of tissues following incubation with varying dilutions of proteinase.
- For salivary gland greater than 50 mg/ml proteinase K caused morphological tissue damage while for normal oesophageal and oesophageal tumours the optimal concentration of proteinase was found to be 20 mg/ml.

6) Posthybridisation

1. Wash in 2 X SSC for 30 min at 37°C for 30 min
2. Incubate with RNAase for 30 min at 37°C
3. Wash in 1 X SSC for 20 min at 55°C
4. Wash in 0.5 X SSC for 20 min at 55°C
5. Wash in 0.1 X SSC for 20 min at 55°C
6. Rinse in fresh TBS for 3 min (3 changes of 1 min each) at room temperature
7. Block non-specific binding sites by 100 ul of 1x blocking buffer added to each slide in humid chamber for 15 min at room temperature
8. Incubate sections with sheep anti-DIG IgG conjugated alkaline phosphatase diluted

1 in 500 in 1x blocking solution. Add 100 ul per slide in humid chamber for 1 h at room temperature
9. Rinse in fresh TBS for 3 min (3 changes of 1 min each) at room temperature
10. Incubate with Chromogen NBT/BCIP (Boehringer Mmannheim) diluted 1:50 in 1 x detection buffer. 100 ul added to each slide and left to develop in a dark humid chamber overnight at room temperature
11. Wash slides in running tap water for 5 min at room temperature
12. Counterstain with 5 drops Mayers Haematoxyllin per slide for 5 min at room temperature
13. Blue in running tap water for 1 h at room temperature
14. Mount - with aqueous glycerol jelly

Isolation of RNA_using the Boehringer Mannheim tri-pure isolation reagent

Schedule 12-Procedure

The following protocol is for 50-100 mg of tissue. The volumes of the reagents will change as the weight of the tissue does.

1. Add 1 ml TriPure Isolation Reagent to a neo tube.
2. Place the frozen tissue on foil and weight.
3. Wrap the tissue in the foil and put into liquid Nitrogen.
4. Mash up the tissue.
5. Place the fresh or frozen tissue into the neo tube and homogenise with a Polytron Homogeniser.

6. Transfer to an eppendorf.
7. *This step is optional:* Centrifuge at 16 000 rpm for 10 min at 4 °C. Remove the fatty top layer (if any). Transfer the supernatant to a new eppendorf (Can be kept at - 70 °C for at least a month).
8. Incubate the supernatant at Room Temperature for 5 min.
9. Add 0.2 ml chloroform, cap and shake vigorously for 15 sec
10. Incubate at Room Temperature for 15 min.
11. Centrifuge at 16 000 rpm for 15 min at 4°C.
12. Transfer the colourless upper aqueous phase to a new eppedorf.
13. Precipitate RNA as follows. Add 0.5 ml isopropanol. Cap and invert for thorough mixing. Incubate at Room Temperature for 10 min to all the RNA precipitate to form.
14. Centrifuge at 16 000 rpm for 10 min.
15. Discard the supernatant
16. Add 1 ml of 75 % ethanol and vortex.
17. Centrifuge at 10 000 rpm for 5 min at 4°C.
18. Discard the supernatant.
19. Remove excess ethanol by air drying.
20. Resuspend in DEPC treated RNAse free water.

2.6.3 Determination of RNA Purity (Schedule 13)

Standard assay

Ratio of $Abs_{260/280}$nm > 1.8-2.0 (Free of protein contamination)

Ratio of $Abs_{260/240\ nm}$ > 1.8 (No contamination by compounds used to isolate RNA)

Ratio of $Abs_{260/280}$nm = $\frac{\text{RNA } Abs_{260\ nm} - H_2O\ Abs_{260\ nm}}{\text{RNA } Abs_{280\ nm} - H_2O\ Abs_{280\ nm}}$

Electrophoresis of RNA (schedule 14)

Gel preparation

Step 2

Dissolve 2g of Agarose D1LE in 100 ml of 1XTE buffer.
Boil until clear in a microwave on medium high
Cool to 50°C and pour
Set for 1 h.

Glyoxal/Dmso Gel Loading Buffer (Sample Buffer)

Mix

Step 3

50% Glyerol (Glycerine)
0.1 M NaH_2PO_4
0.25% Bromophenol Blue
0,25% Xylene cyanole FF

APPENDIX (B)

Regents and buffers

Reagents for sample collection, processing, fixation and storage.

1 Sterile normal saline (NS) (0.9% NaCl, w/v, pH 7, Sabax)

2 1% Alcian blue, pH 2.5 (Sigma Chemicals, St. Louis)

3 5% formal saline (41% formaldehyde / 0.9% NaCl, 1:8 v/v) – dilute formaldehyde (35%, Saarchem, SA) 1:7 in 0.9% NaCl

4 TK cocktail 40 mM Tris, pH 8 - dissolve 4.8 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml dH_2O, adjust pH to 8.0 with HCl and adjust volume to 1 l.

5 Kinin generating cocktail: (60 mM EDTA, 6 mM phenanthraline, 10 uM captopril and 10 uM phosphoramidon) – all w/v and dry reagents purchased from Sigma Chemicals, St. Louis

6 Kinin inhibitor cocktail : (60 mM EDTA, 6 mM phenanthraline, 10 uM captopril, 10 uM phosphoramidon, 10 uM SBTI and 10 uM aprotonin) – all w/v and dry reagents purchased from Sigma Chemicals, St. Louis.

H&E Staining of wax embedded tissue reagents

1 Absolute ethanol (99% ethanol, Saarchem, SA)

2 Xylene (AR, Saarchem, SA)

3 Mayer's Hematoxylin (Sigma Chemicals, St. Louis)

4 Eosin (Sigma Chemicals, St. Louis)

5 Entellen mounting medium (Merck, Germany)

Enzymic assay (Amidolytic microassay) reagents

1 Amidase buffer (0.2 M Tris-HCl, pH 8.2) - dissolve 24.22 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml dH_2O, adjust pH to 8.0 with HCl and adjust volume to 1 l.

2 Assay buffer - dissolve 300 ug SBTI (Sigma Chemicals, St. Louis) and 375 mg EDTA (Saarchem, SA) in 1 ml amidase buffer.

3 1.5 mM S2266 Solution - dissolve 25 mg S2266 (Chromogenix, Sweden) in 28 ml dH_2O

4 SBTI/Bacitracin - dissolve 1.47 g SBTI (Sigma Chemicals, St. Louis; 2720units/mg) and 40 mg Bacitracin (Sigma Chemicals, St. Louis) in 1 ml Amidase buffer

Reagents for measurement of Total protein (Bradford, 1976)

1 Bovine Serum Albumin (BSA, Fraction V, Boehringer Mannheim)

2 Biorad Protein Assay Reagent (Biorad, UK)

Reagents and immunochemicals for TK ELISA

1 Coating buffer ($Na_2CO_3/NaHCO_3$, pH 9.6) - dissolve 1.59 g Na_2CO_3 (Saarchem, SA) and 2.93 g $NaHCO_3$ (Saarchem, SA) in 1 l dH_2O

2 Substrate buffer (5 mM $MgCl_2$/10% Diethanolamine, pH 9.8) - dissolve 0.10163 g $MgCl_2$ (Saarchem, SA) in 80 ml dH_2O, add 10 ml Diethanolamine (Sigma Chemicals, St. Louis), adjust pH to 9.8 with HCl (Saarchem, SA) and make up to 100 ml with dH_2O

3 0.01 M PBS, pH 7.4 - dissolve 1 PBS tablet (Sigma Chemicals, St. Louis) in 200 ml dH_2O

4 5% (w/v) Milk protein blocker - dissolve 5g Country Pasteur fat free milk powder (Nutritional Foods, SA) in 100 ml PBS. Make fresh.

5 0.01 M PBS/ 0.5% (v/v) Tween - dilute 100 ul Tween 20 (Sigma Chemicals, St. Louis) in 200 ml 0.01M PBS

6 Human Urinary Kallikrein (HUK, Calbiochem, USA) - dissolve 1 mg HUK in 833 ml NS. Store 200 ul aliquots at -20°C

7 Goat anti-human TK IgG (30 ng/ml) - from 2.4.3

8 Rabbit anti-human TK IgG (25 ng/ml) - from 2.4.3

9 Anti-rabbit IgG alkaline phosphatase (Sigma Chemicals, St. Louis)

10 Disodium paranitrophenyl phosphate substrate (pNPP, 5 mg tablets, Sigma Chemicals, St. Louis) - dissolve 1 tablet in 5 ml substrate buffer. Make fresh and use within 90 min.

Reagents Immunocytochemical localisation of TK by immunoprecipitation

1 Poly-L-lysine (Sigma Chemicals, St. Louis) - dilute 1:10 in dH_2O in plastic-ware and store at 4°C

2 Xylene (Saarchem, SA)

3 100% EtOH (Saarchem, SA)

4 0.1 M Sodium-Citrate, pH 6.0 (w/v) – dissolve 29.4 g Tri-sodium citrate (Saarchem, SA) in 800 ml dH2O, adjust to pH 6 with HCl and make up to 1 l.

5 10% H_2O_2 /90% MeOH (v/v) – mix 9 ml MeOH (Saarchem, SA) and 1 ml H_2O_2 (Saarchem, SA) just before use.

6 0.01 M PBS, pH 7.4 (w/v) - dissolve 1 PBS tablet (Sigma Chemicals, St. Louis) in 200 ml dH_2O

7 1% non-immune goat serum

8 1^{o} Ab - goat anti-human rTK IgG from 2.4.3

9 Anti-goat IgG Biotin link (LSAB K0690, Dako, UK)

10 2^{0} Ab - Strepavidin-peroxidase (LSAB K0690, Dako, UK)

11 Chromogen - liquid DAB (K3465, Dako, UK)

12 Counterstain - Mayers' Heamotoxylin (Sigma Chemicals, St. Louis)

13 Entellen (Merck, Germany)

Reagents Immunocytochemical localisation of TK by immunofluoresence

1 Poly-L-lysine (Sigma Chemicals, St. Louis) - dilute 1:10 in dH_2O in plastic-ware and store at $4^{o}C$

2 Xylene (Saarchem, SA)

3 100% EtOH (Saarchem, SA)

4 0.1 M Sodium-Citrate, pH 6.0 (w/v) – dissolve 29.4 g Tri-sodium citrate (Saarchem, SA) in 800 ml dH2O, adjust to pH 6 with HCl and make up to 1 l.

5 0.01 M PBS, pH 7.4 - dissolve 1 PBS tablet (Sigma Chemicals, St. Louis) in 200 ml dH_2O

6 1% Human IgG (Sigma Chemicals, St. Louis)

7 1^{o} Ab - goat anti-human rTK IgG from 2.4.3

8 Anti-goat IgG Biotin link (LSAB K0690, Dako, UK)

9 2^{0} Ab - FITC conjugated anti-goat IgG (Sigma Chemicals, St. Louis)

10 Mount - 10% PBS / 90% glycerol (Saarchem, SA)

Detection of TK mRNA by in situ hybridisation

Reagents for Cleansing of Apparatus, Linearisation, Purificarion and Agarose gel Electrophoresis of Plasmids

1 0.1M HCl (v/v) dilute 10 ml 9 M (32%) HCl (Saarchem, SA) with 990 ml DEPC treated H_2O

2. RNAase-Away (Molecular Bio-Products, USA)

3. Absolute ethanol (Saarchem, SA)

4. 0.1% v/v DEPC treated H_2O. 1 ml diethylpyrocarbonate (DEPC, Sigma Chemicals, St. Louis) was added to 1 l dH_2O, placed in a 37°C shaking waterbath overnight and autoclaved.

5. 200 mM Phosphate buffer. 17.02 g Na_2HPO_4 (Saarchem, SA) and 12.48 g NaH_2PO_4 (Saarchem, SA) was dissolved in 1 l DEPC treated H_2O to make a stock solution of 200 mM Phosphate buffer. Required volumes were diluted to 100 mM with DEPC treated dH_2O.

6. 10x Buffer A (Boehringer Mannheim)

7. 10x Buffer H (Boehringer Mannheim)

8. Restriction enzyme -ACC 1 (10 U/ul, Boehringer Mannheim)

9. Restriction enzyme -NOT 1 (10 U/ul, Boehringer Mannheim)

10. Phenol (Saarchem, SA) saturated with Tris, pH 8

11. Chloroform (Saarchem, SA)

12. 3 M NaOAc, pH 5.2

13. T-E Buffer (10mM Tris HCl, 1mM EDTA). Dissolve 0.61g Trizma base (Sigma Chemicals, St. Louis) and 0.19 g EDTA (Saarchem, SA) in 400 ml sterile dH_2O. Adjust to pH 8 with HCl and make up to 500ml. Store at 20 ^{O}C.

*Do not over dry the pellet, as this reduces solubility of the DNA in dH_2O. Also, ensure that all of the ethanol is removed as ethanol will interfere with the PCR in the generation of DIG labelled cRNA sense and anti-sense transcripts

Reagents DIG RNA-Labelling by in vitro transcription

1 The control DNA (PSPT 18-Neo/Pvu II), 10x NTP labelling mix, 10x Transcription buffer, SP6 and T7 RNA polymerases (2 U/ul) were provided in the DIG RNA Labelling Kit (SP6/T7), (Boehringer Mannheim, Cat. No. 1 175 025).

2 The T3 Polymerase (2 U/ul) was purchased separately (Boehringer Mannheim).

3 0.5 M EDTA, pH 8. 18.61 g EDTA (Saarchem, SA) was dissolve in ±400ml DEPC treated dH_2O with stirring while drops of concentrated NaOH was added unti the pH was adjusted to 8. The volume was then corrected to 500 ml and the solution stored at RT. 4 ul of this 0.5 M EDTA stock was diluted with 6 ul DEPC treated dH_2O to obtain 0.2 M EDTA.

4 4 M LiCl - dissolve 8,478 g LiCl (Sigma Chemicals, St. Louis) in 50ml DEPC treated H_2O and store at room temperature.

5 70% EtOH - dilute 7 ml absolute ethanol (Saarchem, SA) with 3 ml DEPC treated dH_2O and keep in ice.

Reagents estimation of minimal probe dilution that can be detected

1 The DIG labelled control RNA (10 ug/100µl) was provided in the DIG RNA Labelling Kit (SP6/T7), (Boehringer Mannheim, Cat. No. 1 175 025).

2 The blocking solution, wash, detection and maleic acid buffers were provided in the DIG Wash and Block buffer set (Boehringer Mannheim, Cat. No. 1585 762) as 10x concentrated and stored at 4°C.

3 The 10x concentrated blocking solution (10% blocking reagent in maleic acid buffer) was stored in aliquots at -20°C to preserve the milk protein. Amounts of 1x blocking solution, sufficient to last approximately 4 weeks, were prepared by diluting 1 ml of the 10x maleic acid buffer in 8 ml dH2O to which was added 1 ml of the 10x concentrated blocking solution. This 1x diluted blocking solution was stored at 4°C but brought to RT before each use.

4 The 10x concentrated washing buffer {10x concentrated maleic acid buffer (1 M Maleic Acid, 1.5 M NaCl), pH 7.5 with 3% Tween 20} was diluted to 1x with dH_2O.

5 The 10x concentrated detection buffer (10 mM Tris, 10 mM NaCl, 5 mM Mg Cl_2) was diluted to 1x with dH_2O stored at 4°C but brought to RT before each use.

6 Sufficient amounts of the sheep anti-DIG IgG conjugated to alkaline phosphatase (Boehringer Mannheim) stored at 4°C was diluted 1:500 with 1x blocking solution just before use.

7 Sufficient amounts of the Nitro blue tetrazolium chloride (18.75 mg/ml) and 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (9.4 mg/ml) in 67% DMSO (v/v) (NBT/BCIP,

Boehringer Mannheim) stored at 4°C was diluted 1:50 with 1x detection buffer just before use.

Reagents for pretreatment of glass slides for ISH

1 0.1M HCl (v/v) dilute 10 ml 9 M (32%) HCl (Saarchem, SA) with 990 ml DEPC treated H_2O

2 2% v/v Silane - Dilute 2 ml 3-aminopropyltriethoxysilane (Sigma Chemicals, St. Louis) in 100 ml dry acetone solution (AR, Saarchem, SA). Make fresh.

Reagents for pre-hybridisation treatment of tissue sections (ISH)

1 Xylene (Saarchem, SA)

2 Absolute Ethanol (Saarchem, SA) - dilute to 90%, 70% and 50% with DEPC treated H_2O

3 0.1% v/v DEPC treated H_2O. 1 ml diethylpyrocarbonate (DEPC, Sigma Chemicals, St. Louis) was added to 1 l dH_2O, placed in a 37°C shaking waterbath overnight and autoclaved.4% w/v PFA (Saarchem, SA)

4 200 mM Phosphate buffer. 17.02 g Na_2HPO_4 (Saarchem, SA) and 12.48 g NaH_2PO_4 (Saarchem, SA) was dissolved in 1 l DEPC treated H_2O to make a stock solution of 200 mM Phosphate buffer. Required volumes were diluted to 100 mM with DEPC treated dH_2O.

5 4% (w/v) paraformaldehyde (PFA) - dissolve 10 g PFA (Saarchem, SA) in 200 ml 100 mM phosphate buffer by slow heating to 60°C, in a laminar flow, until solution is clear. Prepare fresh each day and store at 4°C until use.

6 1 M Tris pH 7.5 – dissolve 121 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml DEPC treated H_2O, adjust to pH 7.5 with HCl and make up to 1 l.

7 5 M NaCl (w/v) – dissolve 292.2 g NaCl (Saarchem, SA) in 1 l DEPC treated H_2O

8 TBS – dilute 50 ml 1 M Tris pH 7.5 and 30 ml 5 M NaCl to 1 l with DEPC treated H_2O.

9 200 mM HCl (v/v) dilute 5 ml 9 M (32%) HCl (Saarchem, SA) with 245 ml DEPC treated H_2O

10 1 M Tris pH 8 – dissolve 121 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml DEPC treated H_2O, adjust to pH 8 with HCl and make up to 1 l.

11 0.5% Acetic anhydride in 100 mM Tris, pH 8 (v/v) – dilute 25 ml 1 M Tris pH 8 with 225 ml DEPC treated H_2O to obtain 250 ml of 100 mM Tris and add 1.25 ml of 100% acetic anhydride (Saarchem, SA) just before use.

12 Proteinase K (Boehringer Mannheim) – dissolve 10 mg proteinase K in 1 ml DEPC treated H_2O. Store 100 ul aliquots at –20°C. Dilute with TBS to obtain required concentrations of proteinase K for each tissue.

13 Chloroform (Saarchem, SA)

14 40% Dextran (w/v) – dissolve 40 g Dextran sulphate Na salt (MW 500 000, Sigma Chemicals, St. Louis) in 70 ml DEPC treated H_2O in a volumetric flask and heat in a 68 °C water bath for 3-4 h. Make up to 100 ml and store at 4°C.

15 10% Sodium dodecyl sulphate (SDS, w/v) – dissolve 10 g SDS (Amresco, Ohio, USA) in 100 ml DEPC treated H_2O

16 Hybridisation Buffer – mix 1 ml 20x SSC, 2.5 ml 40% dextran, 20 ul 10% SDS, 5 ml formamide (Sigma Chemicals, St. Louis) and 1.4 ml DEPC treated H_2O. Store 100 ul aliquots at –20°C.

Reagents for post-hybridisation washes and immunolocalisation of bound DIG labelled probes

17 20x Sodium citrate (SSC, w/v) – dissolve 175.3 g NaCl (Saarchem, SA) and 88.2 g Trisodium Citrate (Saarchem, SA) in 800 ml DEPC treated H_2O, adjust to pH 7 with HCl amd make up to 1 l. Required volumes were diluted with DEPC treated dH_2O.

18 0.5 M EDTA (w/v) – dissolve 18.61 g EDTA (Sigma Chemicals, St. Louis) in 80 ml DEPC treated water, adjust to pH 8 with HCl and make up to 100 ml.

19 RNAase (Boehringer Mannheim) – dissolve 10 mg RNAase in 1 ml dH_2O, place in boiling water bath for 10 min and store 100 ul aliquots at –20°C. For RNAase digestion mix 2 ul RNAase stock, 100 ul 5 m NaCl, 10 ul 1 M Tris pH 8, 2 ul 0.5 M EDTA and 886 ul dH_2O. Add 200 ul to each slide.

20 TBS – dilute 50 ml 1 M Tris pH 7.5 and 30 ml 5 M NaCl to 1 l with DEPC treated H_2O.

21 1x blocking buffer (Boehringer Mannheim) see A – 2.5.5.3

22 Sheep anti-DIG IgG conjugated alkaline phosphatase (Boehringer Mannheim)

23 1x detection buffer (Boehringer Mannheim) see A – 2.5.5.3

24 Chromogen - NBT/BCIP (Boehringer Mannheim)

25 Hematoxylin (Sigma Chemicals, St. Louis)

26 Mount - with aqueous glycerol jelly

Reagents Kinin ELISA

1 13 uM SPDP in 0.1M phosphate buffer pH 8.6 / 20% ethanol. Dissolve 5 mg SPDP (Sigma Chemicals P3415, St. Louis) in 200 ul absolute ethanol, then add 500 ul phosphate buffer

2 10 uM Cytochrome C in 0.1 M phosphate buffer pH 8.6 – dissolve 1 mg cytochrome C (Sigma Chemicals C6913, St. Louis) in 833 ul phosphate buffer

3 1 mM BK. 1 mg BK (Sigma) was dissolved in 943.2 ul 0.01M PBS pH 7.4

4 Kinin generating cocktail: kinin cocktail excluding SBTI and Aprotinin inhibitor

5 Kinin inhibitor cocktail : kinin generating cocktail with SBTI and Aprotinin

6 Acid-Alcohol: absolute ethanol/0.003% HCl (v/v)

7 Rabbit IgG (Sigma Chemicals 8140, St. Louis)

8 CNBr-activated Sepharose 4B (Sigma Chemicals, St. Louis)

9 0.2 M Tris-HCl pH 7.2 (w/v) - dissolve 24 g Trizma base (Sigma Chemicals, St. Louis) in 800 ml dH_2O, adjust to pH 7.2 with HCl and make up to 1 l.

10 1 mM HCl (v/v) dilute 0.1 ml 9 M (32%) HCl (Saarchem, SA) with 1 l dH_2O

11 Coupling buffer (0.2 M $NaHCO_3$/0.5 M NaCl, pH 8.6, w/v) – dissolve 16.8 g $NaHCO_3$ (Saarchem, SA) and 29.22 g NaCl (Saarchem, SA) in 800 ml dH_2O, adjust to pH 8.6 with HCl and make up to 1 l.

12 0.2 M Glycine, pH 8.0 (w/v) – dissolve 15.01 g glycine (BDH, UK) in 800 ml dH_2O, adjust to pH 8 with NaOH and make up to 1 l.

13 0.1 M Na-Acetate/0.5 M NaCl, pH 4.5 (w/v) – dissolve 6.81 g Na-Acetate (Sigma Chemicals, St. Louis) and 14.61 g NaCl (Saarchem, SA) in 400 ml dH_2O, adjust to pH 4.5 with HCl and make up to 500 ml.

14 0.1 M Tris-HCl/0.5 M NaCl, pH 8.5 (w/v) – dissolve 12.1 g Trizma base (Sigma Chemicals, St. Louis) and 29.22 g NaCl (Saarchem, SA) in 800 ml dH_2O, adjust to pH 8.5 with NaOH and make up to 1 l.

15 Blanks - Non-specific binding to the plate was determined by pre incubating SBK1, diluted 1/800 in PBS, with an equal volume of PBS; and adding 100 ul to the wells coated with coating buffer only, and no conjugate.

CHAPTER 3: RESULTS

PREAMBLE

The expression of tissue kallikrein and the kinin B_1 and B_2 receptors in malignant diseases suggests a role for the kallikrein-kinin system. The aims of the present investigation have been to elucidate the role of the serine protease, tissue kallikrein that forms the vasoactive, inflammatory peptides called kinins, and their receptors in oesophageal carcinoma.

1. At first, the cellular orientation of tissue kallikrein, and kinin B1 and B2 receptors in oesophageal carcinoma was determined by immunocytochemistry (ICC). Next, molecular studies were performed to examine the expression of tissue kallikrein by *in situ* hybridisation (ISH) and reverse transcription-polymerase chain reaction (RT-PCR).

2. The question whether holistic changes occur in patients with carcinoma was examined by determining the concentration of renal tissue kallikrein (enzyme-linked immunosorbent assay), and its enzymic (amidase) and kininogenase (kinin formation) activities in the urine of three patient groups (oesophageal cancer, non-oesophageal cancer and non-cancer patients) (Jenzano *et al.*, 1986).

3.1 HISTOPATHOLOGY

3.1.1 Normal morphology of the oesophagus

The lumen of the normal oesophagus is lined by the mucosal layer composed of a stratified, non-keratinising squamous epithelium. The underlying lamina propria is relatively condensed and contains scattered lymphoid aggregations. Underlying this segment is the muscularis mucosae. The submucosa is highly vascular and contains oesophageal glands and ramifying lymphatic plexus in a loose connective tissue network, which accounts for the early and extensive submucosal spread of oesophageal carcinoma (Figure 2). The submucosal glands are mainly confined to the lower third of the oesophagus (Burkit *et al.,* 1993; Morson & Dawson, 1979). The muscularis propia is composed of a well-developed inner circular and outer longitudinal layers of smooth muscle. Since the first part of swallowing is under voluntary control, fasciculi of skeletal muscle predominate in the muscularis of the upper third of the oesophagus, with a gradual change to smooth muscle in the middle part; in the lower third both coats are entirely smooth muscle (Burkit *et al.,* 1993; Morson & Dawson, 1979). A well marked myenteric plexus is present between the circular and longitudinal muscle coats at all levels. Two types of neurones are present. One is argyrophil, multi-axonal and sends numerous dendrites and axons to surround other neurones in the same and adjacent ganglia, but does not supply muscle cells directly. The second is non-argyrophil and cholinergic and supplies the muscle of the oesophagus. It is probable that the first acts as a controlling mechanism to co-ordinate muscular movements in swallowing, while the second is motor in function. There are two sets of lymphatic channels, one in the submucosa, and one in the muscle layers. This dual drainage is important in the early submucosal spread of oesophageal cancer (Morson & Dawson, 1979).

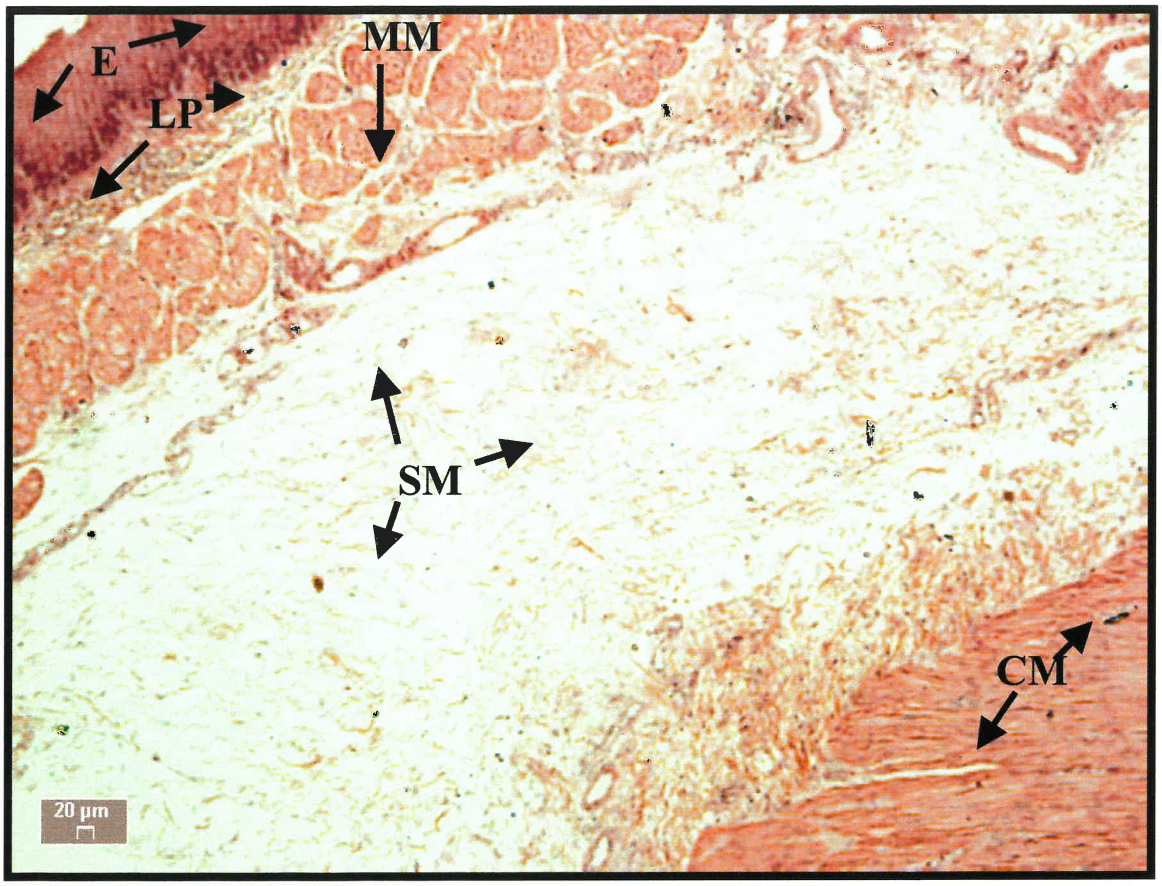


Figure 2 Photomicrograph of the histology of normal oesophagus. The lumen of the oesophagus is lined by a thick protective stratified squamous epithelium (**E**). The underlying lamina propria (**LP**) is relatively condensed and is underlined by muscularis mucosae (**MM**). The submucosa (**SM**) is highly vascular and relatively loose. Allowing for considerable distension during passage of a food bolus. The submucosa also contains small mucous glands which aid lubrication and are mainly confined to the lower third of the oesophagus. The muscularis propia is thick and inner circular (**CM**) and outer longitudinal (not visible in the figure) layers of smooth muscle (X50).

3.1.2 Squamous cell papilloma and papillomatosis

The only benign tumour of the squamous epithelium of the oesophagus is the papilloma and is rare (Livingstone, 1989). Squamous cell papilloma may occur anywhere in the oesophagus but is found most commonly in the lower third as a single, well dermacated sessile intraluminal tumour, usually less than 1.5 cm in size. The aetiology of these lesions is not clear, although some are related to chronic irritation, and others to human papilloma infection (Livingstone, 1995). Histologically, the papilloma consists of finger-like projections of delicate fibrous tissue supporting layers of stratified squamous epithelium (Livingstone, 1989). There may be papilloma-virus associated changes, including koilocytosis and multinucleation.

3.1.3 Squamous cell carcinoma

This is the most common malignant tumour of the oesophagus. Histologically it represents between 72% and 96% of the published cases of oesophageal malignancy (Morson & Dawson, 1979; Livingstone, 1989). Squamous cell carcinomas occur usually in the middle or lower third of the oesophagus, and present clinically with progressive dysphagia, weight loss, anaemia or, rarely, oesophageal perforation (Livingstone, 1995). Squamous cell carcinoma of the oesophagus shows a range of differentiation from abundantly keratinised, well-differentiated lesions containing prominent intercellular bridges to poorly differentiated, virtually anaplastic, large or small cell tumours in which morphologic evidence of squamous differentiation can only be identified after prolonged searching (Figs.3, 4 & 5).

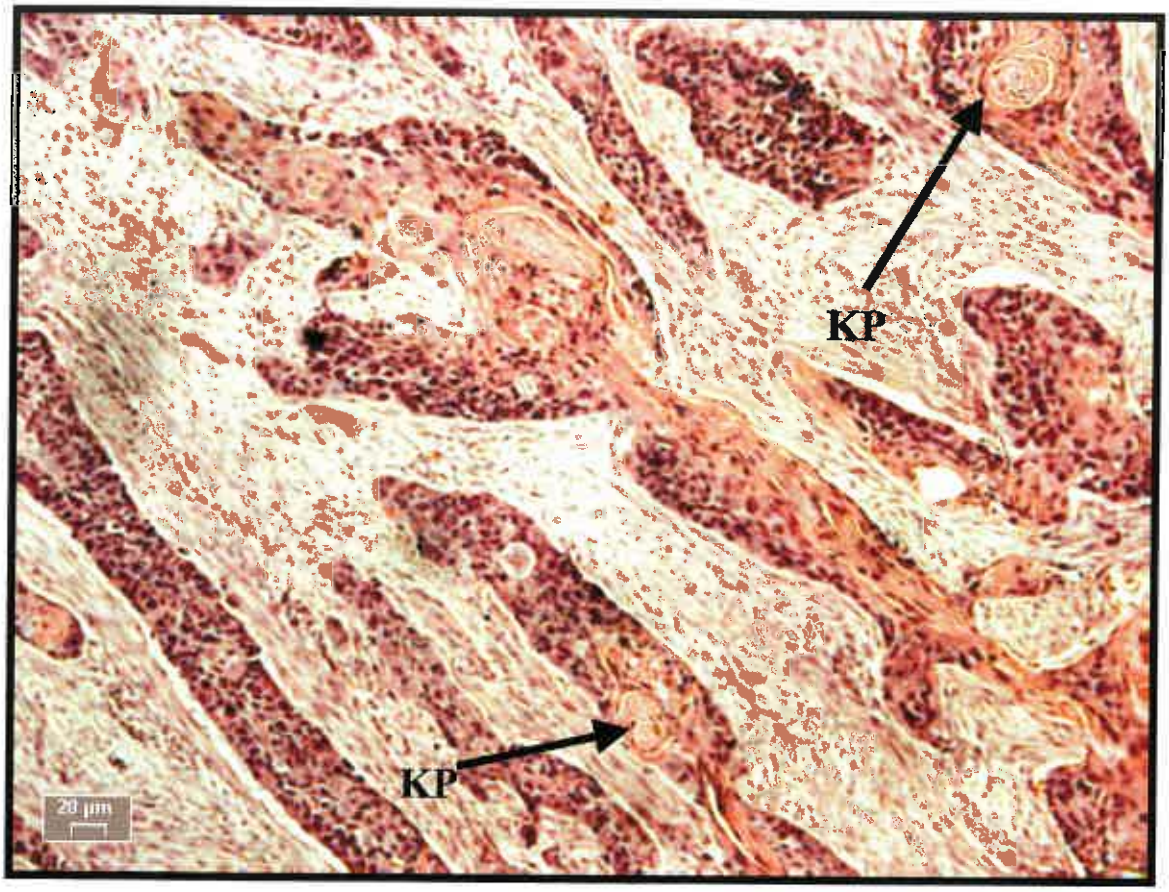


Figure 3 Photomicrograph of well differentiated, keratinising squamous cell carcinoma. Lamellated pink-stained masses known as keratin pearls (**KP**) can be seen. Haematoxylin and Eosin stain (X200).

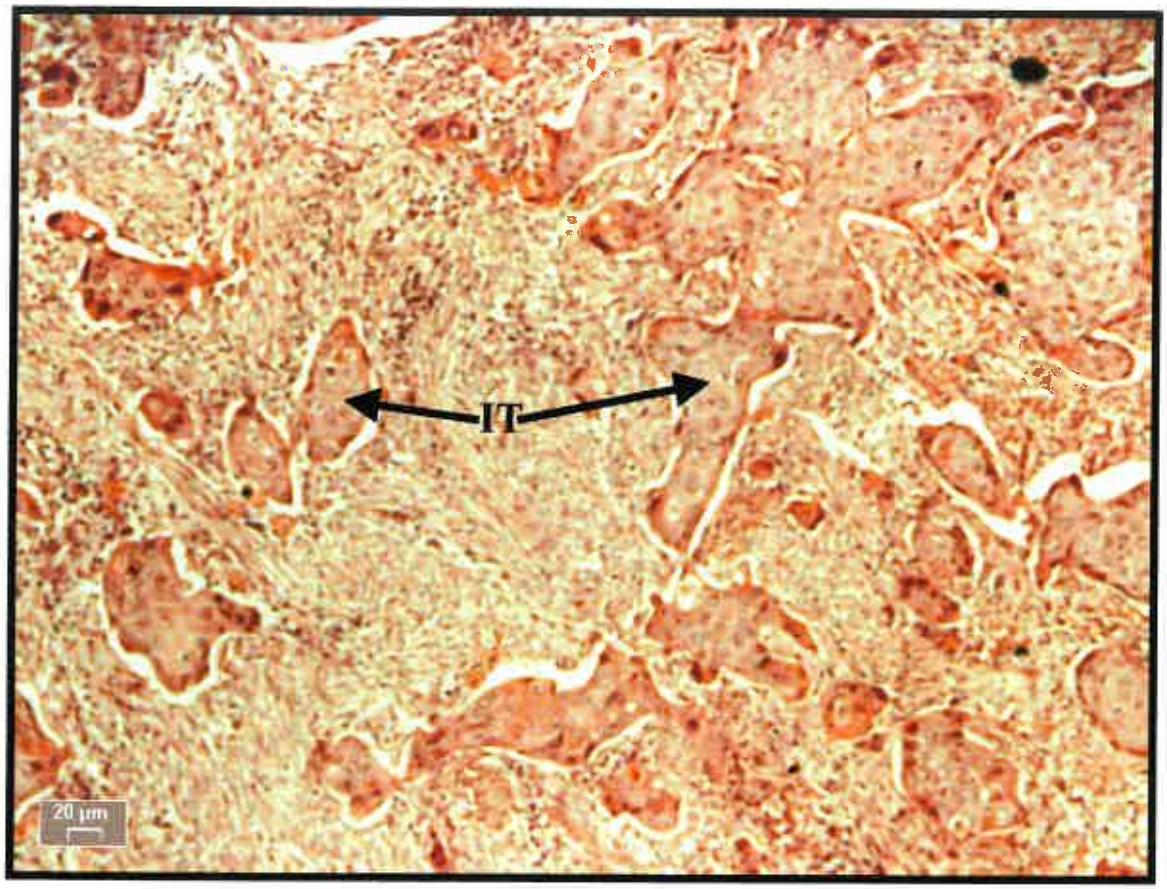


Figure 4 Photomicrograph of moderately differentiated squamous cell carcinoma. Infiltration of the muscular wall by islands of tumours (**IT**) with no keratin. Haematoxylin and Eosin stain (X200).

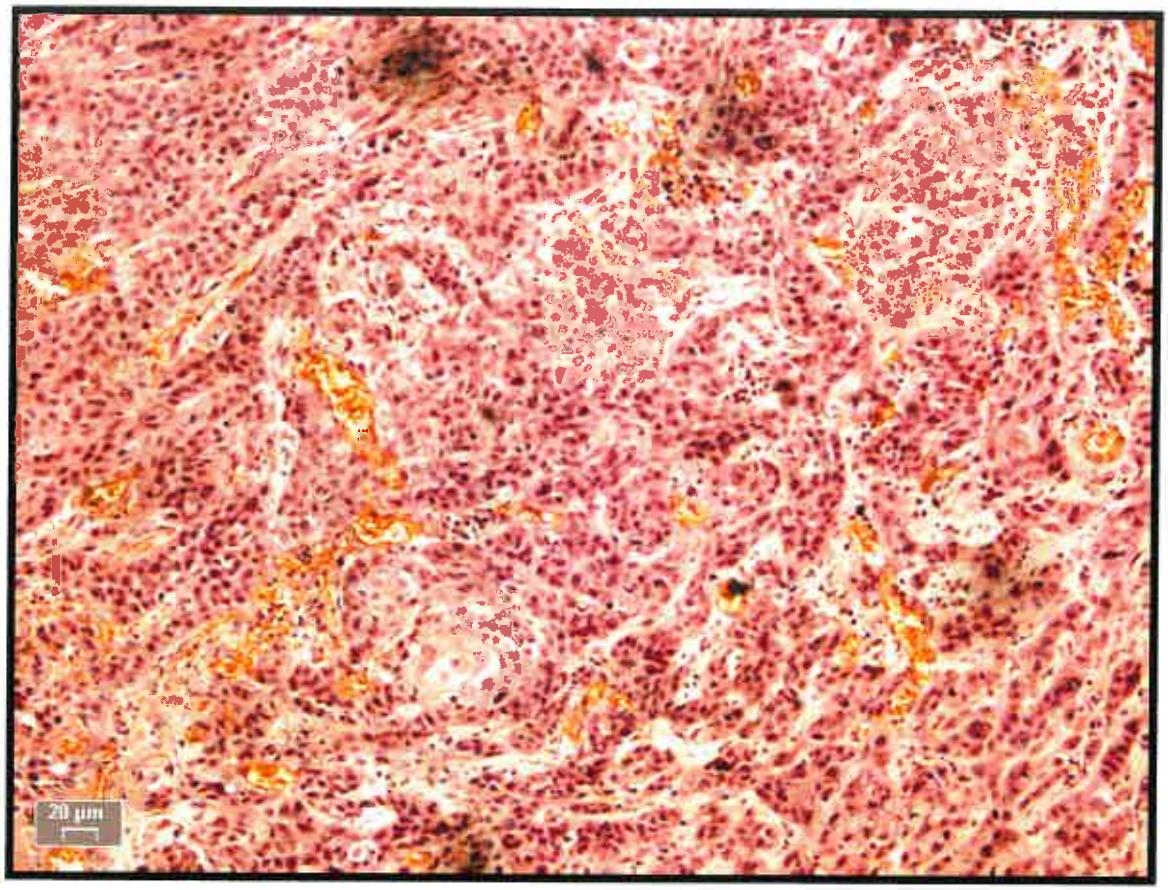


Figure 5 Photomicrograph of poorly differentiated non-keratinising squamous cell carcinoma. Haematoxylin and Eosin stain (X200).

3.1.4 Adenocarcinoma of the oesophagus

The vast majority of primary oesophageal adenocarcinomas arise in the lower third of the oesophagus against a background of glandular (columnar) metaplasia of Barrett's oesophagus, and hence most cases are associated with chronic gastro-oesophageal reflux. Before a diagnosis of primary oesophageal adenocarcinoma is made it is often necessary to exclude the possibility that the lesion is a gastric carcinoma extending upwards to involve the lower oesophagus (Livingstone, 1995; Livingstone, 1989). Oesophageal adenocarcinomas occurring in the absence of Barrett's oesophagus are very rare (Figure 6)

3.1.5 Adenosquamous carcinoma

An adenosquamous carcinoma is one in which both squamous and glandular elements are present (Morson & Dawson, 1979). This type of tumour is not uncommon as it was thought to be, and the outcome is also poor. Groups of cells mimicking the ducts of submucosal glands are usually observed (Livingstone, 1989). Co-existence of independent squamous cell and adenocarcinomas has been reported (Livingstone, 1995) (Figure 7).

3.1.6 Smooth muscle tumours

These are uncommon tumours that present most often in middle age with dysphagia or heartburn. They originate from the smooth muscle either of muscularis mucosae or muscle coats. Benign leiomyomas greatly outnumber leiomyosarcomas, which tend to occur in the older age group (Livingstone, 1995). Most oesophageal smooth muscle tumours resemble

similar tumours elsewhere in the body, and are composed of interlacing bundles of smooth muscle cells intermingled with variable amounts of collagen. The cells have blunt-ended, cigar-shaped nuclei and eosinophilic cytoplasm. Leiomyosarcomas may show anaplastic features with marked nuclear polymorphism, tumour giant cells and abnormal mitotic figures, although they are more often difficult to separate from leiomyomas on the basis of histology alone (Livingstone, 1995) (Figure 8).

3.1.7 Connective tissue tumours

A whole range of benign and malignant connective tissue tumours of the oesophagus have been reported. Benign lesions include neurilemmomas and neurofibromas arising from nerve plexuses, rhabdomyomas of the striated muscle of the proximal oesophagus, hemangiomas and lymphongiomas, lipomas, chondromas and osteochondromas. (Livingstone, 1995).

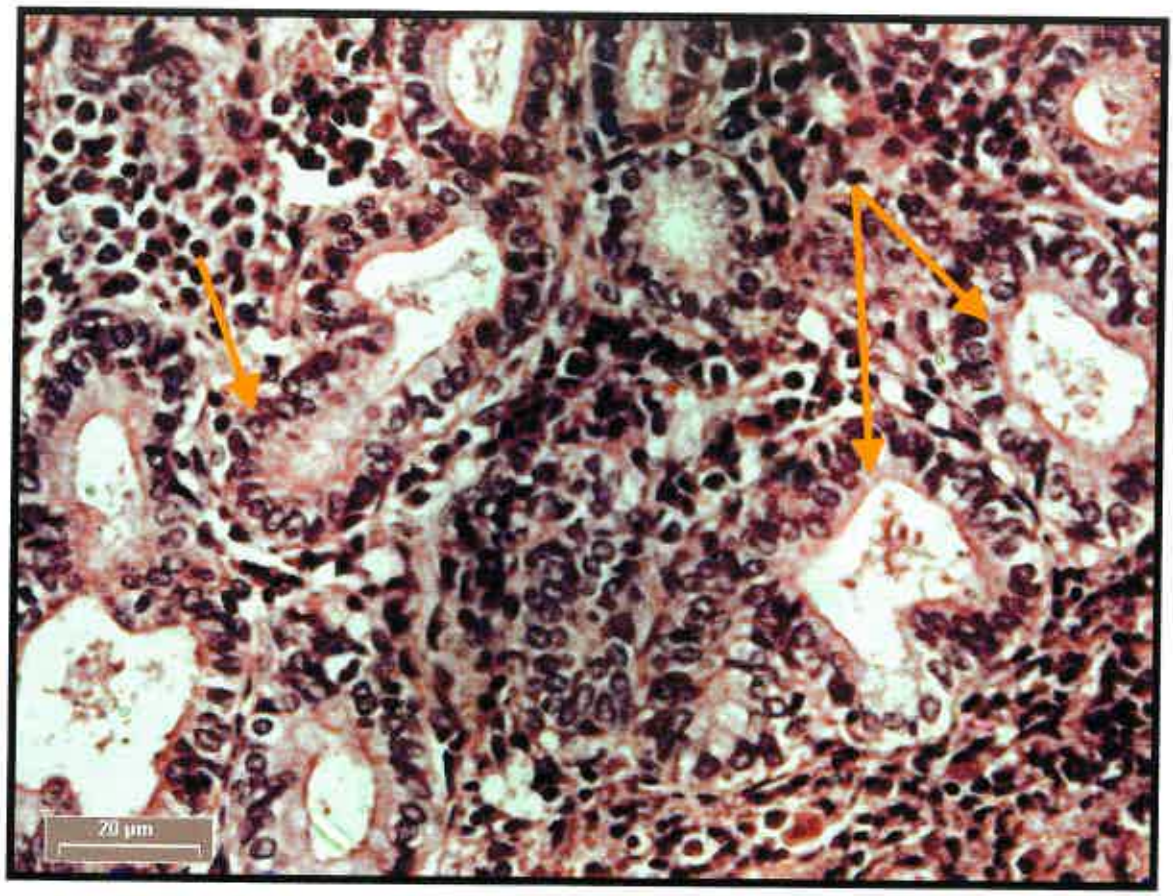


Figure 6 Photomicrograph of adenocarcinoma of the oesophagus. Cuboidal or columnar cells form tubular or acinar structures. The tumour is very well differentiated and mimics dysplasia of cardiac glands. Haematoxylin and Eosin stain (X400).

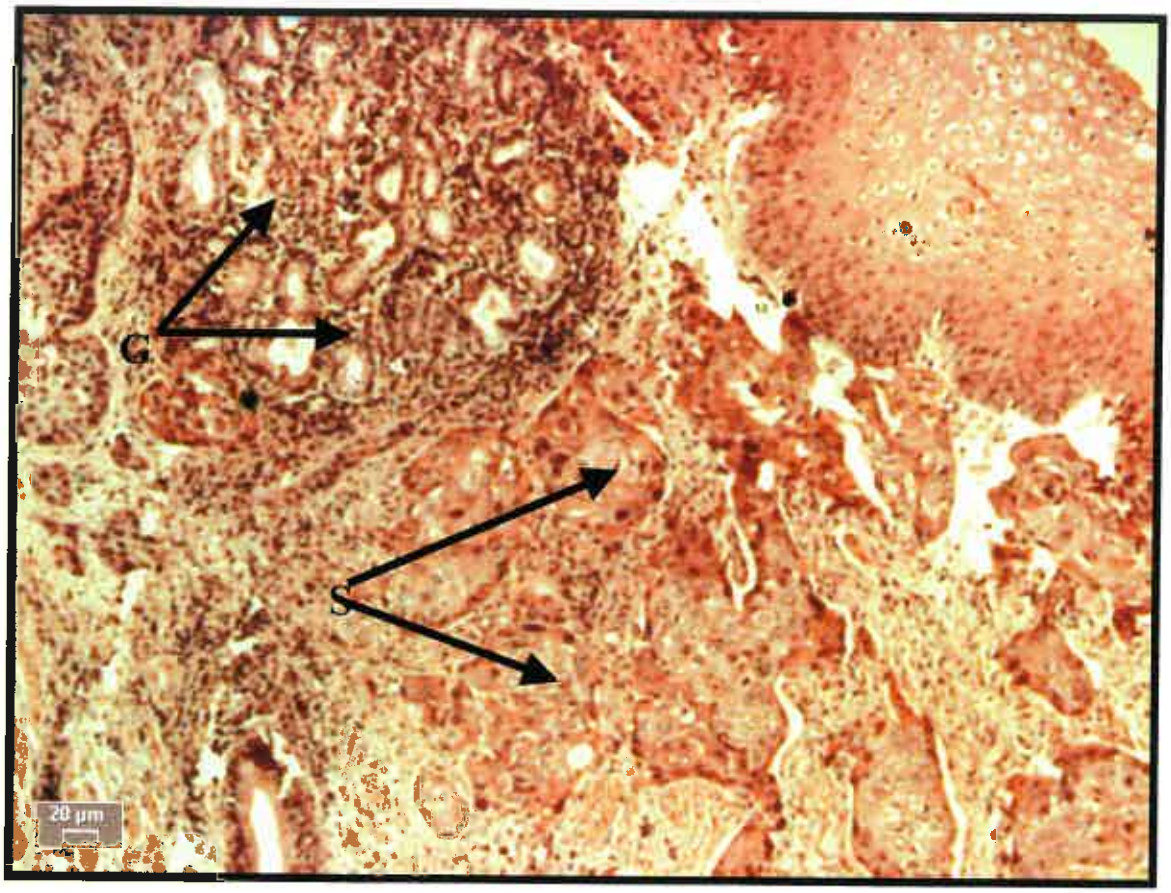


Figure 7 Photomicrograph of malignant squamous (**S**) and glandular (**G**) elements which are both present in adenosquamous carcinoma. Haematoxylin and Eosin stain (X200).

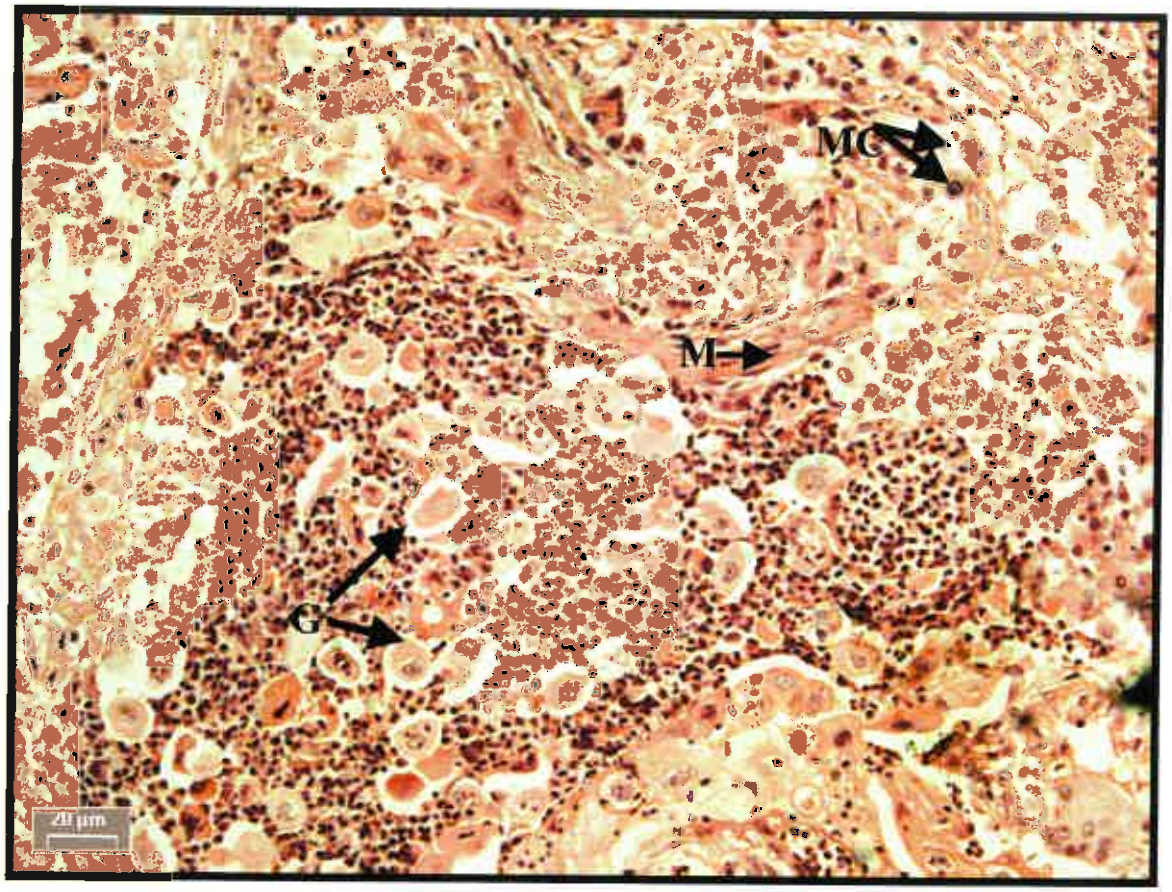


Figure 8 Photomicrograph of smooth muscle carcinoma of the oesophagus. Bizzare giant cells (**G**) and mast cells (**MC**) can be seen infiltrating the muscle layer (**M**). Haematoxylin and Eosin stain (X200).

3.2 Cellular localisation of tissue kallikrein in oesophageal carcinoma

3.2.1 Patient demographics

Out of 50 oesophageal cancer patients selected, 33 were biopsies and 17 were resection specimens (Annexe D). The histological grading of these patients showed that they all were squamous cell carcinoma (SCC) and predominantly infiltrating or invasive moderately differentiated. Well and poorly differentiated squamous cell carcinoma was very rare.

3. 2. 1. 1 Immunolocalisation of tissue kallikrein using 3 3'-diaminobenzidine (DAB)

When DAB is used as a chromogenic substrate in immunohistochemical experiments, a brown precipitate is observed. That is, the immunolocalisation of an antibody-labelled target protein for example tissue kallikrein is specifically and exclusively indicated by brown colour in the tissue, as is demonstrated in the following results. The human salivary gland was used as a positive control tissue for cellular localisation of TK, which is specifically synthesised and stored in the ducts of the gland. This finding is illustrated in **figure 9A**, and **figure 9B** provides the method control where the antibody was replaced with phosphate buffered saline or preabsorbed with an excess of antigen.

A

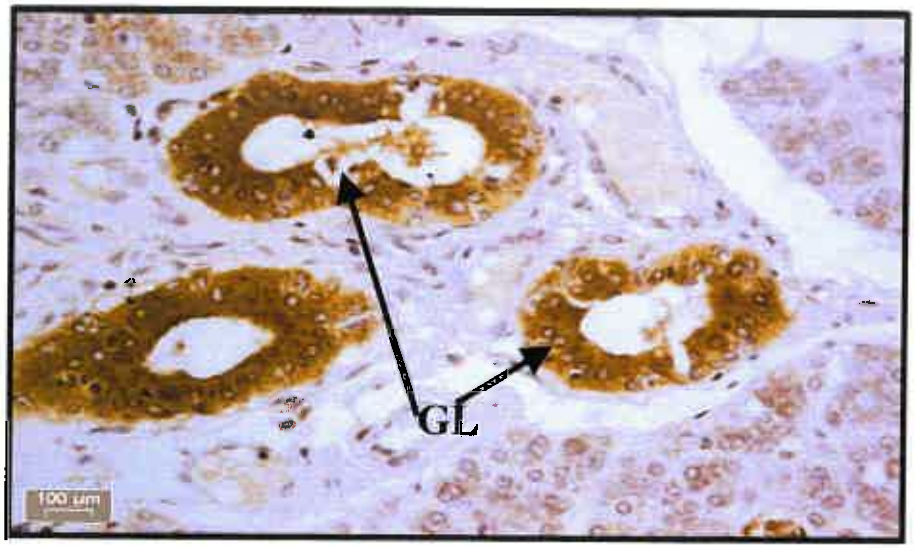


Figure 9 A Photomicrograph showing salivary gland.

(A) Tissue kallikrein positive control. Note that the glandular structures (**GL**) are distinctly labelling for TK (X400).

B

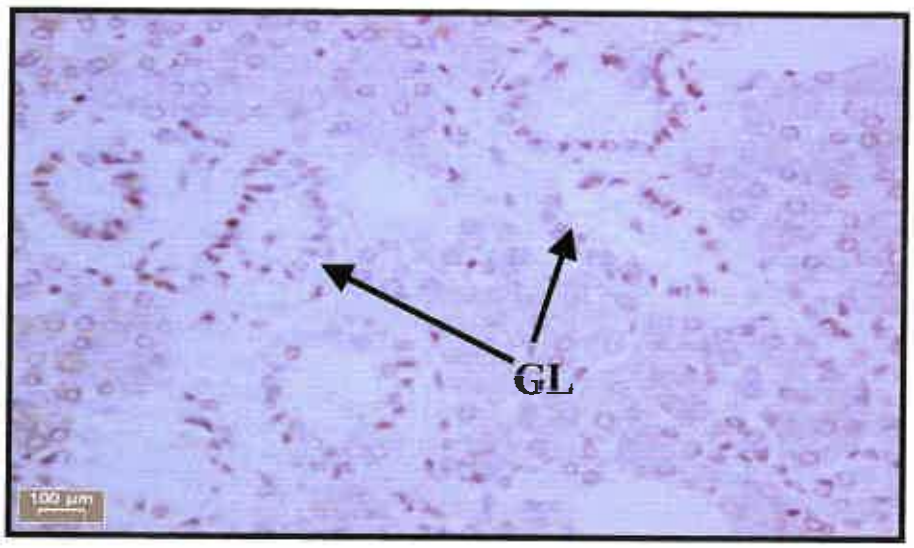


Figure 9 B Photomicrographs showing salivary gland.

(B) method control where the primary antibody was replaced with phosphate buffered saline or preabsorbed with the antigen. This photomicrograph shows no labelling in glandular structures (**GL**) (X400).

Figure 10 below is the normal epithelium of the oesophagus (mucosa) immunolabelled with DAD for TK immunolocalisation. Note that TK immunolabels intensely on the upper epithelium (**UE**) of the mucosa and the basal (**BE**) part of the mucosa is negative for TK. The submucosa (**S**) labels negative for TK

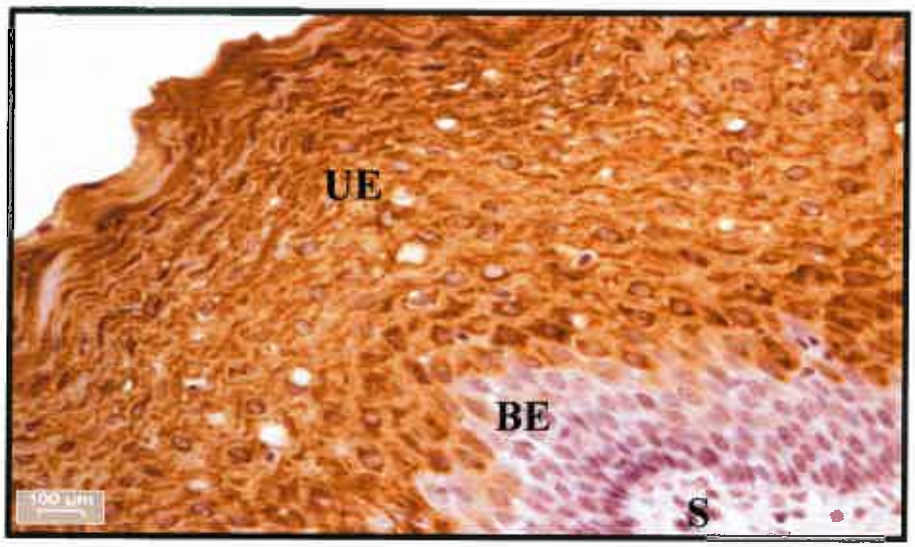


Figure 10 Photomicrograph of a normal oesophageal epithelium (X400).

The following **figure 11** is a photomicrograph of a oesophageal tumour epithelium. The squamous cells have lost their squamous shape and now they vary in shape and size i.e. they show pleomorphism and they now also show a high nuclei to cytoplasm ratio. The epithelium is disintergrating. The disintegrating upper epithelium (**UE**) is labelling for TK.

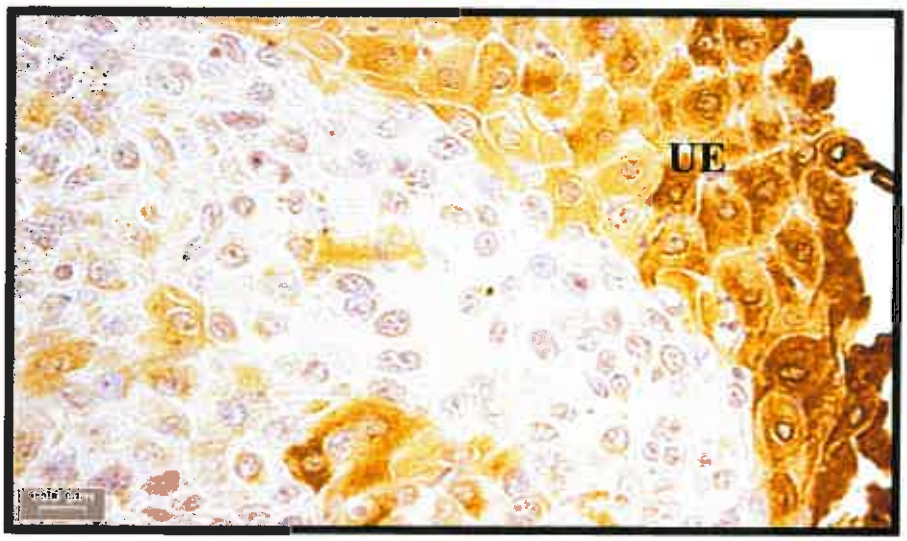


Figure 11 Photomicrograph of a tumour epithelium (X400).

Figure 12 is a photomicrograph of oesophageal tumour muscularis mucosa showing the infiltration by tumour giant cells (G). Note the intense labelling for TK in the giant cells. The intensity of labelling is not the same in all the giant cells seen in the photomicrograph. There is also some infiltration of some of the giant cells by neutrophils tumour is infiltrating the muscular layer. Note the infiltration of the muscular layer by degenerating giant cells (G) which immunolabel for TK.

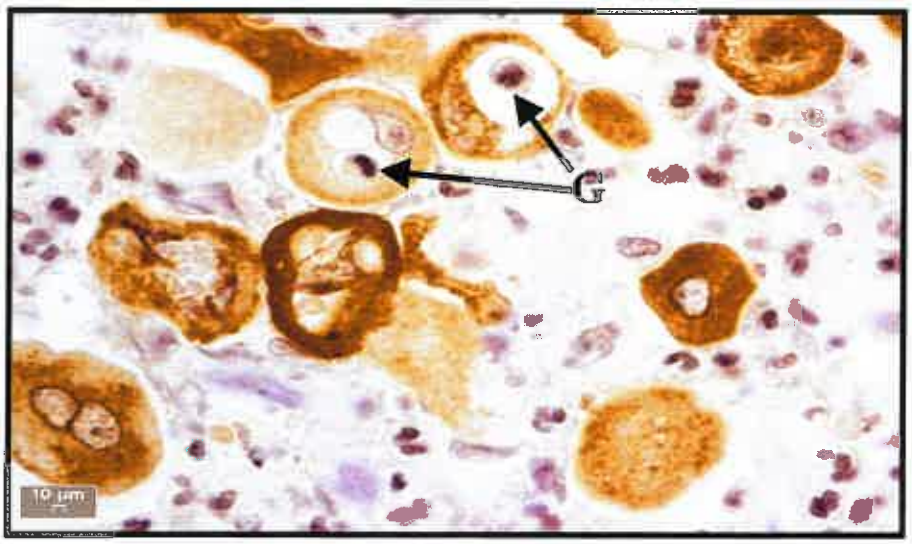


Figure 12 Photomicrograph of oesophageal muscularis mucosa. (X1000).

Figure 13 is the normal oesophageal muscularis propia (inner and outer muscular layer) and it appears negative for TK immunolabelling.

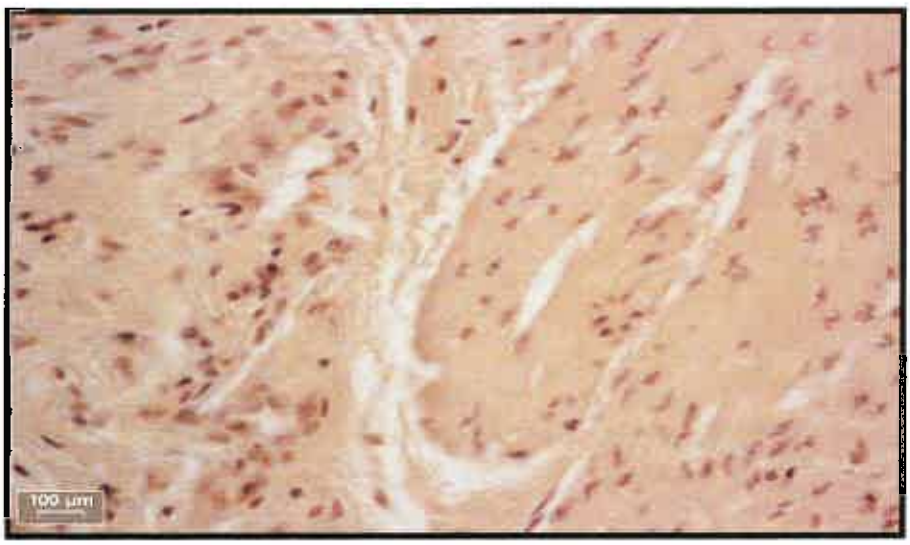


Figure 13 Photomicrograph of normal oesophageal muscularis propria.

(X400).

Figure 14A is a photomicrograph of oesophageal tumour in the submucosa showing the intense labelling for TK in mast cells (**M**). **Figure 14B** is the Giemsa stain used as a histological indicator for mast cells. The dark purplish stained mast cells are seen on this photomicrograph against a bluish background.

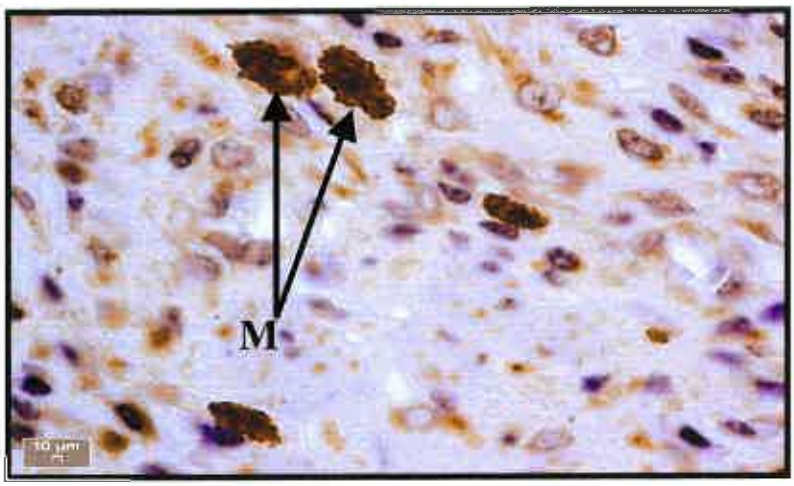


Figure 14 A Photomicrograph of oesophageal tumour in the submucosa (X1000).

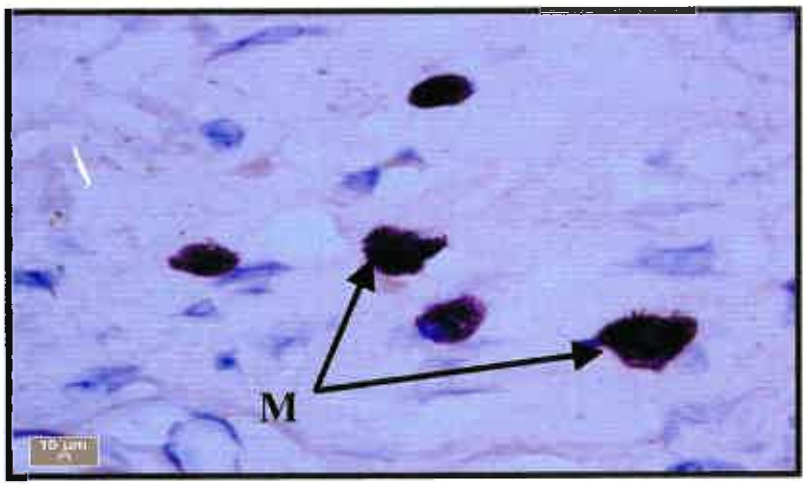


Figure 14 B Photomicrograph of Giemsa staining of oesophageal tumour (X1000).

Figure 15 is a photomicrograph of oesophageal tumour showing a submucosa with a well differentiated keratinising squamous cell tumour showing a TK labelling in keratin pearls (**KP**) in the submucosa.

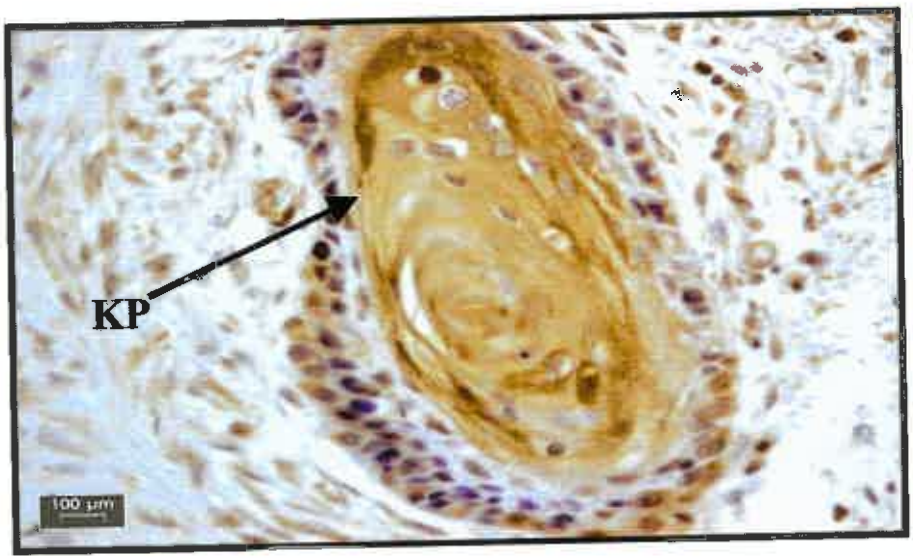


Figure 15 Photomicrographs of well differentiated keratinising oesophageal tumour (X400).

Figure 16 is the photomicrograph of oesophageal submucosal tumour showing the method control where the antibody was replaced with a phosphate buffered saline or preabsorbed with excess antigen. This photomicrograph serves as a negative control for TK labelling.

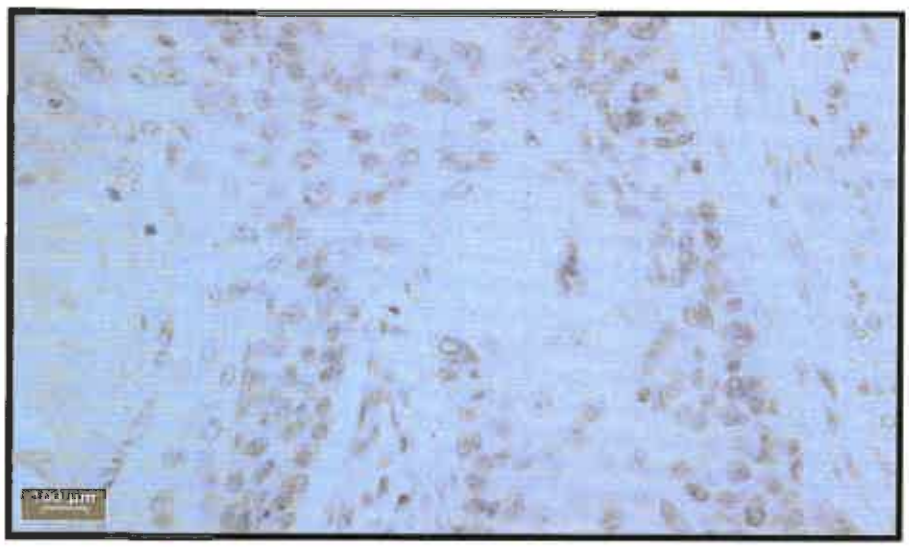


Figure 16 Photomicrographs of oesophageal tumour submucosa method control (X400).

3.2 Cellular localisation of tissue pro-kallikrein (TproK) immunolocalisation using 3 3'-diaminobenzidine (DAB)

Figure 17 is a photomicrograph of tissue prokallikrein (TproK) positive control. Note the immunoreactive tissue pro-kallikrein in tubular structures. The intensity of TproK labelling was not as strong as the TK labelling in the salivary gland.

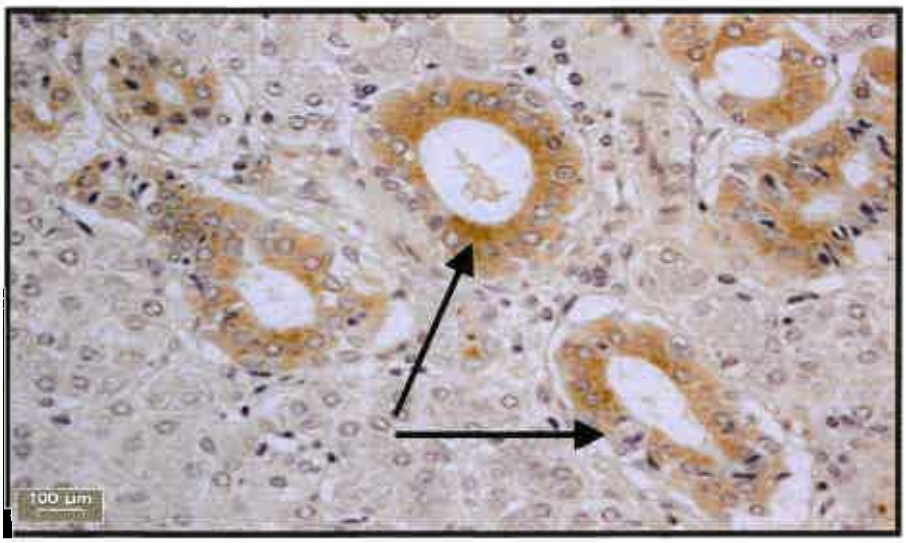


Figure 17 Photomicrograph of a salivary gland positive control (X400).

Figure 18 is the normal epithelium of the oesophagus (submucosa) immunolabelled for tissue prokallikrein (TproK). Note that the intensity of DAB labelling was higher towards the periphery of the mucosa and the basal epithelium appeared negative for TproK immunoreactivity.

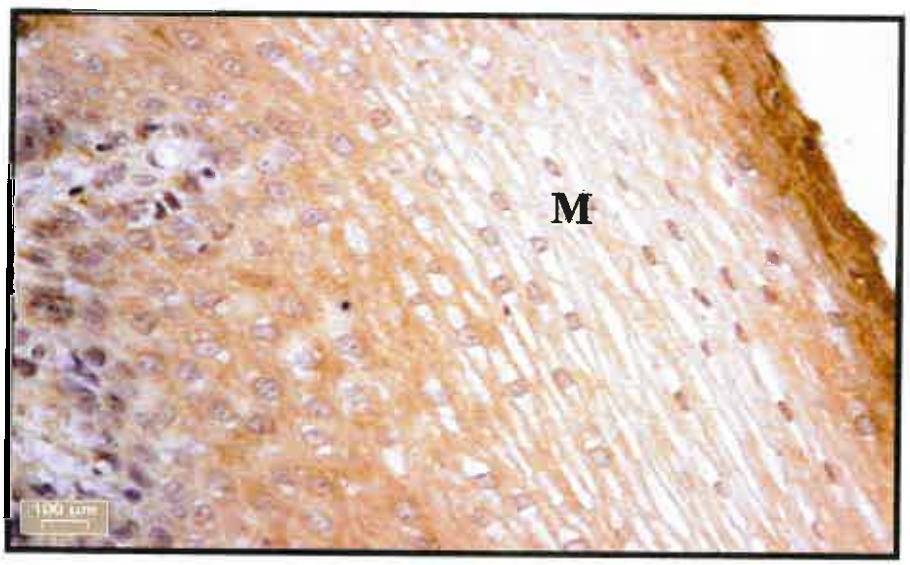


Figure 18 Photomicrograph of a normal oesophageal mucosa (**M**) (X400).

Figure 19 is a photomicrograph of oesophageal tumour epithelium. . The squamous cells have lost their squamous shape and now they vary in shape and size i.e. they show pleomorphism and they now also show a high nuclei to cytoplasm ratio. Labelling for TproK is seen on the surface epithelium. Note the tissue prokallikrein labelling on the periphery of the epithelium (E). There is no clear separation of mucosa and submucosa as is the case in the normal epithelium.

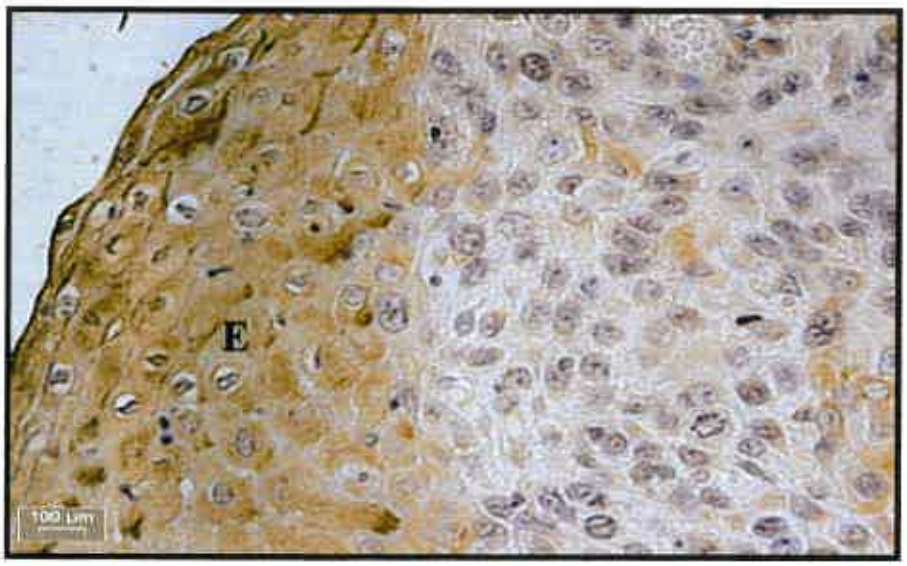


Figure 19 Photomicrograph of a tumour in the oesophageal mucosa (X400).

Figure 20 is a photomicrograph of oesophageal tumour submucosa showing the intense labelling for TproK in the activated mast cells. Note the intense of DAB labelling for tissue prokallikrein in activated mast cells (**M**).

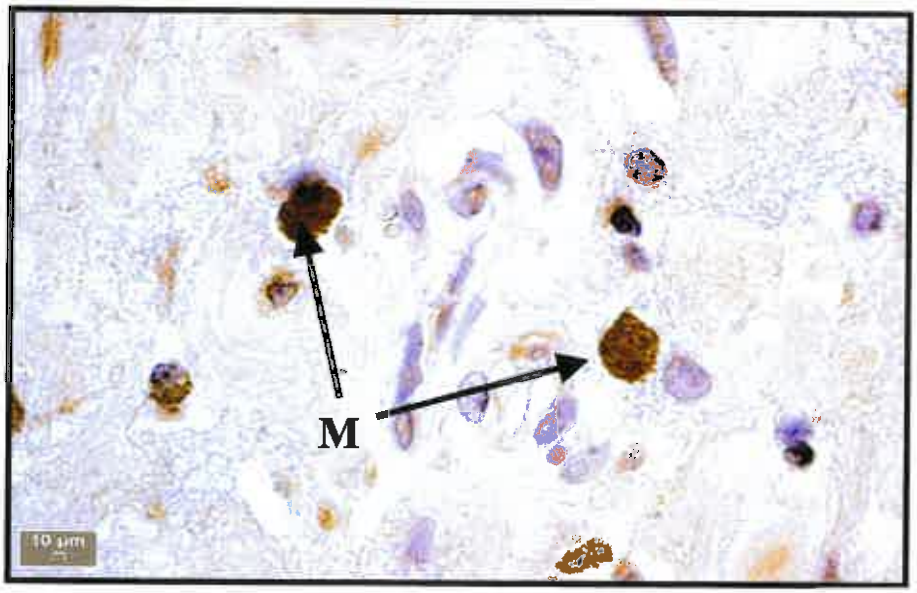


Figure 20 Photomicrograph of oesophageal tumour in the submucosa (X1000).

Figure 21 is a photomicrograph of oesophageal tumour muscularis mucosae showing the infiltration by tumour giant cells (G). Note the intense labelling for TproK in the giant cells. There is also an infiltration of tumour giant cells by neutrophils seen in this photomicrograph.

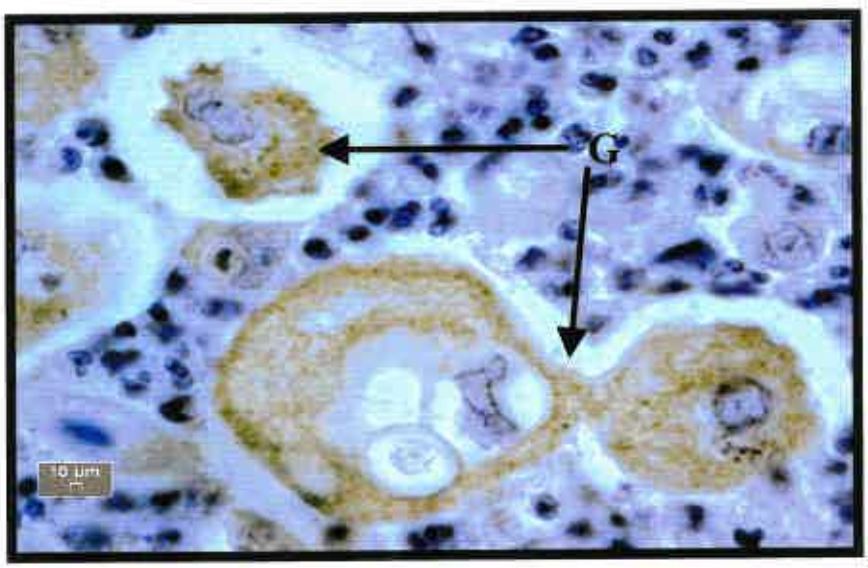


Figure 21 Photomicrograph of oesophageal submucosal tumour (X1000).

Figure 22 is a photomicrograph of a well differentiated keratinising squamous cell oesophageal tumour submucosa showing a TproK labelling in a keratin pearls (KP).

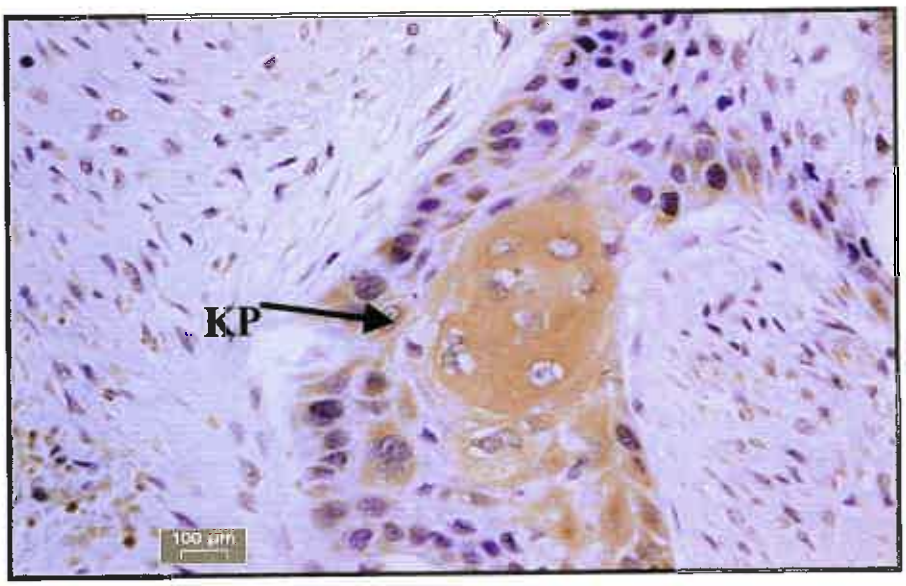


Figure 22 Photomicrograph of oesophageal infiltrating keratinising well differentiated squamous cell carcinoma (X400).

Figure 23 is the normal oesophageal muscularis propia immunolabelled for TproK. The section appears to show no immunoreactivity for the tissue prokallikrein in this photomicrograph.

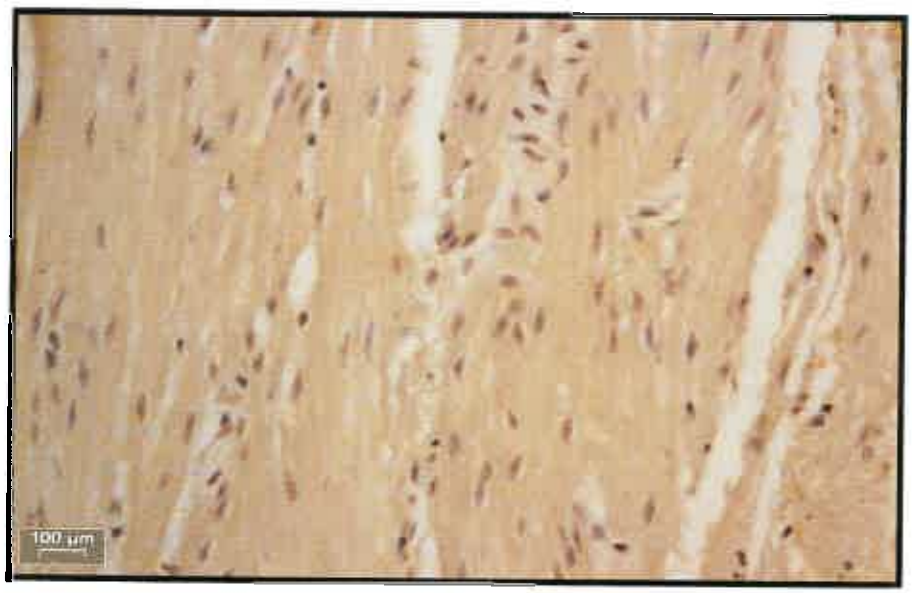


Figure 23 Photomicrograph of a muscularis propria of normal oesophageal tissue (X400).

3.4 Cellular localisation of kinin B1 and B2 receptors

3.4.1 Kinin B1 receptor immunolocalisation using 3,3'diaminobenzidine (DAB)

Figure 24 is a photomicrograph a section of the human spinal cord served as positive control tissue for B1 receptor immunolocalisation (Bhoola, 1996). Although immunolabelling for B1 receptors was observed in some interneurons in the intermediate area of the spinal cord grey matter, the most intense labelling was observed in neurones within laminae 1 and 2. This latter region corresponds to substantia gelatinosa of the spinal cord, which is an area that has important significance in pain modulation and is also the site of other important putative peptide neurotransmitters viz. serotonin and substance P. For these reasons and because of the constant intense immunolabelling of B1 receptors observed in these neurones, this tissue was used as the positive control tissue in all future experiments for the localisation of immunoreactive kinin B1 receptor (Raidoo, 1999 personal communications).

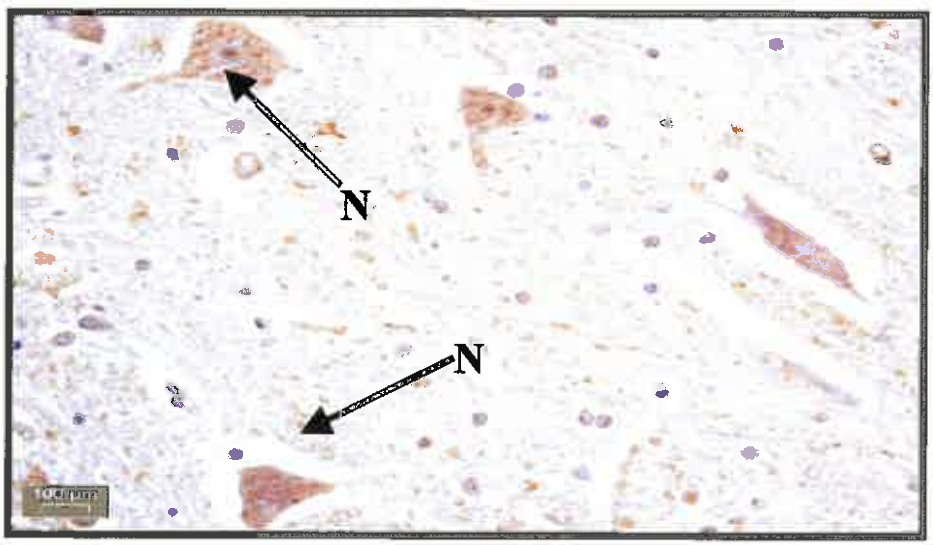


Figure 24 Photomicrograph of the grey matter (anterior horn of the spinal cord) Note B1 DAB immunolabelling in neurons (**N**) (X1000).

Figure 25 is a method control photomicrograph showing a loss of immunolabelling following pre-absorption of the antibody with an excess of the antigen and the replacement of the primary antibody by PBS.

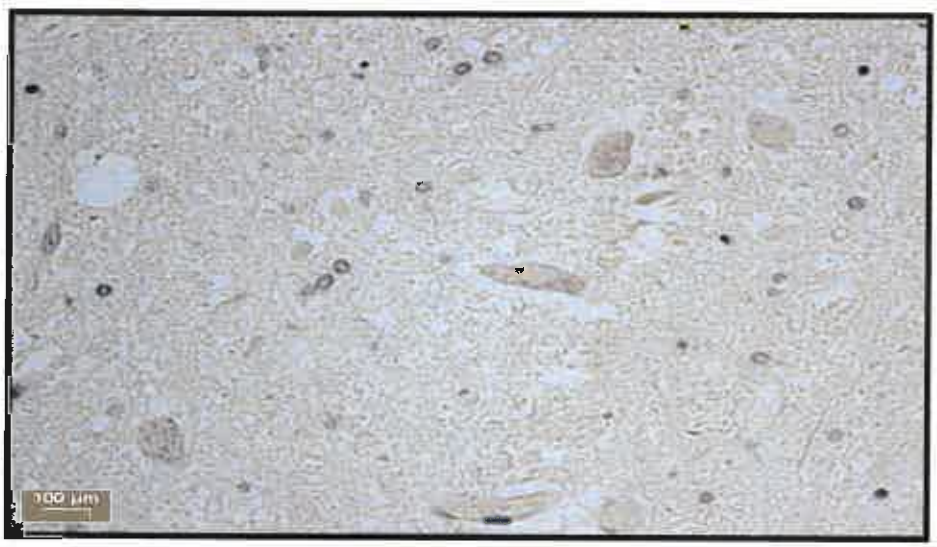


Figure 25 Photomicrograph of a spinal cord method control (400X).

Figure 26 is the normal epithelium of the oesophagus (submucosa) immunolabelled for the kinin B1 receptor. Note the intense cytoplasmic DAB labelling on the periphery of the submucosa and the basal epithelium is negative for the kinin B1 receptor.

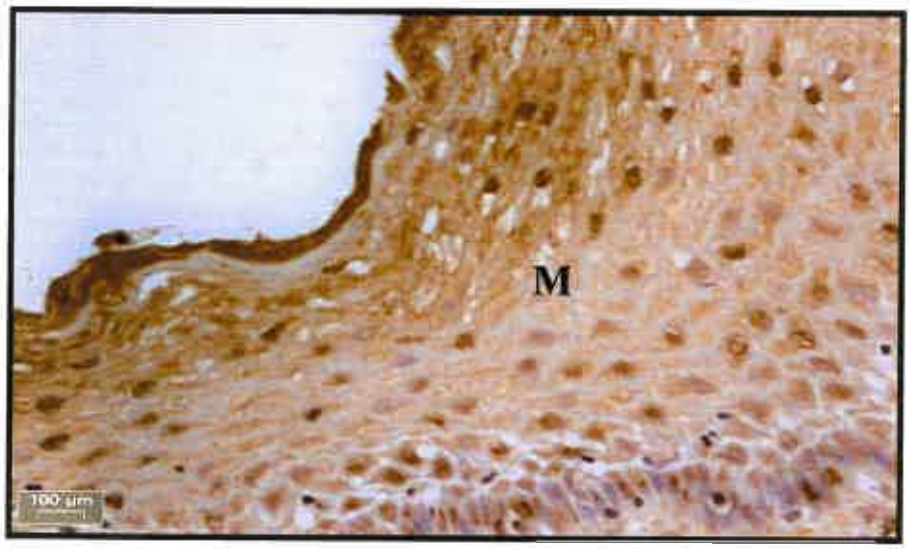


Figure 26 Photomicrograph of the mucosa (M) of a normal oesophageal tissue (400X).

Figure 27 is a photomicrograph of a oesophageal tumour epithelium. The squamous cells are disintegrating. And there is no clear dermacation between the basal and upper epithelium. Labelling for the kinin B1 is seen on the periphery of the mucosa (M).

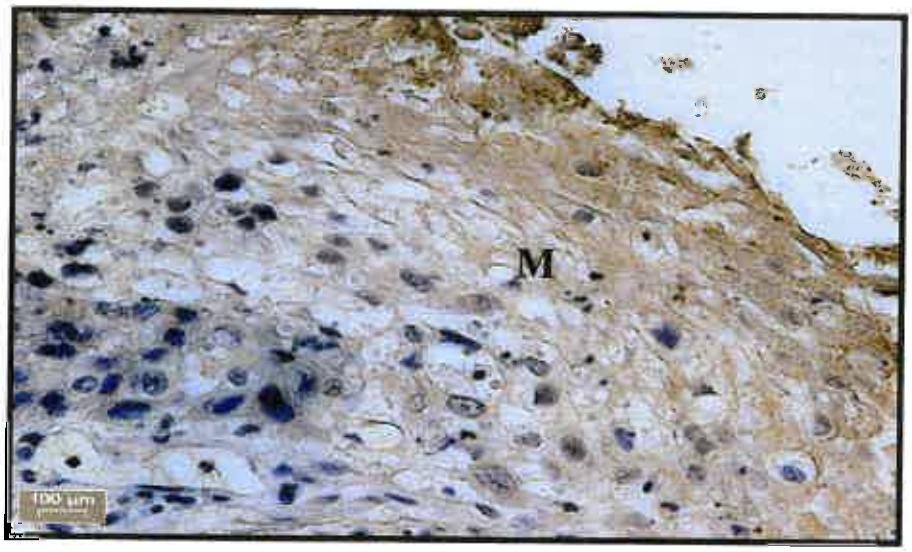


Figure 27 Photomicrograph of oesophageal tumour mucosal layer (M) (X400).

Figure 28 is a photomicrograph of oesophageal tumour submucosa showing the immunoreactive kinin B1 receptor in mast cells. Note the activated mast cells (**M**) intensely labelling for kinin B1 receptor.

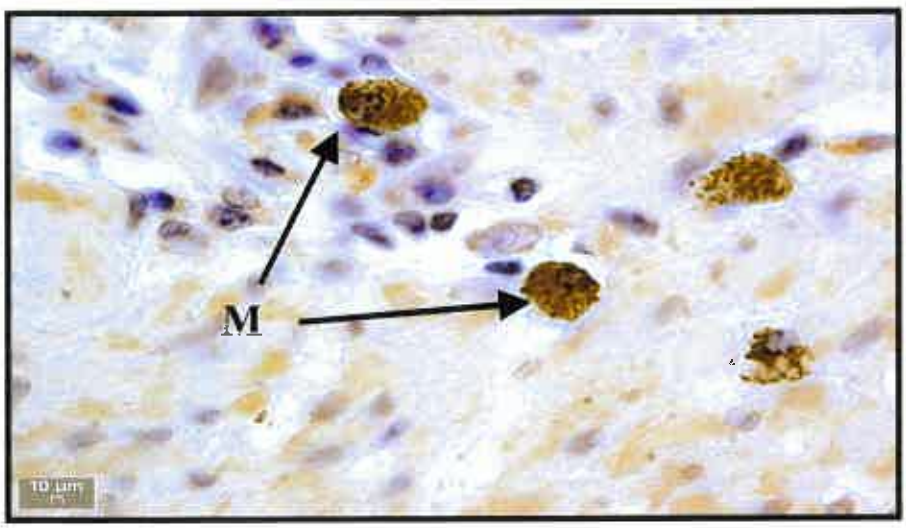


Figure 28 Photomicrograph of the submucosa of oesophageal tumour (X1000).

Figure 29 is a photomicrograph of oesophageal tumour muscularis mucosa showing the infiltration by tumour giant cells (G). Note the intense labelling for kinin B1 in the giant cells.

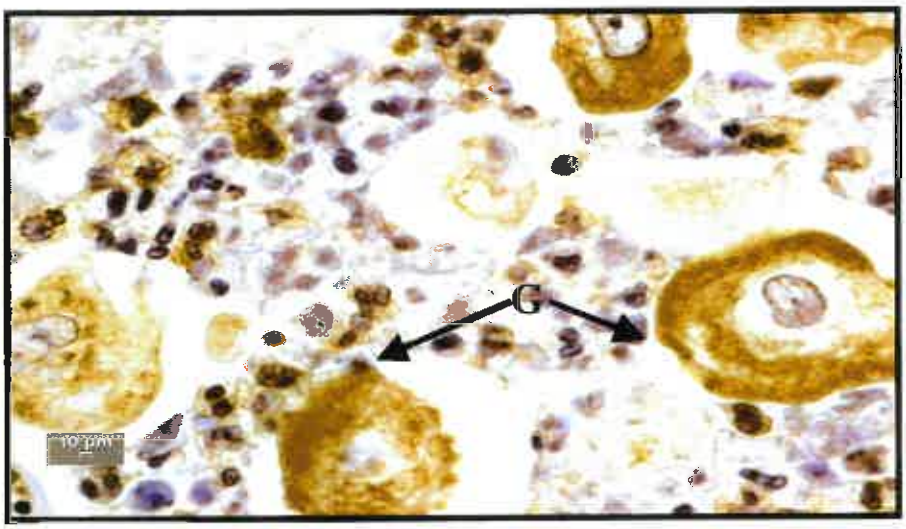


Figure 29 Photomicrograph of the submucosa of oesophageal tumour immunolabelled for kinin B1 receptor (X1000).

Figure 30 is a photomicrograph of oesophageal submucosa with a well differentiated keratinising squamous cell tumour showing the kinin B1 receptor immunoreactivity in keratin pearl (KP).

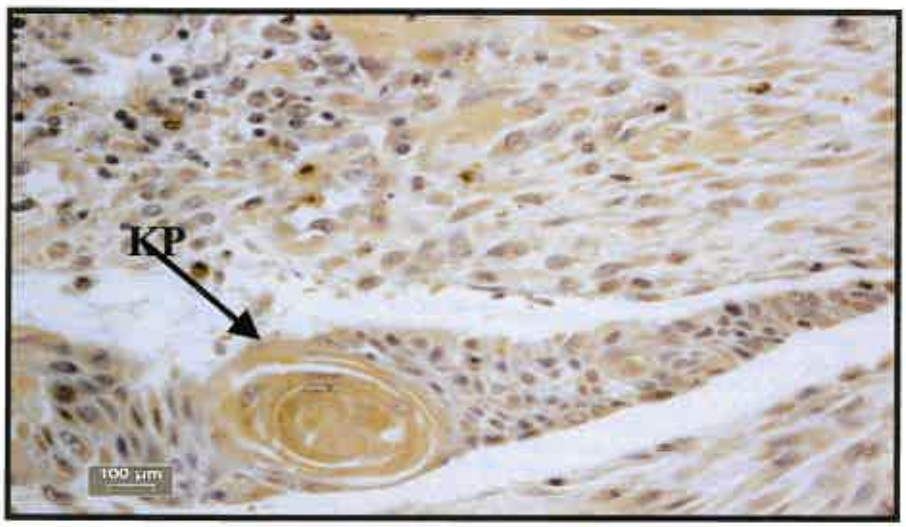


Figure 30 Photomicrograph of the submucosa of oesophageal tumour (400X).

Figure 31 is the normal oesophageal muscularis mucosa immunolabelled for the kinin B1 receptor and it appears negative for the kinin B1 receptor.

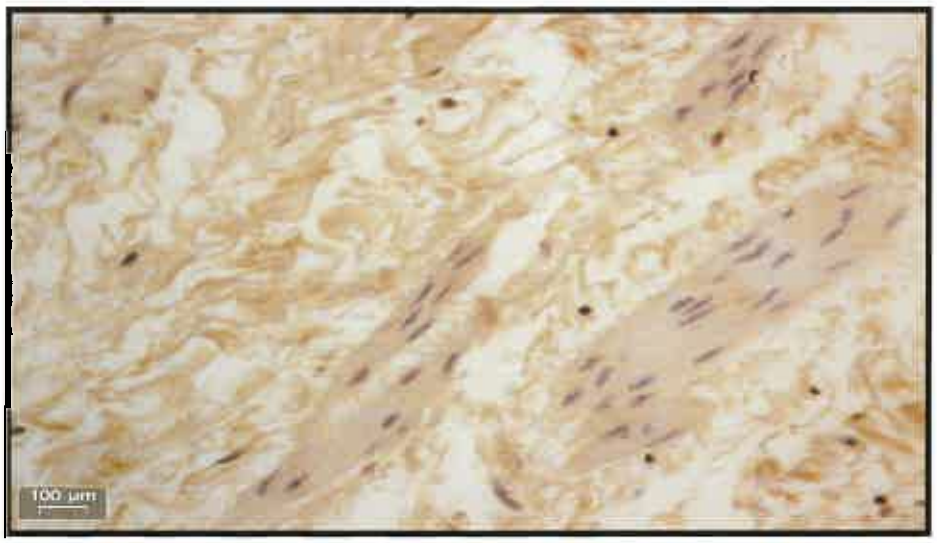


Figure 31 Photomicrograph of the submucosa of normal oesophageal tissue (400X).

3.4.2 Kinin B2 receptor immunolocalisation using 3,3'diaminobenzidine (DAB)

It has been found that kinin B2 receptors are abundant in the ducts of the salivary gland. (Personal communications). Samples of fresh normal salivary gland collected at post-moterm and fixed in 5% formal saline was used as an appropriate positive control during each immunoprecipitation and that is demonstrated by **figure 32** in the following photomicrograph. Note the intensity of B2 DAB labelling in the ducts (**D**) of the salivary gland

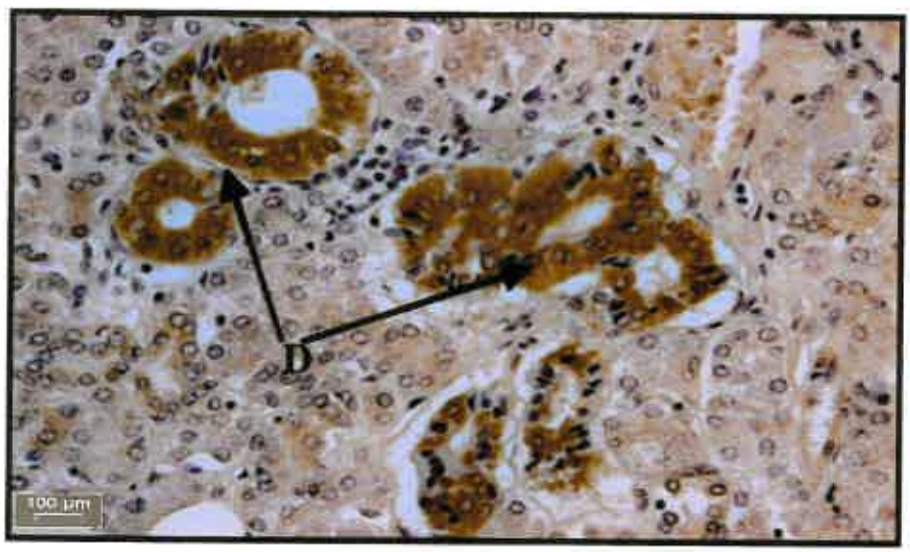


Figure 32 Photomicrograph of the salivary gland used as a positive control (400X).

Figure 33 is the normal epithelium of the oesophagus immunolabelled for the kinin B2 receptor. Note the immunolabelling for kinin B2 receptor is more intense on the periphery of the mucosa and the basal epithelium is negative for the kinin B2 receptor.

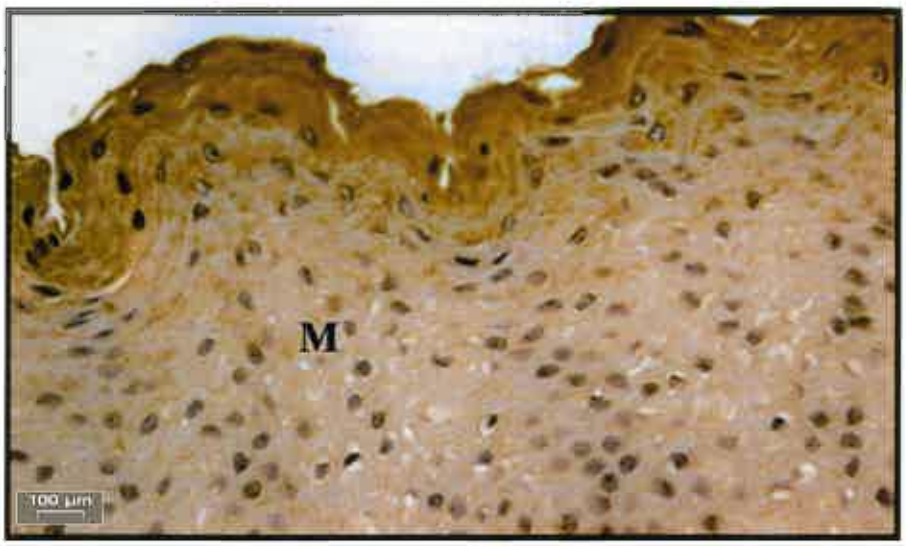


Figure 33 Photomicrograph of the epithelium of oesophageal tissue (400X).

Figure 34 is a photomicrograph of a oesophageal tumour epithelium. The squamous cells are disintergrating and now appear oval in shape rather than flattened and there is no clear dermacation between the basal and upper epithelium. Immunoreactive kinin B1 receptor is seen on the surface epithelium.. The surface cells cytoplasm to nuclei ratio has decreased

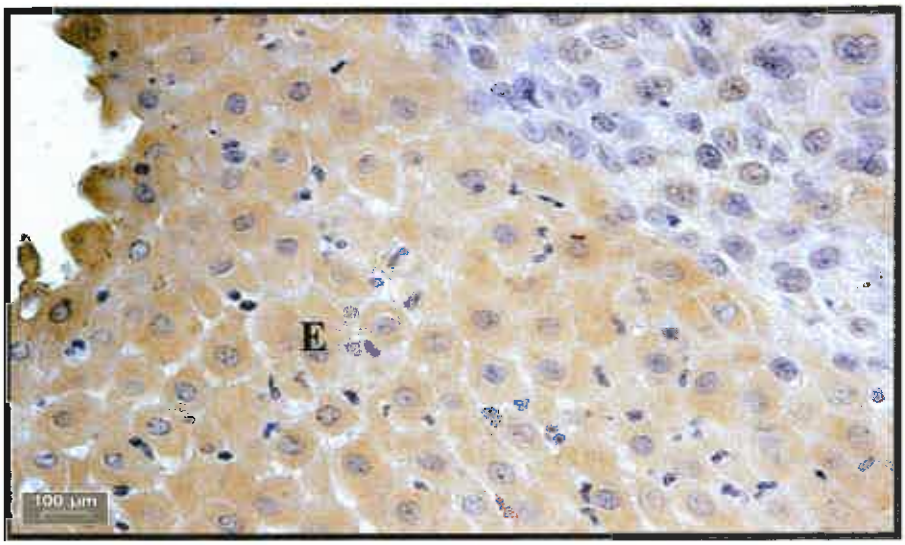


Figure 34 Photomicrograph of an epithelium of the oesophageal tumour (400X)

Figure 35 is a photomicrograph of oesophageal tumour submucosa immunolabelled for the kinin B2 receptor. The high intensity immunolabelling is seen in the mast cells.

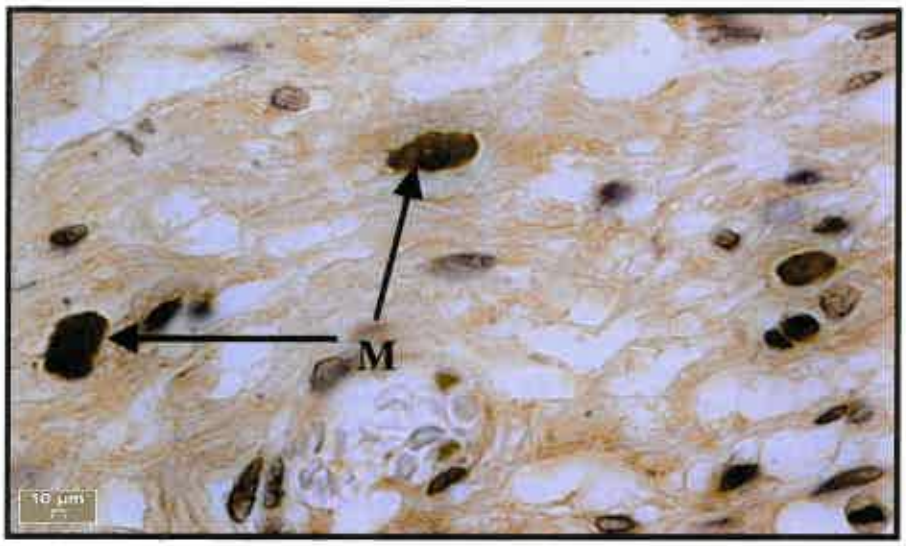


Figure 35 Photomicrograph of the submucosa of the oesophageal tumour labelling for kinin B2 receptors (1000X).

Figure 36 is a photomicrograph of oesophageal tumour muscularis mucosae showing intense immunoreactivity of the kinin B2 receptor in the tumour giant cells (G).

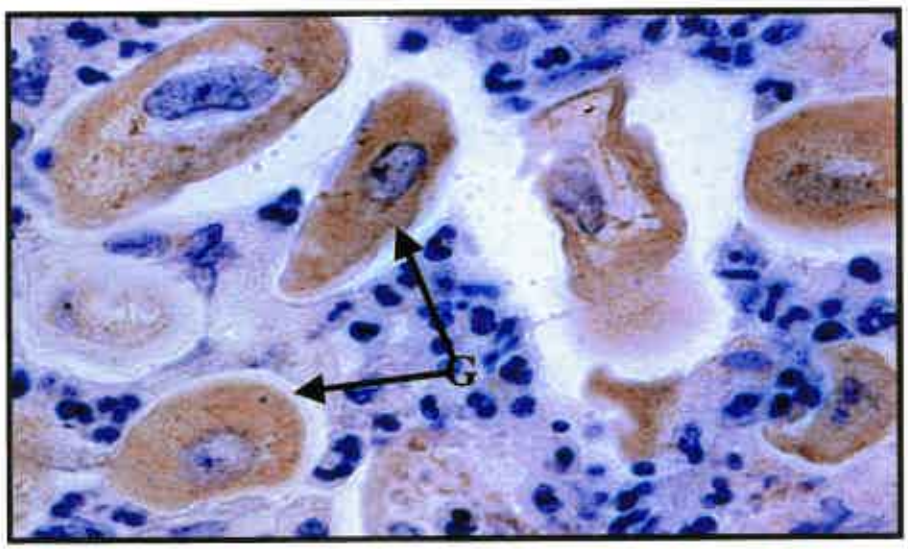


Figure 36 Photomicrograph of the submucosa of the oesophageal tumour B2 receptor labelling (1000X).

Figure 37 is a photomicrograph of oesophageal submucosa with a well differentiated keratinsing squamous cell tumour showing the kinin B2 receptor labelling in a keratin pearls (KP).

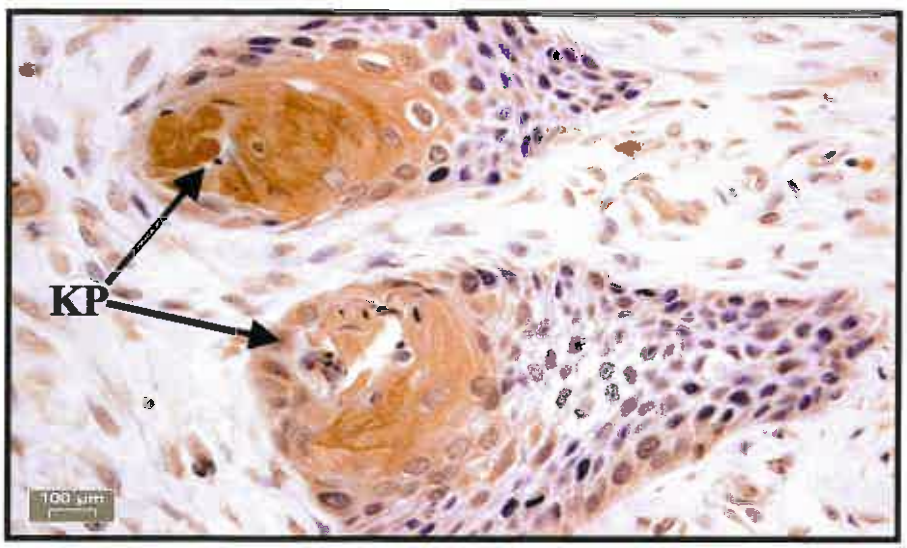


Figure 37 Photomicrograph of an infiltrating keratinising well differentiated squamous cell oesophageal tumour B2 receptor labelling (400X).

Figure 38 is the normal oesophageal muscularis propria (inner circular muscular layer) immunolabelled for the kinin B2 receptor and it shows negative immunoreactivity for B2 receptors.

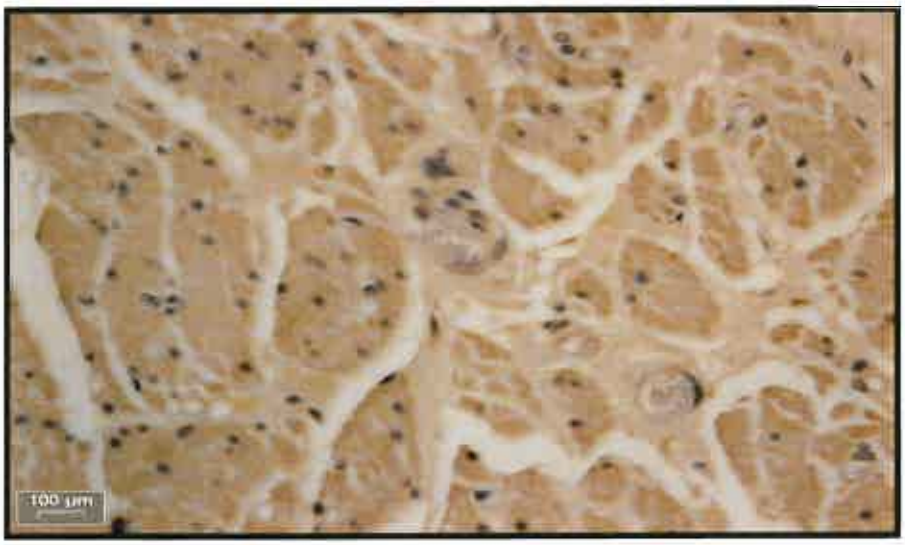


Figure 38 Photomicrograph of a muscularis propria of the oesophageal normal tissue labelling for the kinin B2 receptor (400X).

3.5 Image analysis of immunolabelled TK, TproK and the kinin receptors

Images were generated from diaminobenzidine (DAB) stained sections of biopsies and resections specimens from normal tissues and tumours. The sections were viewed in a Nikon Optiphot microscope (Nikon, Japan) which was interphased to a 3 CCD digital camera system (Sony Corp., Japan). The quantitative image analyser used to determine labelling intensity of DAB immunolocalisation was the Kontron Elektronic KS 300 (Zeiss GmbH, Germany), running on Windows 95™, (Microsoft Corp., USA). The pixel density for these 24 bit true colour images were 768 x 582.

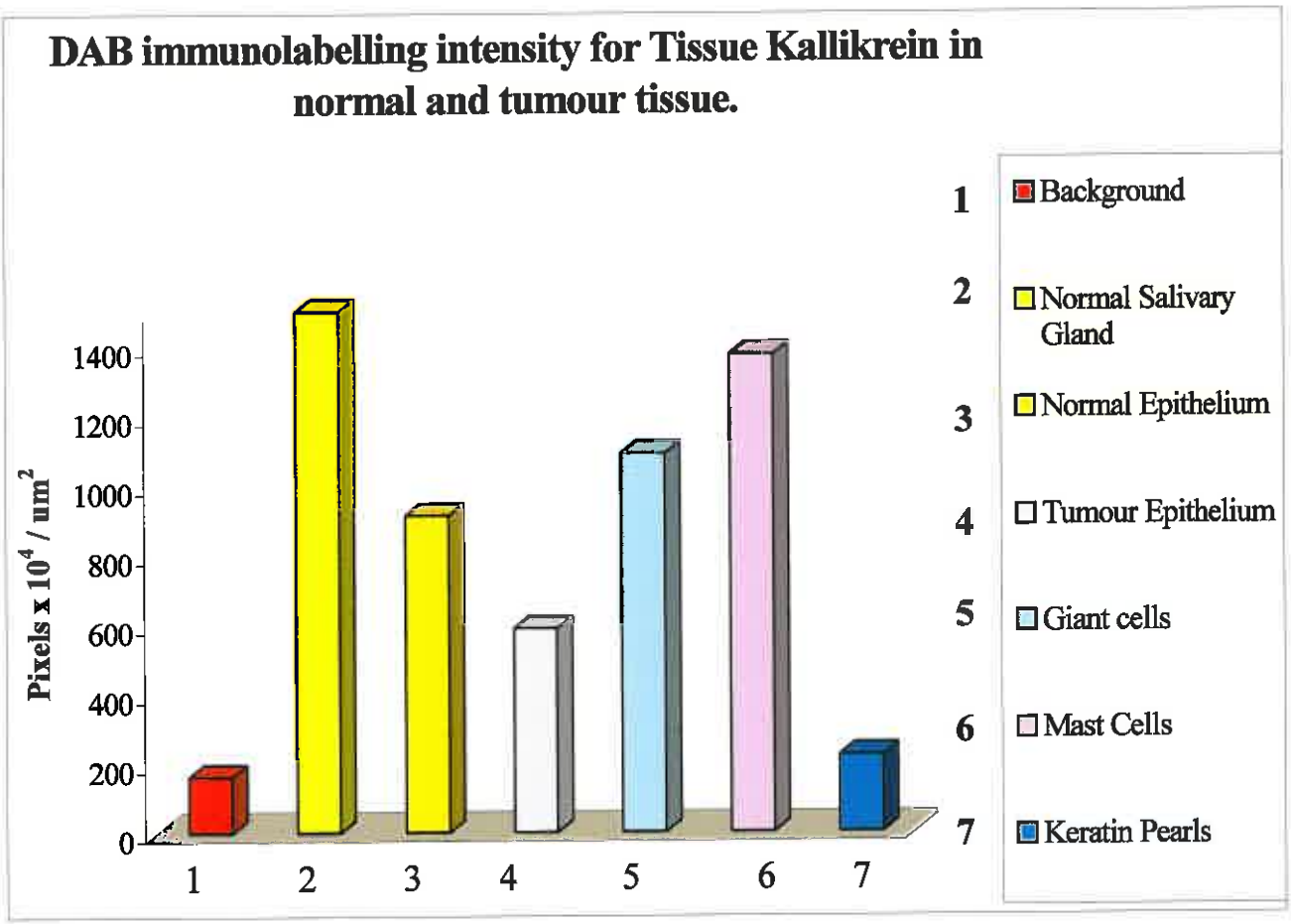
For image analysis the images were converted to 8 bit false colour images with a pixel density of 225x225 pixels, expressed as a grey scale that ranged from 0 to 256, and was divided into 8 equal phases (POLI Look-Up-Table), with each phase having a lower and upper threshold value on the grey scale (see Table 3). Pixels with a grey scale value between 156 and 256 were considered to indicate high immunolabelling intensity.

The amount of antigen was estimated by analysis of the computer-generated images. Using the Analysis 2.1 Pro system (Soft-Imaging Software GmbH, 1996, Germany) the regions of interest (ROI) in each image were encircled. Within these areas the number of pixels falling within each phase, as well as the area analysed, was established. This data, exported to Microsoft Excel was computed to give an indication of the relative intensity of immunolabelling in (n) number of circled areas, by calculating the mean value of the grey scale range 155 – 256, using the unit pixel x $10^4/\mu m^2$.

Table 3 POLI Look-Up-Table showing upper and lower threshold values on the grey scale

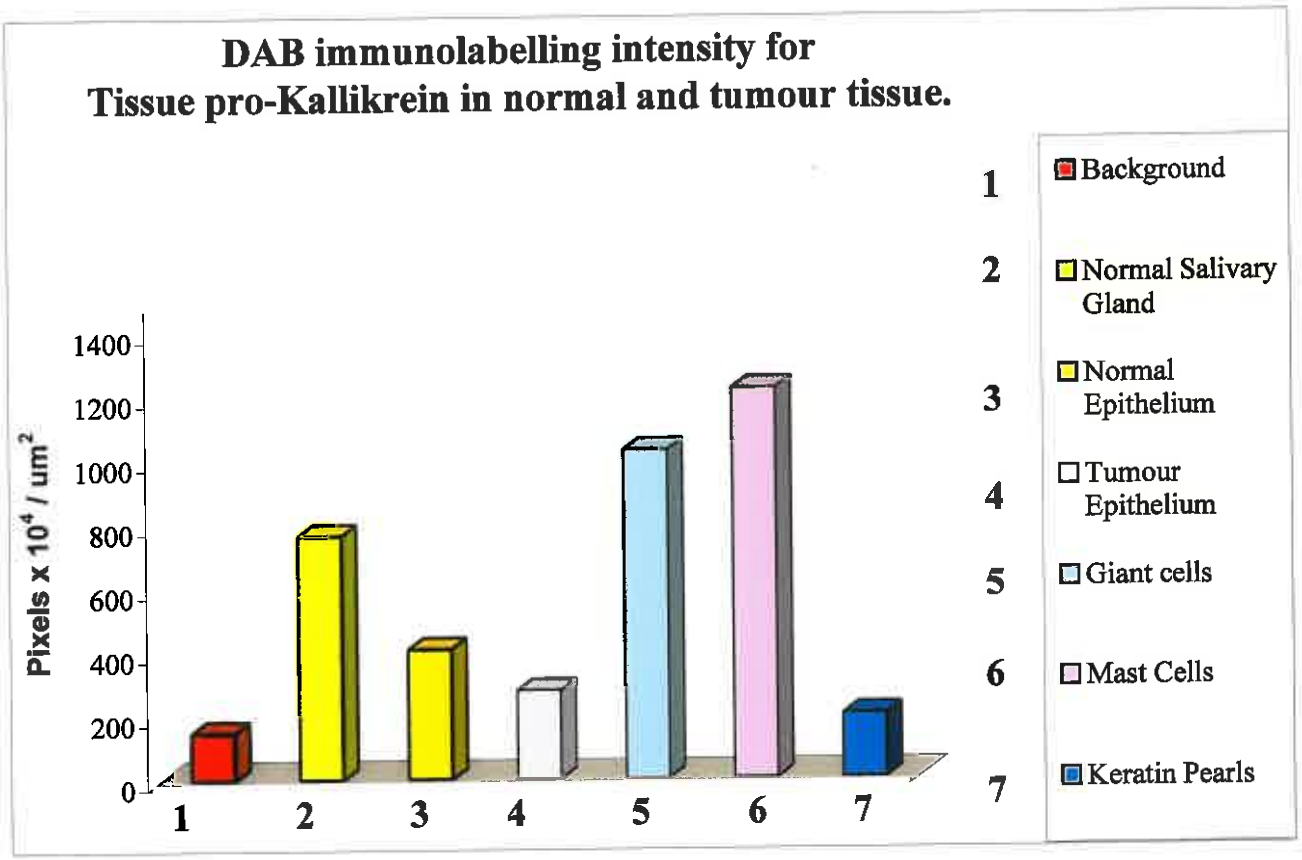
GREY VALUES FOR THE 8 GREY SCALE VALUES	REPRESENTATIVE IMMUNOLABELLING INTENSITY
(8) 221-253 (7) 188-220 (6) 155-187	HIGH
(5) 122-154 (4) 89-121	MEDIUM
(3) 56-88 (2) 33-55	LOW
(1) 0-32	ZERO IMMUNOLABELLING

The following histogram shows the intensity of labelling for TK. The intensity of DAB labelling for TK is the greatest in the salivary gland positive control followed by the mast cells. Note that TK labelling is more intense in the normal oesophageal epithelium and less in tumour epithelium and this is also confirmed by figures 10 and 11. Giant cells also show a very intense labelling for TK. Keratin pearls show the least value for intensity of TK labelling.



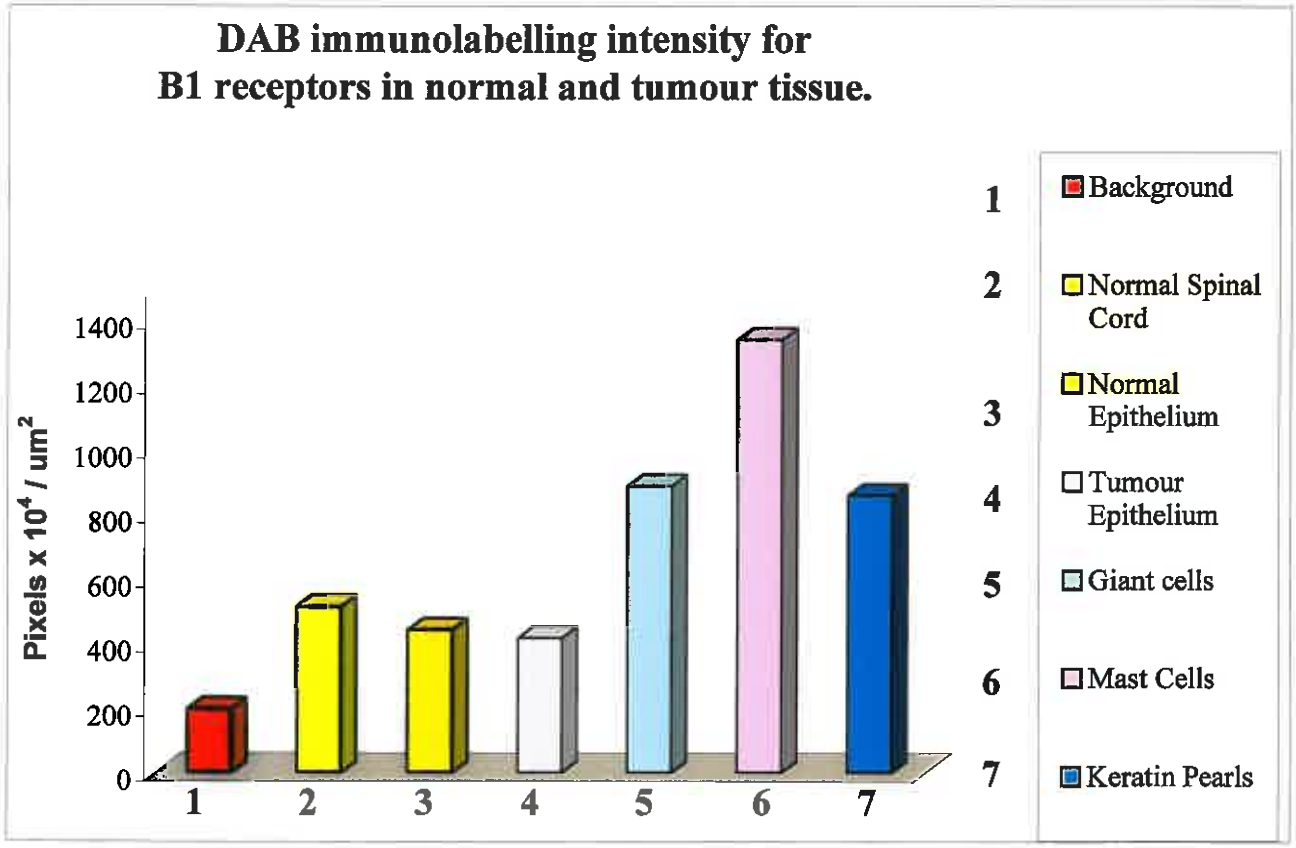
Mean of the grey scale values 155-256

The histogram below shows the intensity of labelling for TproK. The intensity of DAB labelling for TproK is the greatest in the mast and giant cells. Note that TproK labelling is more intense in the normal oesophageal epithelium and less in tumour epithelium and this is also confirmed by figures 18 and 19. Keratin pearls show the smallest value for intensity of TproK labelling. The normal muscularis propria show some positivity although the image appeared negative for TproK.



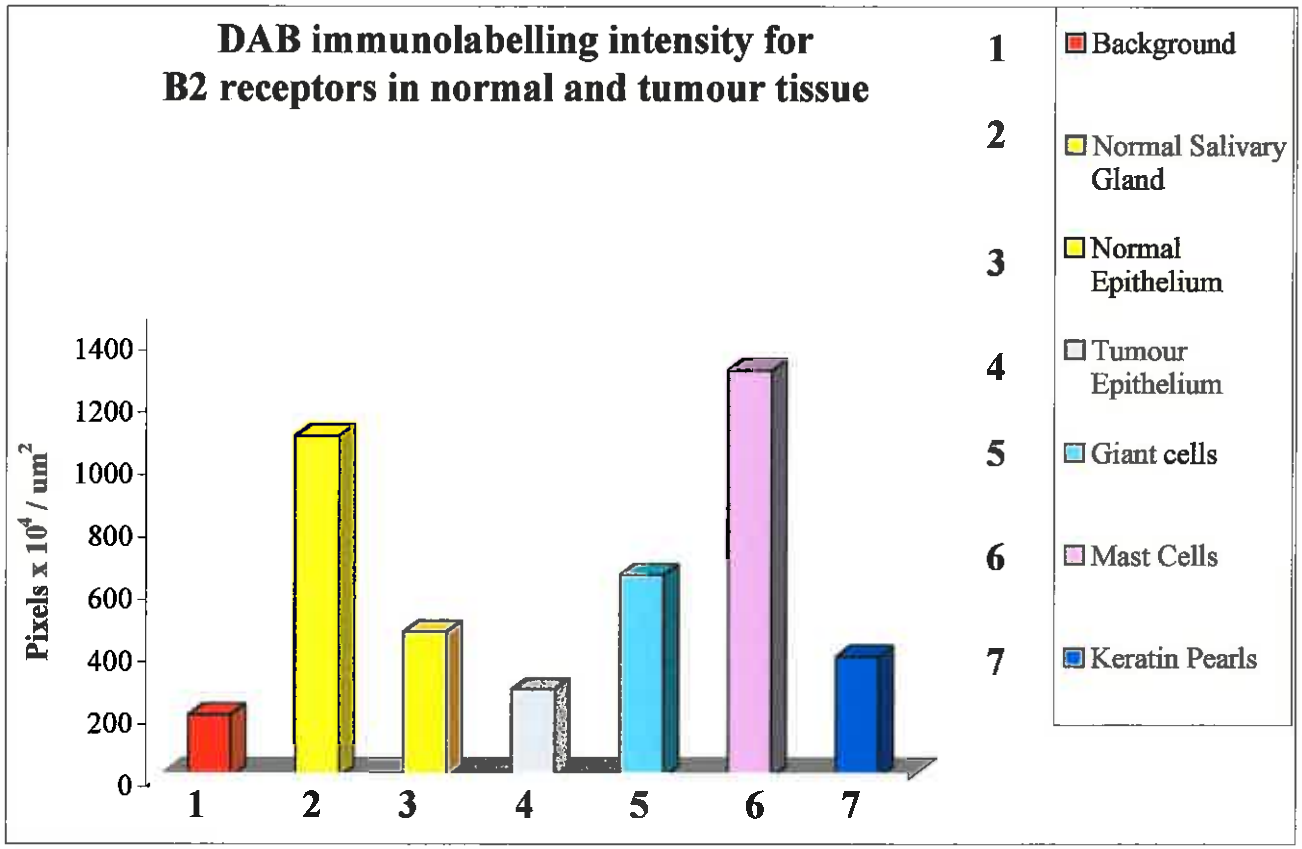
Mean of the grey scale values 155-256

The following histogram shows the intensity of labelling for kinin B1. The intensity of DAB labelling for the kinin B1 is the largest in the mast cells. Followed by the giant cells and then the keratin pearls. Note that kinin B1 labelling is more intense in the normal oesophageal epithelium and less in tumour epithelium and this is also confirmed by figures 26 and 27. The normal muscularis propia show some positivity although the image appeared negative for B1.



Mean of the grey scale values 155-256

The following histogram shows the intensity of labelling for kinin B2. The intensity of DAB labelling for kinin B2 is the largest in the mast cells followed by the salivary gland positive. Note that kinin B2 labelling is more intense in the normal oesophageal epithelium and less in tumour epithelium and this is also confirmed by figures 33 and 34.Giant cells also show a very intense labelling for kinin B2 receptor. The normal muscularis propia show some positivity although the image appeared negative for TK.



(Mean of the grey scale values 155-256)

3.6 *IN SITU* HYBRIDISATION

Fresh resection specimens were collected on ice from local hospitals immediately after oesophagectomy. These samples were then fixed in 5% formal saline for 24 hours and paraffin wax embedded. They were then used for *in situ* hybridisation experiments.

3.6.1 Detection of tissue kallikrein messenger RNA by *in situ* hybridisation

In order to establish the synthesis sites for tissue kallikrein in oesophageal tissue immunolocalisation of KLK1 mRNA was performed by *in situ* hybridisation. KLK1 cDNAs which had been subcloned into the Not 1/Cla 1 site of the circular plasmid, Bluescript (Stratagene) (Figure 39), were linearised by cleavage with restriction enzymes ACC-1 or NOT-1 which identify the sites on the plasmid (Figure 40A and 40B). The linearised plasmids were used to generate digoxigenin (DIG)-labelled cRNA transcripts (see section 2.4.2.1), which were used to localise TK mRNA in tissue samples by *in situ* hybridisation.

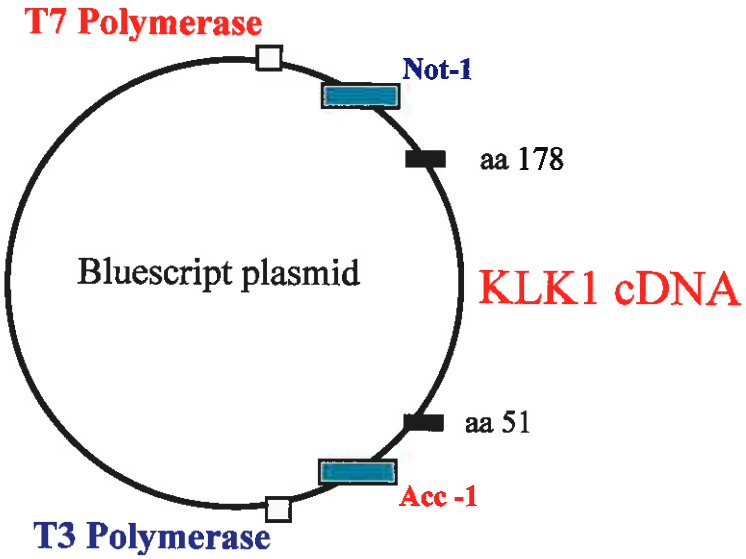


Figure 39 KLK1 cDNA subcloned into a Not-1/Cla-1 site of a Bluescript Stratagene plasmid.

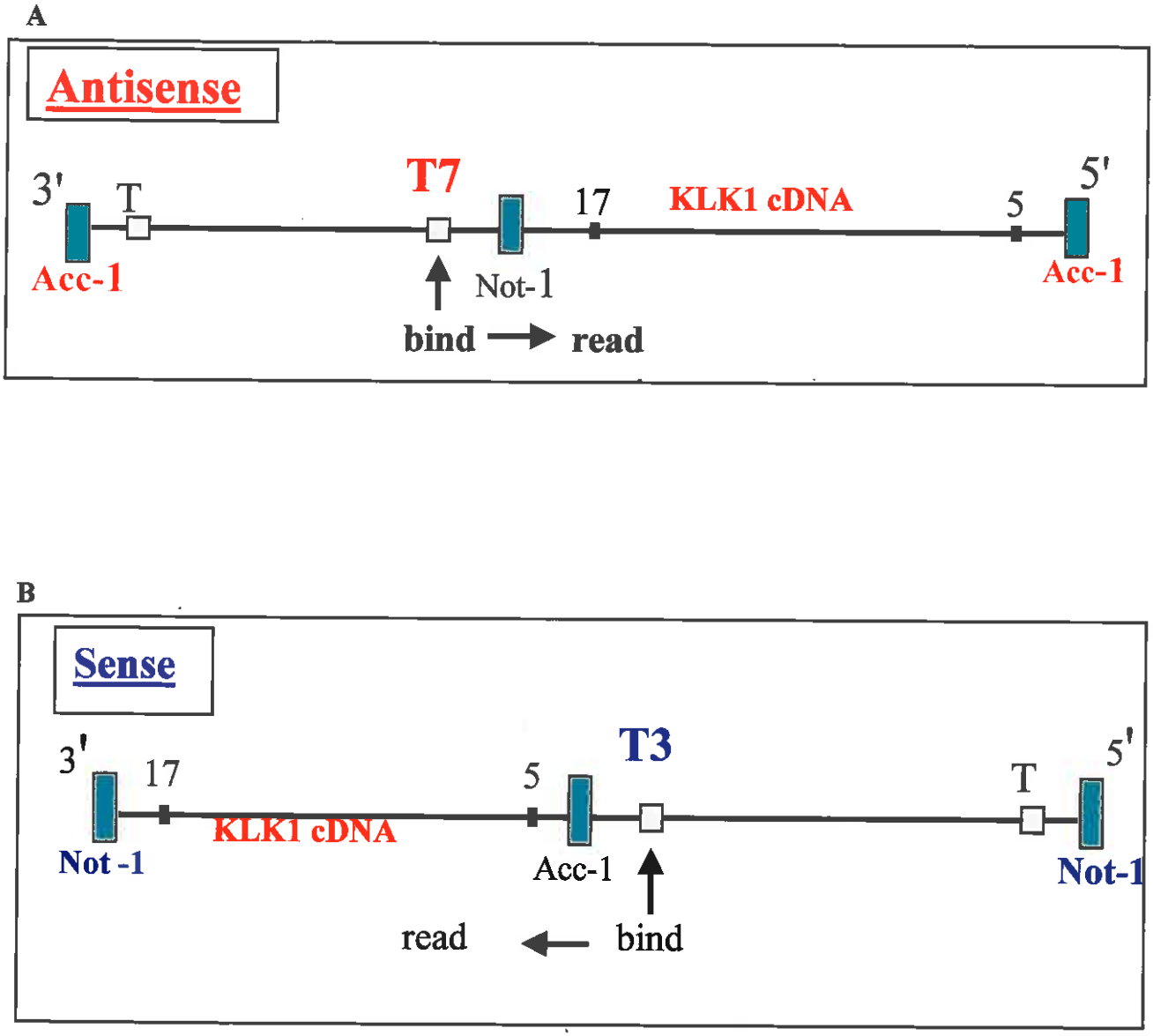


Figure 40 Synthesis of KLK1 cRNA probes.

(A) antisense cRNA synthesis

(B) sense cRNA synthesis

In order to establish tissue specific expression of TK in oesophageal carcinoma samples, it was first necessary to establish tissue specific expression of TK in human salivary gland. This tissue

is known to contain large amounts of TK. The following photomicrograph shows the localisation of the TK mRNA by *in situ* hybridisation **(figure 41).**

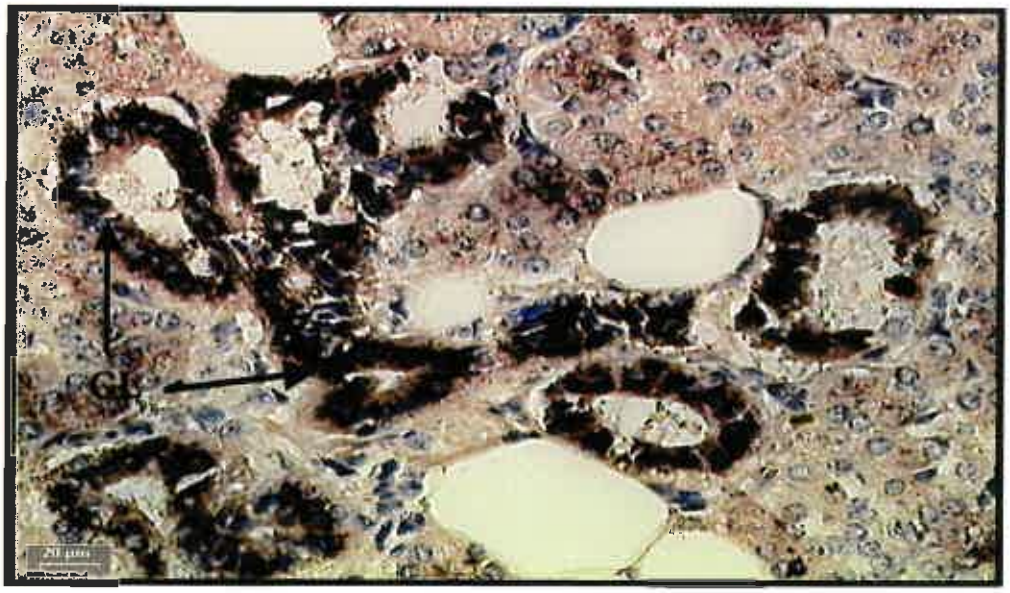


Figure 41 Photomicrograph of normal salivary gland tissue labelled with an antisense TK mRNA probe. Glandular (**GL**) structures show immunoreactivity for the TK mRNA (X400).

Figure 42 is a photomicrograph of a normal salivary gland labelled with an antisense cRNA probe. This serves as a negative control. The cRNA probe is not complementary to the TK mRNA and this is clearly shown by the negative TK mRNA immunoreactivity in **figure 40.**

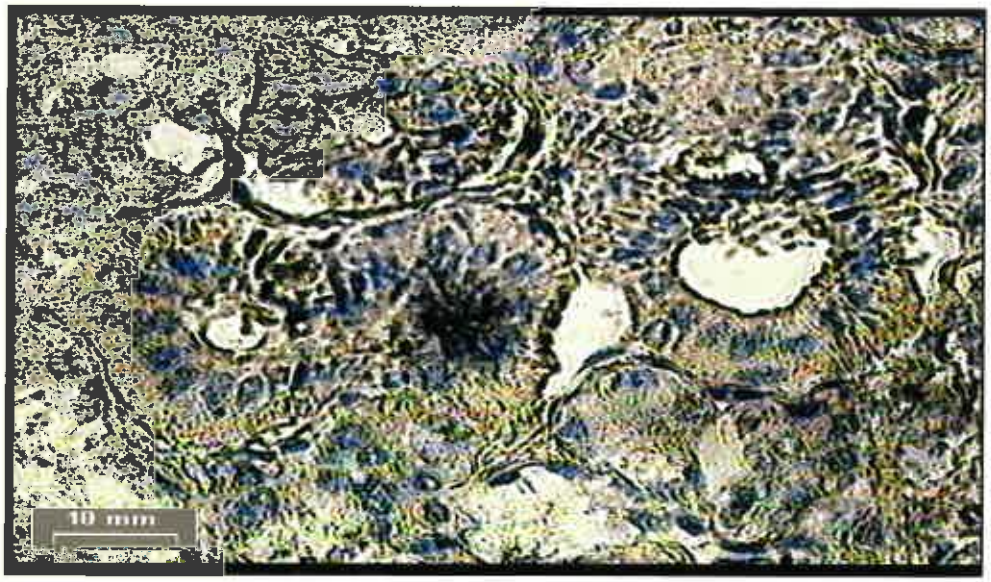


Figure 42 Photomicrograph of salivary gland labelled with a sense mRNA probe. There is no mRNA labelling in the ducts as opposed to the antisense labelled salivary gland (X400).

3.6.1.1 Localisation of TK mRNA in oesophageal normal and tumour tissue

Figure 43 is a photomicrograph of a normal oesophageal epithelium labelled with an antisense TK mRNA probe. Note the immunostained cells in the mucosal layer.

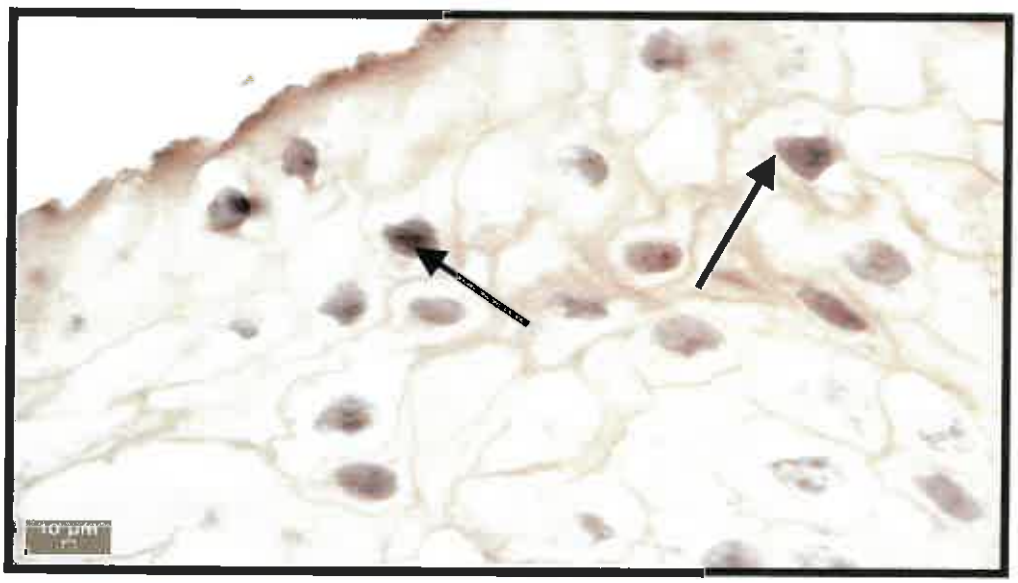


Figure 43 Photomicrograph of a normal oesophageal epithelium labelled with an mRNA antisense probe (X1000).

Figure 44 is a normal oesophageal epithelium labelled with a sense mRNA probe. Note that the mucosal cells of the squamous epithelium are negative for the TK mRNA labelling.

Figure 44 Photomicrograph of a normal oesophageal epithelium labelled with an sense mRNA probe (X1000).

Figure 45 is a photomicrograph of a oesophageal tumour epithelium labelled with an antisense TK mRNA. There positive labelling in the pleomorphic cells of the tumour epithelium.

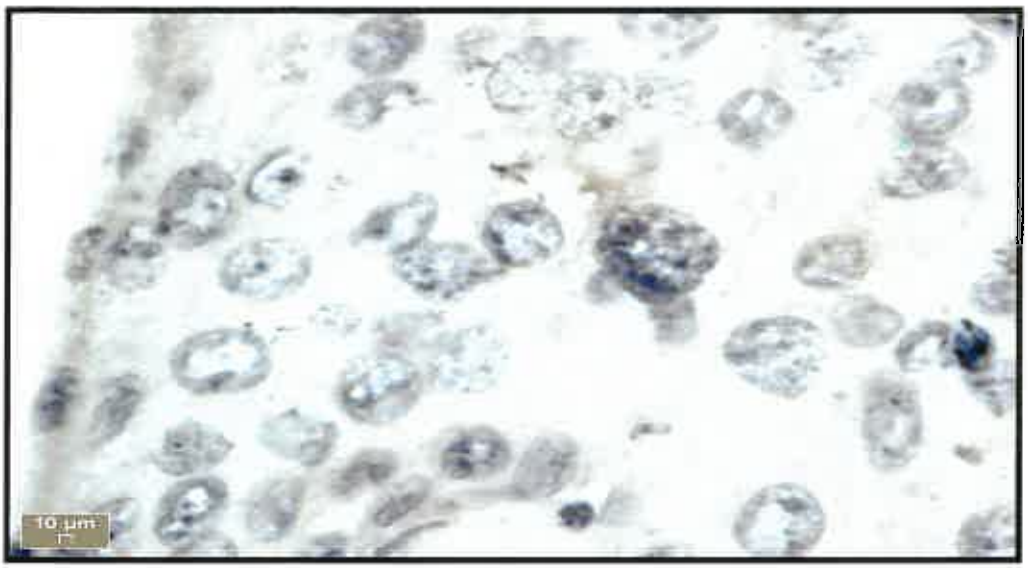


Figure 45 Photomicrograph of oesophageal tumour epithelium labelled for antisense TK mRNA (X1000).

Figure 46 is a photomicrograph of oesophageal tumour epithelium labelled with a sense TK mRNA probe. The photomicrograph is negative for TK mRNA.

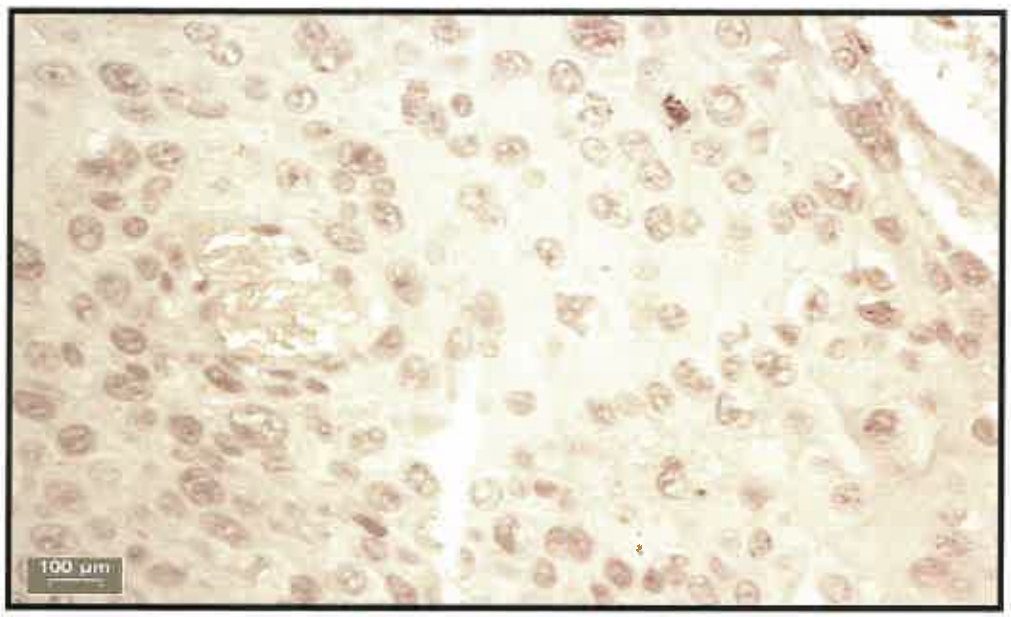


Figure 46 Photomicrograph of oesophageal tumour epithelium labelled for sense TK mRNA.(X400).

Figure 47 is a photomicrograph showing TK mRNA detection in the inflammatory cells of the oesophageal carcinoma in situ. There is a positive TK mRNA detection in these cells, which are probably neutrophils with some plasma cells in the inflammatory cell infiltrate.

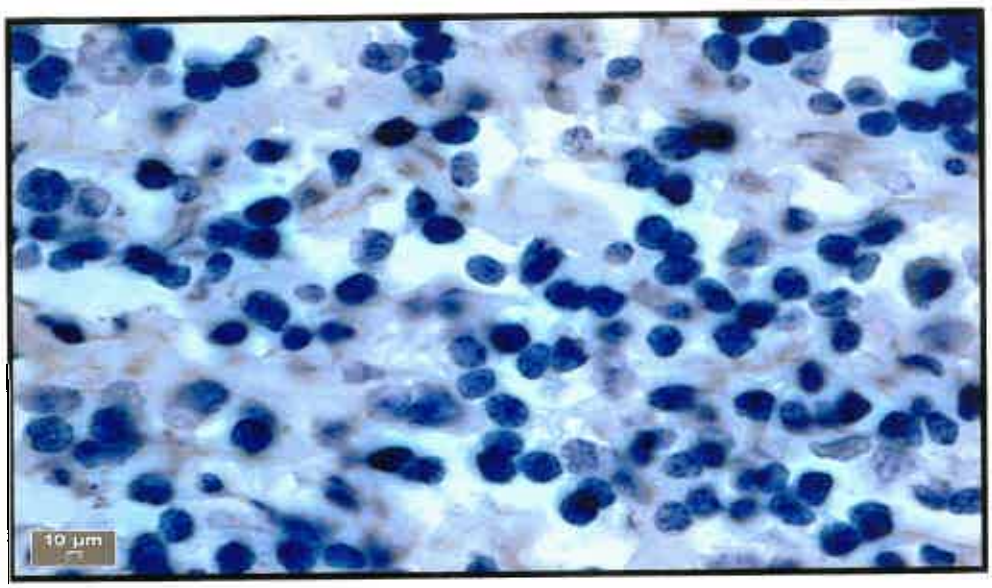


Figure 47 Photomicrograph of oesophageal tumour submucosa showing carcinoma in situ. with inflammatory cells labelled with an antisense TK mRNA probe (X1000).

Figure 48 is an oesophageal tumour photomicrograph showing TK RNA labelling in the mast cells. Note that the mast (**M**) cells in the connective tissue show positive labelling for TK mRNA.

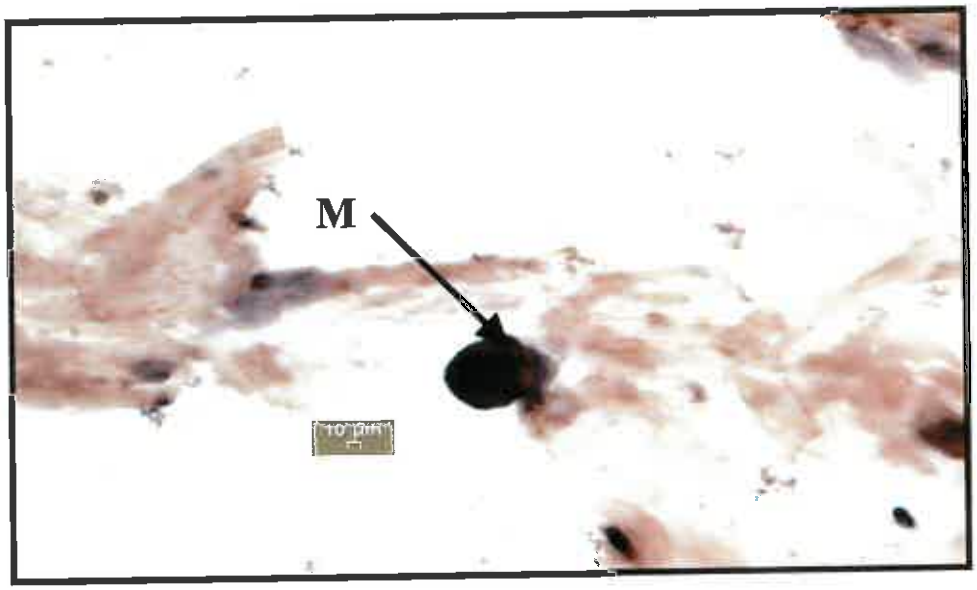


Figure 48 Photomicrograph of oesophageal tumour tissue labelled with an antisense TK mRNA probe. (X1000).

Figure 49 is the normal muscularis propria of the oesophagus. There is no labelling for TK mRNA seen in these photomicrographs. Note that there is no positive labelling for TK mRNA in both photomicrographs. This confirms DAB experiments that there seems to be no localisation of TK in the normal muscularis propria.

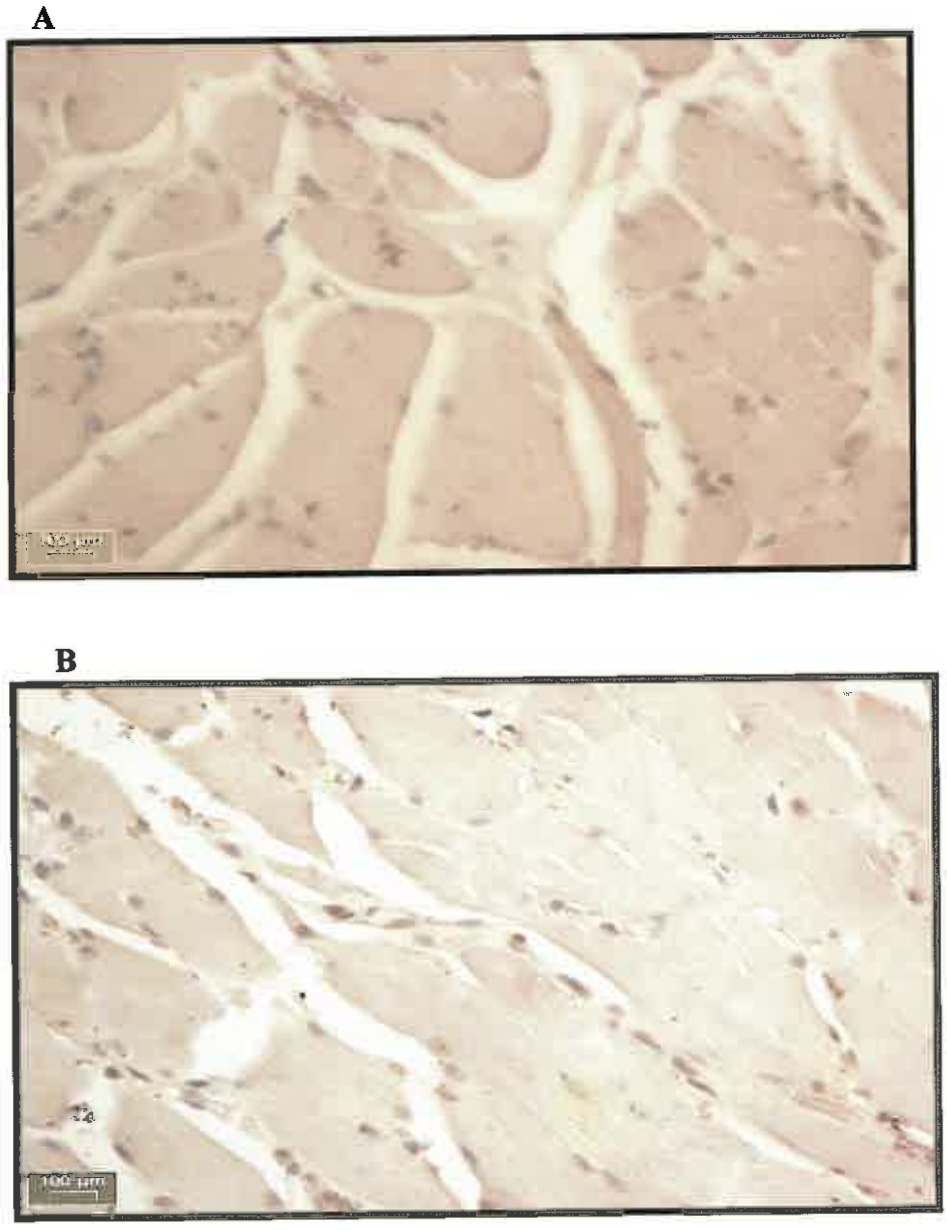


Figure 49 Photomicrographs of normal oesophageal muscularis propria labelled with an antisense (A) and sense (B) probes (X1000).

3.7 REVERSE TRANSCRIPTASE POLYMERASE-CHAIN REACTION (RT-PCR)

To determine if the KLK1 gene was expressed in oesophageal carcinoma, RT-PCR using KLK1 gene specific primers derived from two highly conserved regions of the KLK1 gene to amplify a 150bp KLK1 gene product by reverse transcriptase-chain reaction (RT-PCR) in oesophageal tissue was carried out.

3.7.1 Total RNA extraction

Total RNA was isolated from 100mg oesophageal carcinoma tissue using TriPure Isolation Reagent (Roche) according to the isolation procedure outlined in schedule 12 of the material and methods section. Toatal RNA concentration was dertemined as in Schedule 13 of the materials and methods section. This was then run on a 2% agarose gel in the presence of DNAse, RNAse and its absence. **(Figure 50)**. Total RNA when treated with RNAse showed a total degradation of the RNA resulting in no visible bands. (figure 50, lanes 14-17). The absence of bands also indicate that the isolated RNA was free from DNA.

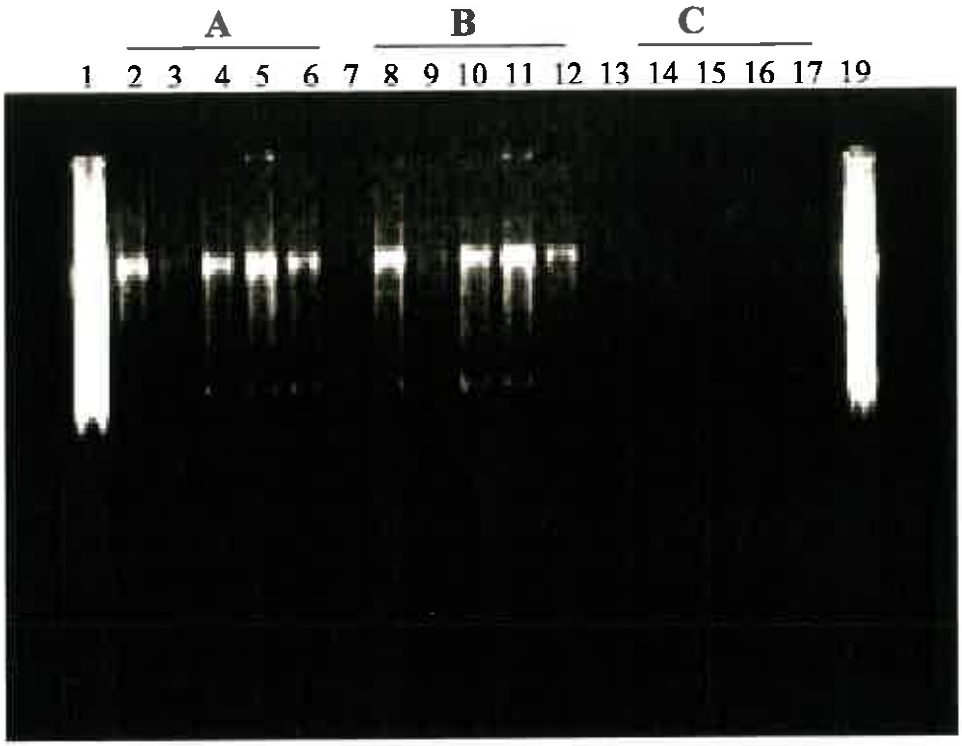


Figure 50 Isolated totak RNA from oesophageal resection specimens run on a 2% agarose gel.

(A) Lanes 1-19 represent 5µl Ribosomal RNA marker (Sigma chemicals).

(B) Lanes 2-6 total RNA without DNAse or RNAse treatment

(C) Lanes 8-12 are total RNA samples treated with DNAse

(D) Lanes 14-17 are total RNA samples treated with RNAse

3.7.2 RT-PCR

5µg of total RNA was used per each reverse transcriptase reaction to prepare cDNA from the KLK1 gene using the enzyme M-MLV (Promega) and the reverse primer (hKLK1-790R) was utilised for the synthesis ofcDNA. CDNA was performed by nested PCR. The initial PCR was carried out with the primers hKLK1-160F and hKLK1-790R yielding a PCR product of 630bp. The second PCR was performed using hKLK1-160F and hKLK1-310 (internal primer) to yield a product of 150bp. A band of 150bp RT-PCR product was observed in the positive control (salivary gland, lane 20). No band was seen in the negative control (lane 3). Only a faint band was observed in normal samples (lane 4). In tumour samples only lanes 8 and 9 showed a 150bp RT-PCR band.

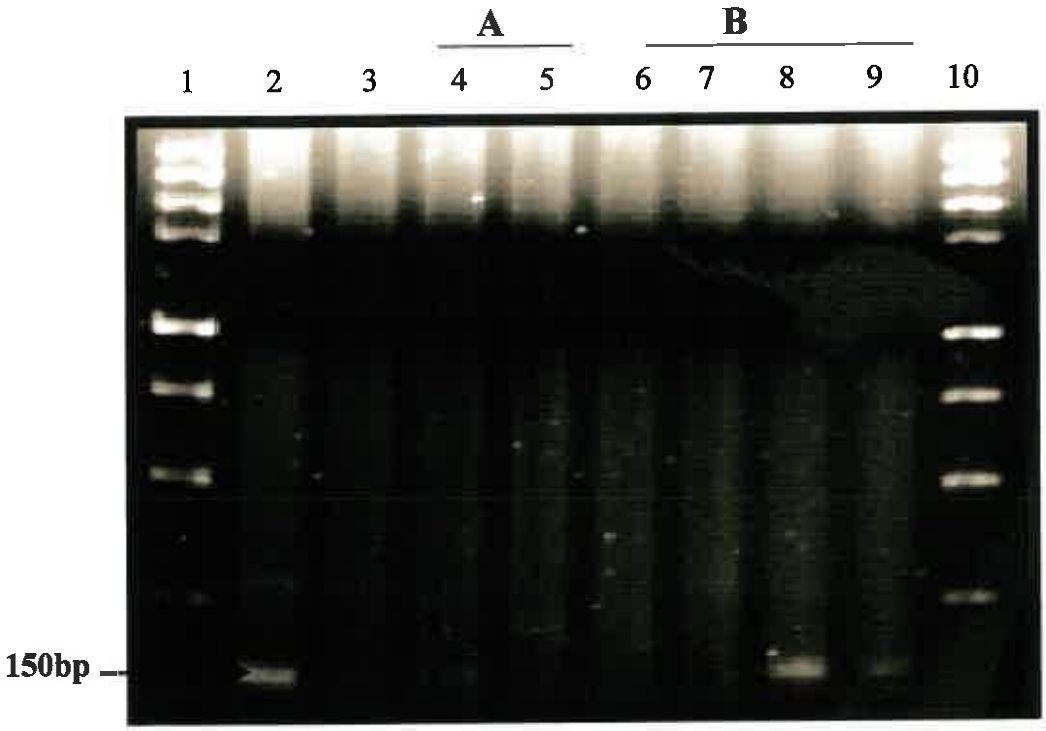


Figure 51 Oesophageal resections RT PCR product ran on a 2% agarose gel.

Lanes 1-10 is a 100bp Ladder (marker) (6µl).

Lane 2 is the salivary gland

Lane 3 is a negative control where the DNA template was replaced with DEPC H_20.

Lanes 4-and 5 (A) are normal resection specimens

Lanes 6-9 (B)are tumour resection specimens

3.5 PATIENT DEMOGRAPHICS AND EPIDEMIOLOGY

Out of the 208 cases recruited 87 were oesophageal carcinoma patients, and the remainder composed two groups namely, 61 non oesophageal cancers (cancers other than oesophageal; non-oesophageal) and 60 non cancer (patients suffering from ailments other than cancer) (see Table 4). Information from a comprehensive questionnaire (Appendix B), completed by a trained nursing sister on interview of each patient the three major groups were divided into seven subgroups according to whether, patients had a family history of oesophageal carcinoma, those whose eyes watered if they were burning wood to cook their meals, patients who had smoked previously or were still smoking, patients who were or had smoked and drunk alcohol, those who had eaten maize with mould, patients who had drank home made beer made from mouldy maize, and finally sex and age of the patients. Table 4 shows the age and sex distribution in the three groups.

Table 4 Marginal frequency summary for age in three patient groups

Groups	Mean	SEM (±)	Median	Range	Mode
Oesophageal carcinoma patients (N=87)	56.47	1.10	57.00	47.00	49
Non-cancer patients (N=60)	43.57	1.89	44.00	57.00	46.00
Non-oesophageal cancer patients (N=61)	53.07	1.84	53.00	79.00	59.00

Mean, median, range and mode given in years

SEM Standard error of mean

To compare the age distribution in the three groups a nonparametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 5 shows the comparison of age by group.

Table 5 Kruskal-Wallis 1-Way Anova test for age distribution in oesophageal, non-oesophageal and non-cancer patients

Groups	Mean Rank	Cases (n)	Chi-square	D. F.	Significance
Oesophageal carcinoma patients	89.38	87	27.8308	1	0.0000
Non-cancer patients	51.70	60			
Oesophageal carcinoma patients	78.26	87	1.6243	1	0.2025
Non-oesophageal cancer patients	69.14	61			
Non-oesophageal cancer patients	72.86	61	14.0834	1	0.0002
Non-cancer patients	48.94	60			

D. F degrees of freedom

Table 6 Demographics of sample patient population (n=208)

Groups	Oesophageal Carcinona Patients N=87	Percentage (%)	Non-oesophageal Cancer patients N=61	Percentage (%)	Non-cancer patients N=60	Percentage (%)
Family History	17	19.5	7	11.5	3	5.0
Eyes Water	58	66.7	31	50.8	18	30.0
Smoking (N+P)	49	56.3	15	24.6	16	27.0
Smoking & Alcohol	43	49.4	16	26.2	16	27.0
Infected Maize	17	19.5	1	1.6	1	1.7
Infected Beer	44	50.6	25	41.0	10	16.7
Males	47	54.0	7	11.5	20	33.3
Females	40	46.0	54	88.5	40	66.7

n = Number of cases

The Pearson Chi-Square Test was used to test whether there was no association (null hypothesis) between the three groups membership and other variables e.g. family history, eyes water, smoking, smoking and drinking, infected maize and infected beer subgroups.

Table 7 compares the family history between the three groups of patients using the Pearson Chi-Square Test.

Table 7 compares Chi-Square Test for association in family history between the three groups.

Groups	Fisher's exact test		Pearson Chi-square	D. F.	Significance
Oesophageal Carcinoma patients N=17	**One-Tail**	**Two-Tail**			
Non-cancer patients N=3	0.00882	0.01353	6.38700	1	0.01150
Oesophageal carcinoma patients N=17					
Non-oesophageal cancer patients N=7	0.13884	0.25798	1.71659	1	0.19013
Non-oesophageal cancer patients N=7					
Non-cancer patients N=3	0.16813	0.32271	1.67293	1	0.19587

D. F. degrees of freedom

Table 8 compares the eyes water subgroup between the three groups of patients using the Pearson Chi-Square Test.

Table 8 Pearson Chi-Square Test for association in eyes water between the three groups

Groups	Fisher's exact test		Pearson Chi-square	D. F.	Significance
	One-Tail	Two-Tail			
Oesophagel Carcinoma patients N=17	0.00001	0.00001	19.11872	1	0.00001
Non-cancer patients N=3					
Oesophageal carcinoma patients N=17	0.03870	0.06174	3.75632	1	0.05261
Non-oesophageal cancer patients N=7					
Non-oesophageal cancer patients N=7	0.01562	0.02621	5.44109	1	0.01967
Non-cancer patients N=3					

D. F. degrees of freedom

Table 9 compares the smoking subgroup between the three groups of patients using the Pearson Chi-Square Test.

Table 9 Pearson Chi-Square Test for the association in smoking subgroup between the three groups

Groups	Fisher's exact test		Pearson Chi-square	D. F.	Significance
Oesophagel Carcinoma patients N=17	**One-Tail**	**Two-Tail**			
Non-cancer patients N=3	0.00030	0.00040	1266081	1	0.00037
Oesophageal carcinoma patients N=17					
Non-oesophageal cancer patients N=7	0.00010	0.00018	14.71084	1	0.00013
Non-oesophageal cancer patients N=7					
Non-cancer patients N=3	0.47865	0.83718	0.06844	1	0.79362

D. F. degrees of freedom

Table 10 compares the smoking and alcohol subgroup between the three groups of patients using the Pearson Chi-Square Test.

Table 10 Pearson Chi-Square Test for the association in smoking and alcohol subgroup between the three groups.

Groups	Fisher's exact test		Pearson Chi-square	D. F.	Significance
Oesophagel Carcinoma patients N=17	**One-Tail**	**Two-Tail**			
Non-cancer patients N=3	0.0040	0.0638	7.65500	1	0.00566
Oesophageal carcinoma patients N=17					
Non-oesophageal cancer patients N=7	0.00355	0.00618	8.04798	1	0.00456
Non-oesophageal cancer patients N=7					
Non-cancer patients N=3	0.56006	1.00000	0.00297	1	0.95653

D. F. degrees of freedom

Table 11 compares the infected maize subgroup between the three groups of patients using the Pearson-Chi-Square Test.

Table 11 Pearson Chi-Square Test for the association in infected maize subgroup between the three groups

Groups	Fisher's exact test		Pearson Chi-square	D. F.	Significance
Oesophagel Carcinoma patients N=17	**One-Tail**	**Two-Tail**			
Non-cancer patients N=3	0.00058	0.00070	10.55718	1	0.00116
Oesophageal carcinoma patients N=17					
Non-oesophageal cancer patients N=7	0.00052	0.00065	10.75583	1	0.00104
Non-oesophageal cancer patients N=7					
Non-cancer patients N=3	0.74793	1.00000	0.00014	1	0.99060

D. F. degrees of freedom

Table 12 compares the infected beer subgroup between the three groups of patients using the Chi-Square Test.

Table 12 Pearson Chi-Square Test for the association in infected beer subgroup between the three groups

Groups	Fisher's exact test		Pearson Chi-square	D. F.	Significance
Oesophagel Carcinoma patients N=17	**One-Tail**	**Two-Tail**			
Non-cancer patients N=3	0.00005	0.00007	15.76393	1	0.00007
Oesophageal carcinoma patients N=17					
Non-oesophageal cancer patients N=7	0.21592	0.40575	0.90959	1	0.34022
Non-oesophageal cancer patients N=7					
Non-cancer patients N=3	0.00322	0.00544	8.40672	1	0.00374

D. F. degrees of freedom

3.6 RENAL TISSUE KALLIKREIN

3.6.1 Tissue kallikrein enzyme-linked immunosorbent values

3.6.1 Statistical analysis of results of tissue kallikrein values

Data analysed was from the results of tissue kallikrein measurement by enzyme-linked immunosorbent assay (ELISA), tissue kallikrein amidase enzymic activity by amidase microassay, kinin generation measurement by kinin ELISA, and tissue kallikrein immunolocalisation image analysis. Results were analysed using an analysis of variance to compare the subgroups. Frequency statistics as a measure of central tendency was used to evaluate data relating to each individual group, and presented as mean, median, and mode. Due to the variability in the analysis of the measurement of tissue kallikrein for the subgroups, factors representing a measure of dispersion such as range and the standard error of mean were also employed.

3.6.1.1 Frequency statistics for TK ELISA in urine samples of three patient groups.

Total tissue kallikrein concentration of the three groups was analysed statistically using frequency statistics as a measure of central tendency and measures of dispersion such as range and the standard error of mean.

Table 13 represents marginal frequency summary for the total tissue kallikrein ELISA in three patient groups [oesophageal cancer, non-oesophageal cancer and non-cancer patients].

Table 13 Marginal frequency summary for total tissue kallikrein ELISA in three patient groups

Groups	Mean	SEM (±)	Median	Range	Mode
Oesophageal carcinoma patients (n=87)	162.18	9.43	174.53	305.22	160.71
Non-cancer patients (n=60)	157.02	8.99	156.42	288.30	200.03
Non-oesophageal cancer patients (n=61)	133.71	11.50	105.55	296.40	3.34

Mean, median, range and mode given in ηg/μg protein

SEM Standard error of mean

To compare the tissue kallikrein concentration measured by ELISA in three groups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 14 shows the comparison of total tissue kallikrein concentration by group.

Table 14 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in three patient groups

Groups	Mean Rank	Number of cases (n)	Chi-square	D. F.	Significance
Oesophageal carcinoma N=87	75.93	87	0.4358	1	0.5092
Non-cancer patients N=60	71.21	60			
Oesophageal carcinoma N=87	80.20	87	3.7259	1	0.0536
Non-oesophageal cancer patients N=61	66.38	61			
Non-oesophageal cancer patients N=61	55.66	61	2.8562	1	0.0910
Non-cancer patients N=60	66.43	60			

D. F degrees of freedom

3.6.1.2 Frequency statistics for TK ELISA in urine samples of six oesophageal patient sub-groups

Each of the three groups was subdivided into six subgroups and the tissue kallikrein concentration of these subgroups was analysed statistically using frequency statistics as a measure of central tendency and measures of dispersion such as range and the standard error of mean. Table 15 represents marginal frequency summary for tissue kallikrein ELISA in oesophageal cancer patients subgroups.

Table 15 Marginal frequency summary for tissue kallikrein ELISA in six oesophageal cancer patient subgroups

Groups	Number of cases (n)	Mean	SEM 9±)	Median	Range	Mode
Family History	17	204.10	16.46	216.47	279.48	29.55
Eyes Water	58	166.04	10.95	180.85	287.58	160.71
Smoking	49	166.37	13.175	181.92	295.09	10.49
Smoking & Alcohol	43	172.13	13.74	173.58	298.54	10.49
Infected Maize	17	218.59	10.88	226.95	172.03	112.70
Infected Beer	44	156.41	13.816	189.07	291.16	160.71

Mean, median, range and mode given in ηg/μg protein

SEM standard error of mean

3.6.1.3 Frequency statistics for TK ELISA in urine samples of six non-oesophageal cancer patients sub-groups

Table 16 represents marginal frequency summary for tissue kallikrein ELISA in non-oesophageal cancer patient subgroups.

Table 16 Marginal frequency summary for tissue kallikrein ELISA in six non-oesophageal cancer patient subgroups

Groups	Number of cases (n)	Mean	SEM (±)	Median	Range	Mode
Family History	7	139.20	37.42	112.94	9.06	265.42
Eyes Water	28	142.189	16.75	101.62	290.21	9.53
Smoking	15	117.51	25.47	82.92	3.34	289.37
Smoking & Alcohol	16	132.254	23.16	100.91	283.65	9.01
Infected Maize	1	—	—	—	—	—
Infected Beer	25	148.44	18.22	97.09	279.72	20.02

Mean, median, range and mode given in ηg/μg protein

SEM standard error of mean

— No patients available in this population group

3.6.1.4 Frequency statistics for TK ELISA in urine samples of non-cancer patients sub-groups

Table 17 represents marginal frequency summary for tissue kallikrein ELISA in non-cancer patient subgroups.

Table 17 Marginal frequency summary for tissue kallikrein ELISA in six non-cancer patient subgroups

Groups	Number of cases (n)	Mean	SEM (±)	Median	Range	Mode
Family History	3	131.96	44.12	120.44	151.54	61.95
Eyes Water	18	148.38	15.99	149.69	234.30	21.00
Smoking	16	161.92	17.70	163.09	224.33	68.26
Smoking & Drinking	16	163.73	17.27	163.99	245.17	47.42
Infected Maize	1	—	—	—	—	—
Infected Beer	10	138.96	23.85	138.43	204.67	47.42

Mean, median, range and mode given in ηg/μg protein

SEM standard error of mean

— No patients available in this population group

To compare the tissue kallikrein concentration measured by ELISA in the family history subgroup a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 18 shows the comparison of tissue kallikrein concentration by subgroup.

Table 18 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in the family history subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	11.35	17	2.3557	1	0.1248
Non-cancer patients	5.67	3			
Oesophageal carcinoma	13.76	17	1.8645	1	0.1721
Non-oesophageal cancer patients	9.43	7			
Non-oesophageal cancer patients	5.57	7	0.0130	1	0.9093
Non-cancer patients	5.33	3			

D. F. degrees of freedom

To compare the tissue kallikrein concentration measured by ELISA in the eyes water subgroup a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 19 shows the comparison of tissue kallikrein concentration by subgroup.

Table 19 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in the eyes water subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	40.09	58	1.2635	1	0.2610
Non-cancer patients	33.39	18			
Oesophageal carcinoma	47.07	58	1.0679	1	0.3014
Non-oesophageal cancer patients	41.13	31			
Non-oesophageal cancer patients	524.16	31	0.2908	1	0.5897
Non-cancer patients	26.44	18			

D. F. degrees of freedom

To compare the tissue kallikrein concentration measured by ELISA in the smoking subgroup a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 20 shows the comparison of tissue kallikrein concentration by subgroup.

Table 20 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in the smoking subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	33.61	49	0.2087	1	0.6478
Non-cancer patients	31.13	16			
Oesophageal carcinoma	34.55	49	2.5370	1	0.1112
Non-oesophageal cancer patients	25.80	15			
Non-oesophageal cancer patients	25.80	15	2.5370	1	0.1112
Non-cancer patients	34.55	49			

D. F. degrees of freedom

To compare the tissue kallikrein concentration measured by ELISA in the smoking and alcohol subgroup a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 21 shows the comparison of tissue kallikrein concentration by subgroup.

Table 21 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in the smoking and drinking subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	30.91	43	0.4422	1	0.5061
Non-cancer patients	27.56	16			
Oesophageal carcinoma	32.05	43	2.2512	1	0.1335
Non-oesophageal cancer patients	24.50	16			
Non-oesophageal cancer patients	14.31	16	17401	1	0.1871
Non-cancer patients	18.69	16			

D. F. degrees of freedom

To compare the tissue kallikrein concentration measured by ELISA in the infected maize subgroup a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 22 shows the comparison of tissue kallikrein concentration by subgroup.

Table 22 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in the infected maize subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	9.53	17	0.0093	1	0.9232
Non-cancer patients	9.00	1			
Oesophageal carcinoma	9.59	17	0.0836	1	0.7725
Non-oesophageal cancer patients	8.00	1			
Non-oesophageal cancer patients	2	1	1.0000	1	0.3173
Non-cancer patients	1	1			

D. F. degrees of freedom

To compare the tissue kallikrein concentration measured by ELISA in the infected beer subgroup a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 23 shows the comparison of tissue kallikrein concentration by subgroup.

Table 23 Kruskal-Wallis H test for tissue kallikrein ELISA comparison in the infected beer subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	27.98	44	0.2187	1	0.6400
Non-cancer patients	25.40	10			
Oesophageal carcinoma	34.86	44	0.0056	1	0.9403
Non-oesophageal cancer patients	35.24	25			
Non-oesophageal cancer patients	18	25	0.1920	1	0.6612
Non-cancer patients	16.80	10			

D. F. degrees of freedom

3.6.2 Amidase assay for tissue kallikrein

3.6.2.1 Frequency statistics for amidase assay in urine samples of three patient groups

Amidase activity of the three groups was analysed statistically using frequency statistics as a measure of central tendency such as mean median and mode and measures of dispersion such as range and the standard error of mean. Table 24 represents marginal frequency summary for total tissue kallikrein activity in three patient groups (oesophageal cancer, non-oesophageal cancer and non-cancer patients).

Table 24 Marginal frequency summary for amidase activity in three patient groups

Groups	Mean	SEM(±)	Median	Range	Mode
Oesophageal carcinoma. Patients (n=87)	0.90	0.14	0.59	9.13	0.37
Non-oesophageal cancer patients (n=61)	0.76	0.21	0.410	12.80	0.25
Non-cancer patients (n=60)	1.21	0.21	0.58	8.48	0.30

Mean, median, range and mode given in ηg/μg protein

SEM Standard error of mean

To compare the amidase activity in three groups a nonparametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 25 shows the comparison of amidase activity by group.

Table 25 Kruskal-Wallis amidase activity comparison in three patient groups

Groups	Mean Rank	Cases	Chi-square	D. F.	Significance
Oesophageal carcinoma	78.41	87	1.7546	1	0.0953
Non-cancer patients	68.93	61			
Oesophageal carcinoma	71.70	87	0.6245	1	0.4294
Non-oesophageal cancer patients	77.34	60			
Non-oesophageal cancer patients	54.56	61	4.1520	1	0.0416
Non-cancer patients	67.55	60			

D. F degrees of freedom

3.6.2.2 Frequency statistics for amidase assay in urine samples of six oesophageal cancer patients sub-groups

Table 26 represents marginal frequency summary for tissue kallikrein activity in oesophageal cancer patient subgroups.

Table 26 Marginal frequency summary amidase activity in six oesophageal cancer patient subgroups

Groups	Number of cases (n)	Mean	SEM(±)	Median	Range	Mode
Family History	17	0.76	0.15	0.59	2.17	0.00
Eyes Water	58	0.98	0.21	0.58	9.10	0.08
Smoking	49	0.70	0.09	0.54	2.66	0.81
Smoking & Drinking	43	0.725	0.10	0.54	2.66	0.08
Infected Maize	17	0.910	0.18	0.75	2.66	0.00
Infected Beer	44	1.06	0.27	0.56	9.14	0.08

Mean, median, range and mode given in ηg/μg protein

SEM standard error of mean

3.6.2.3 Frequency statistics for amidase assay in urine samples of six non-oesophageal cancer patients sub-groups

Table 27 represents marginal frequency summary for tissue kallikrein activity in non-oesophageal cancer patient subgroups.

Table 27 Marginal frequency summary for amidase activity in six non-oesophageal cancer patient subgroups

Groups	Number of cases (n)	Mean	SEM(±)	Median	Range	Mode
Family History	7	0.34	0.10	0.30	0.75	0.12
Eyes Water	28	0.92	0.41	0.35	12.74	0.11
Smoking	15	0.62	0.15	0.35	2.09	0.18
Smoking & Alcohol	16	0.59	0.15	0.39	2.17	0.18
Infected Maize	1	—	—	—	—	—
Infected Beer	25	0.526	0.11	0.36	2.11	0.12

Mean, median, range and mode given in ηg/μg protein

SEM standard error of mean

— No patients available in this population group

3.6.2.4 Frequency statistics for amidase assay in urine samples of six non-cancer patients subgroups

Table 28 represents marginal frequency summary for tissue kallikrein activity in non-cancer patient subgroups.

Table 28 Marginal frequency summary for amidase activity in six non-cancer patient subgroups

Groups	Number of cases (n)	Mean	SEM(±)	Median	Range	Mode
Family History	3	1.06	0.56	0.52	1.69	0.49
Eyes Water	18	0.99	0.48	0.31	8.48	0.14
Smoking	16	1.35	0.46	0.64	5.80	0.11
Smoking & Alcohol	16	0.98	0.35	0.58	5.9	0.01
Infected Maize	1	—	—	—	—	—
Infected Beer	10	1.71	0.94	0.44	8.48	0.01

Mean, median, range and mode given in ηg/μg protein

SEM standard error of mean

— No patients available in this population group

To compare the amidase activity in six subgroups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 29 shows the comparison of amidase assay by family history subgroup.

Table 29 Kruskal-Wallis amidase activity comparison in the family history subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	10.29	17	0.1373	1	0.7110
Non-cancer patients	11.67	3			
Oesophageal carcinoma	13.91	17	2.3264	1	0.1272
Non-oesophageal cancer patients	9.07	7			
Non-oesophageal cancer patients	8.33	3	3.7532	1	0.0527
Non-cancer patients	4.29	7			

D. F. degrees of freedom

To compare the amidase activity in six subgroups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 30 shows the comparison of amidase assay by eyes water subgroup.

Table 30 Kruskal-Wallis amidase activity comparison in the eyes water subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	40.09	58	1.2635	1	0.2610
Non-cancer patients	33.09	18			
Oesophageal carcinoma	47.13	58	1.1313	1	0.2875
Non-oesophageal cancer patients	41.02	31			
Non-oesophageal cancer patients	23.92	18	0.1637	1	0.6858
Non-cancer patients	25.63	31			

D. F. degrees of freedom

To compare the amidase activity in six subgroups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 31 shows the comparison of amidase activity by smoking subgroup.

Table 31 Kruskal-Wallis amidase activity comparison in smoking subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	31.19	49	1.8169	1	0.1777
Non-cancer patients	38.53	16			
Oesophageal carcinoma	32.05	49	2.2512	1	0.1335
Non-oesophageal cancer patients	24.50	16			
Non-oesophageal cancer patients	14.31	16	1.7401	1	0.871
Non-cancer patients	18.69	16			

D. F. degrees of freedom

To compare the amidase activity in six subgroups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 32 shows the comparison of amidase activity by smoking and alcohol subgroup.

Table 31 Kruskal-Wallis amidase activity comparison in the smoking and alcohol subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	29.22	43	0.3263	1	0.5678
Non-cancer patients	32.09	16			
Oesophageal carcinoma	30.56	43	0.1675	1	0.6823
Non-oesophageal cancer patients	28.50	16			
Non-oesophageal cancer patients	18.03	16	0.4536	1	0.3555
Non-cancer patients	14.97	16			

D. F. degrees of freedom

To compare the amidase activity in six subgroups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 33 shows the comparison of amidase activity by infected maize subgroup.

Table 33 Kruskal-Wallis amidase activity comparison in the infected maize subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	9.82	17	1.1238	1	0.2891
Non-cancer patients	4.00	1			
Oesophageal carcinoma	9.76	17	0.7523	1	0.3857
Non-oesophageal cancer patients	5.00	3			
Non-oesophageal cancer patients	1	1	1.0000	1	0.3173
Non-cancer patients	2	1			

D. F. degrees of freedom

To compare the amidase activity in six subgroups a non-parametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 34 shows the comparison of amidase activity by infected beer subgroup.

Table 34 Kruskal-Wallis amidase activity comparison in the infected beer subgroup

Groups	Mean Rank	Number of cases (n)	Chi-Square	D. F.	Significance
Oesophageal carcinoma	27.97	44	0.2084	1	0.6480
Non-cancer patients	25.45	10			
Oesophageal carcinoma	36.15	44	0.3976	1	0.5283
Non-oesophageal cancer patients	32.98	25			
Non-oesophageal cancer patients	18.35	10	0.0163	1	0.8983
Non-cancer patients	17	25			

D. F. degrees of freedom

3.7 Kininogenase Assay

The kininogen content of the urine samples was determined by initially generating the release of its kinin moiety with endogenous TK followed by the quantitative analysis of the released kinin by competitive ELISA.

3.7.1 Frequency statistics for kinin ELISA in urine samples of three patient groups

Total basal and generated kinins of the three groups was analysed statistically using frequency statistics as a measure of central tendency such as mean, median, mode and measures of dispersion such as range and the standard error of mean. Table 35 represents marginal frequency summary for total basal and generated kinins in three patient groups (oesophageal cancer, non-oesophageal cancer and non-cancer patients).

Table 35 Marginal frequency summary for kinin ELISA in three patient groups

Groups	Mean SEM±		Mean SEM±		Median		Range		Mode	
Oesophageal carcinoma (n=87)	Basal level		generated		base	gen	base	gen	base	gen
	9.66	0.57	31.32	1.32	7.80	29.70	25.40	49.60	6.40	53.80
Non-oesophageal cancer (n=61)	7.46	0.51	29.23	1.48	6.60	26.30	16.10	47.70	2.80	19.80
Non-cancer patients (n=60)	9.76	0.78	32.67	1.36	7.70	32.90	26.50	45.70	4.80	53.80

Mean, median, range and mode given in ηg/ml

SEM Standard error of mean

base basal kinin concentration

gen generated kinin concentration

To compare the total kininogenase assay in three groups a nonparametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 36 shows the comparison of total basal and generated kinins by group.

Table 15 Kruskal-Wallis 1-Way Anova total kinin ELISA comparison in three patient groups

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	75.39	71.39	87	0.2256	0.8005	1	0.6348	0.3709
Non-cancer patients	71.99	77.78	60					
Oesophageal carcinoma	82.52	77.79	87	7.3845	1.2457	1	0.0066	0.2644
Non-oesophageal cancer	63.07	70.00	61					
Non-oesophageal cancer	54.34	54.70	61	4.4311	3.9736	1	0.0353	0.0462
Non-cancer patients	67.77	67.41	60					

D. F degrees of freedom

base basal kinin concentration

gen generated kinin concentration

3.2.2 Frequency statistics for kinin generation values in urine samples of six oesophageal cancer patients sub-groups

Table 37 represents marginal frequency summary for kinin generation assay in oesophageal cancer patient subgroups

Table 37 Marginal frequency summary for kinin generation assay in six oesophageal cancer patient subgroups

Groups	Mean SEM±		Mean SEM±		Median		Range		Mode	
	Basal level		Generated		Base	gen	base	gen	base	gen
Family History (n=17)	11.45	1.38	34.62	3.52	11.50	32.85	19.90	45.70	8.20	53.80
Eyes Water (n=58)	8.41	0.53	29.04	1.582	7.35	27.75	16.40	49.60	6.40	16.30
Smoking (n=49)	7.64	0.48	26.97	1.64	7.00	25.25	15.60	49.60	6.40	16.30
Smoking & Alcohol (n=43)	7.71	0.526	25.84	1.65	7.00	23.70	15.60	49.60	6.4	16.3
Infected Maize (n=17)	6.98	0.84	25.21	2.80	6.60	22.60	15.30	44.80	3.00	7.10
Infected Beer (n=44)	11.57	0.92	36.67	1.70	10.65	36.85	23.60	45.70	4.80	53.80

Mean, median, range and mode given in ηg/ml

SEM standard error of mean

3.7.3 Frequency statistics for kinin ELISA in urine samples of six non-oesophageal cancer patients sub-groups'

Table 38 represents marginal frequency summary for kinin generation values in non-oesophageal cancer patient subgroups.

Table 38 Marginal frequency summary for kinin generation ELISA in six non-oesophageal patient subgroups

Groups	Mean SEM±		Mean SEM±		Median		Range		Mode	
	Basal level		Generated		Base	gen	base	gen	base	gen
Family History (n=7)	9.47	2.28	36.19	4.246	6.60	36.80	14.10	29.30	4.8	22.10
Eyes Water (n=31)	6.17	0.54	28.83	2.08	5.50	26.70	13.30	44.60	2.8	23.80
Smoking (n=15)	7.41	0.86	30.18	2.77	6.80	28.40	13.20	36.30	2.90	14.90
Smoking & Alcohol (n=16)	7.38	0.81	29.36	2.72	6.85	27.55	13.20	36.30	2.9	14.90
Infected Maize (n=1)	—	—	—	—	—	—	—	—	—	—
Infected Beer (n=25)	9.38	0.86	31.46	2.41	10.40	29.30	15.90	43.70	3.00	20.20

Mean, median, range and mode given in ηg/ml

SEM standard error of mean

— No patients available in this population group

3.7.4 Frequency statistics for kinin generation assay in urine samples of six non-cancer patients sub-groups

Table 39 represents marginal frequency summary for kinin ELISA in non-cancer patient subgroups.

Table 39 Marginal frequency summary for kinin ELISA in six non cancer patient subgroups

Groups	Mean		SEM		Median		Range		Mode	
Family History (n=3)	Basal level		Generated		Basal	Gen	basal	gen	basal	Gen
	9.10	1.42	29.03	3.94	9.70	30.80	4.80	13.30	6.40	21.50
Eyes Water (n=18)	8.13	1.174	30.98	1.99	6.100	29.00	16.60	32.40	4.40	17.10
Smoking (n=16)	7.14	0.98	30.11	2.11	5.6	28.35	13.10	32.40	4.40	17.10
Smoking & Drinking (n=16)	7.14	0.98	30.11	2.11	5.6	28.35	13.10	28.35	4.40	17.10
Infected Maize (n=1)	—	—	—	—	—	—	—	—	—	—
Infected Beer (n=10)	6.99	0.69	31.31	1.68	6.3	32.45	7.4	18.20	5.6	21.50

Mean, median, range and mode given in ηg/ml

SEM standard error of mean

— No patients available in this population group

To compare the kininogenase assay in six subgroups a nonparametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 40 shows the comparison of basal and generated kinins by family history subgroup.

Table 40 Kruskal-Wallis 1-Way Anova kinin ELISA comparison in the family history subgroup

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	9.68	9.62	17	2.1994	2.5229	1	0.1381	0.1122
Non-cancer patients	15.17	15	3					
Oesophageal carcinoma	14.21	11.09	17	3.3967	2.3254	1	0.0653	0.1273
Non-oesophageal cancer	8.36	15.93	7					
Non-oesophageal cancer	8.33	6.67	3	3.7761	0.6402	1	0.0.520	0.4236
Non-cancer patients	4.29	5.00	7					

D. F degrees of freedom

base basal kinin concentration

gen generated kinin concentration

To compare the kininogenase assay in six subgroups a nonparametric statistical analysis was employed using Kruskal-Wallis !-Way Anova. Table 41 shows the comparison of basal and generated kinins by eyes water subgroup.

Table 41 Kruskal-Wallis 1-Way Anova kinin ELISA comparison in the eyes water subgroup

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	8.44	37.12	58	0.0018	0.9558	1	0.9659	0.3283
Non-cancer patients	38.69	42.94	18					
Oesophageal carcinoma	50.72	48.24	58	8.1515	2.6213	1	0.0043	0.1054
Non-oesophageal cancer	34.31	38.94	31					
Non-oesophageal cancer	21.28	21.32	31	5.4940	5.5922	1	0.0191	0.0180
Non-cancer patients	31.28	31.32	18					

D. F **degrees of freedom**

base **basal kinin concentration**

gen **generated kinin concentration**

To compare the kininogenase assay in six subgroups a nonparametric statistical analysis was employed using Kruskal-Wallis !-Way Anova. Table 42 shows the comparison of basal and generated kinins by smoking subgroup.

Table 41 Kruskal-Wallis 1-Way Anova kinin ELISA comparison in the smoking subgroup

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	34.64	32.90	49	1.5038	0.0058	1	0.2201	0.9393
Non-cancer patients	27.97	33.31	16					
Oesophageal carcinoma	34.42	33.09	49	2.2205	0.2113	1	0.1362	0.6458
Non-oesophageal cancer	26.23	30.57	16					
Non-oesophageal cancer	16.56	15.63	16	0.0014	0.2785	1	0.9699	0.5977
Non-cancer patients	16.44	17.38	16					

D. F degrees of freedom

base basal kinin concentration

gen generated kinin concentration

To compare the kininogenase assay in six subgroups a nonparametric statistical analysis was employed using Kruskal-Wallis !-Way Anova. Table 43 shows the comparison of basal and generated kinins by smoking and alcohol subgroup.

Table 43 Kruskal-Wallis 1-Way Anova kinin ELISA comparison in the smoking and alcohol subgroup

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	31.48	30.43	43	1.1729	0.0611	1	0.2788	0.8047
Non-cancer patients	26.03	29.09	16					
Oesophageal carcinoma	30.97	29.73	43	0.5008	0.0385	1	0.4791	0.8445
Non-oesophageal cancer	27.41	30.72	16					
Non-oesophageal cancer	16.56	15.63	16	0.0014	0.2785	1	0.9699	0.5977
Non-cancer patients	16.44	17.38						

D. F degrees of freedom

base basal kinin concentration

gen generated kinin concentration

To compare the kininogenase assay in six subgroups a nonparametric statistical analysis was employed using Kruskal-Wallis 1-Way Anova. Table 44 shows the comparison of basal and generated kinins by infected maize subgroup.

Table 44 Kruskal-Wallis 1-Way Anova kinin ELISA comparison in the infected maize subgroup

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	9.00	9.00	17	2.6842	2.6842	1	0.1013	0.1013
Non-cancer patients	18	18	1					
Oesophageal carcinoma	9.65	17	9.47	0.2322	0.0093	1	0.6299	0.932
Non-oesophageal cancer	7.00	1	10.00					
Non-oesophageal cancer	2	2	1	1.0000	1.0000	1	0.3173	0.3173
Non-cancer patients	1	1	1					

D. F degrees of freedom

base basal kinin concentration

gen generated kinin concentration

To compare the kininogenase assay in six subgroups a nonparametric statistical analysis was employed using Kruskal-Wallis !-Way Anova. Table 45 shows the comparison of basal and generated kinins by infected beer subgroup.

Table 45 Kruskal-Wallis 1-Way Anova kinin ELISA comparison in the infected beer subgroup

Groups	Mean Rank		Cases (n)	Chi-square		D. F.	Significance	
	base	Gen		base	Gen		base	Gen
Oesophageal carcinoma	26.72	27.32	44	0.5904	0.0317	1	0.4423	0.8586
Non-cancer patients	30.95	28.30	10					
Oesophageal carcinoma	39.59	36.74	44	6.3620	0.9122	1	0.0117	0.3395
Non-oesophageal cancer	26.92	31.94	25					
Non-oesophageal cancer	24.00	20.75	10	4.8013	1.0089	1	0.0284	0.3152
Non-cancer patients	15.60	16.90	25					

D. F degrees of freedom

base basal kinin concentration

gen generated kinin concentration

CHAPTER 4: DISCUSSION

4.1 Histopathological changes in oesophageal tumours

Keratin pearls

Well differentiated tumours of cervix, endometrium and bladder show intercellular bridges of keratin pearls as visualised in the oesophageal carcinoma. The resection specimens usually demonstrated invasion into, and often through, the muscle coats, and infiltrated also the extramural mediastinal tissues to a variable degree. The extent of the local spread is an important prognostic factor.

Giant cells

Giant cell carcinomas are mostly poorly differentiated carcinomas. These neoplasia consists predominantly of giant cells. Many giant cells were observed in the oesophageal tumour specimens. The dominance of giant cells is indicative of aggressive neoplasms with relatively poor prognosis, as was the case with oesophageal tumours. The examples of giant cell formation are subependymal giant cell astrocytoma (SEGA), giant cell granuloma and tumours of the bladder, lung, endometrium.

Mast cells

The mast cell infiltrate in some tumours may be associated with prominent proliferation of capillaries (angiogenesis) or venules. This might be due to the subsequent release of the potent

angiogenic factor, VEGF that enhances the progression of tumours. Increased mast cell prevalence is a predisposing factor for basal cell carcinoma in humans. In lung cancer, there appears to be a direct correlation between the number of mast cells and tumour angiogenesis, and this relationship appears to be independent of vascular endothelial growth factor. The unique finding of intense labelling of mast cells by specific antibodies to tissue kallikrein, the proenzyme and the kinin receptors is the first indication of a possible role of the kallikrein-kinin cascade in carcinogenic angiogenesis.

Squamous cells

The squamous cell carcinoma (SCC) is the predominant histological type of oesophageal cancer world-wide (Powell *et al.,* 1990; Blot *et al.,* 1993). Heamatoxylin and Eosin stains carried out on 50 oesophageal specimens (biopsies and resections) during the present study showed that the most prevalent type was the moderately differentiated squamous cell oesophageal carcinomas (Appendix D). The normal oesophageal specimens showed an intact oesophageal epithelium composed of the mucosa, submucosa and muscularis propria. The squamous cell carcinoma was shown to appear, at least in three histological gradings, namely, well and moderately differentiated and poorly differentiated squamous cell carcinoma. Of these the moderately differentiated squamous cell carcinoma was the most prevalent. The poorly differentiated (dedifferentiated), non-keratinising squamous cell carcinomas formed a very small percentage of the oesophageal tumours. The present findings, therefore, support previous reports on the prevalence of the squamous cell carcinoma in humans. Adenocarcinoma and adenosquamous carcinoma were only visualised in a few patients. Of

the tumours examined only one specimen was of a smooth muscle carcinoma that comprised bizzare tumour giant cells infiltrating the smooth muscle layer.

4.2 Cellular visualisation of tissue kallikrein and prokallikrein in oesophageal carcinoma

To elucidate the cellular orientation of tissue kallikrein in oesophageal carcinomas immunocytochemistry (ICC) was performed using recombinant tissue kallikrein antibody as the primary antibody. Tissue kallikrein was histochemically immunolocalised both in normal and tumour oesophageal epithelium. The human salivary gland was used as the positive control tissue in which TK is specifically synthesised and stored in the ducts of the gland; there was absence of labelling when the primary antibody was replaced with phosphate buffered saline or preabsorbed with an excess of antigen (method control). In normal tissue TK was immunolabelled only in the epithelial layer of the submucosa. The basal epithelium showed no immunoreactivity for tissue kallikrein. In tumour oesophageal epithelium tissue kallikrein was seen, mostly concentrated in the upper pleomorphic cells of the epithelium. The labelling in tumour epithelium was not as intense as was in the normal epithelium. High intensity immunoreactive TK was seen also in bizarre, degenerating giant tumour cells, infiltrating the muscular tissue. These cells showed a spread of variability in the intensity of labelling. An explanation may be that as the cancer cells degenerate they loose the capacity to synthesise and store more TK. The muscularis propria (inner and outer muscular layers) appeared to be normal as there was no infiltration by any of the TK containing giant tumour cells. Very intense labelling was seen in the activated mast cells (verified by the Giemsa

stain). The intensity of labelling was almost as great as that in the normal epithelium of the oesophagus. Immunoreactive TK was visualised also in the keratin pearls of the well differentiated, keratinising squamous cell carcinomas, even though the labelling was less intense than that observed in the epithelial cells. Weak TK immunoreactivity was observed in islands of well differentiated, non-keratinising squamous cell carcinomas (data not shown). In the method control sections where the TK antibody was replaced with phosphate buffered saline or preabsorbed with an excess of antigen no labelling for TK had occurred.

Because tissue prokallikrein is synthesised in the ducts of the human salivary gland it was used as a positive control tissue. Immunoreactive tissue pro-kallikrein (TproK) was localised in the normal epithelial cells of the oesophageal mucosa, but was more intensely sited towards the periphery of the epithelium. The basal epithelium did not react to the tissue prokallikrein antibody. TproK was localised also in the pleomorphic cells of the tumour epithelium. The intensity of reactivity for TproK was less in tumour epithelium when compared to the normal epithelium, probably because as cells disintegrate they loose their ability to synthesise the proenzyme. High intensity labelling for TproK was a new discovery in the activated mast cells. However, the intensity in giant tumour cells was less. Week reactivity TproK was seen in the keratin of the well differentiated, keratinising squamous cell carcinomas. Week labelling for TproK was observed also on the island of well differentiated, non-keratinising squamous cell carcinomas (data not shown). The normal muscularis propria of the oesophagus showed no labelling for TproK.

4.2.1. Image analysis of immunolabelling of TK and TproK

The intensity of the labelled antigen was determined by analysis of the computer-generated images, which quantified the relative intensity of the labelling by calculating the mean value of the grey scale range 155-256 for each area, using the unit pixel x $10^4/\mu m^2$. The higher range was taken since it indicated specific labelling. The POLI Look-Up-Table (see Table 3) provided the lower and the upper threshold values on the grey scale.

Although photomicrograph of the normal muscularis propria showed no labelling, the histogram values represented non-specific background. The normal muscularis propria of the oesophagus gave a mean intensity of 162 X 10^4 pixels/μm^2. The mean intensity for the salivary gland of 1668 X 10^4 pixels/μm^2 (positive control) was very high. This was an expected finding since the salivary gland has a high concentration of TK in the duct cells. For normal epithelium the mean intensity was 913 X 10^4 pixels/μm^2, which was also a high value, confirming the TK labelling results for the epithelium. The mean intensity labelling in the tumour epithelium of 587 X 10^4 pixels/μm^2 was lower than that of the normal epithelium. The disintegrating tumour epithelium indicated loss of TK. The mean intensity for the giant calls was found to be 1088 X 10^4 pixels/μm^2 which was a very similar to that observed in the salivary ducts cells. Although TK immunoreactivity in the giant cells varied, most showed very high intensity. For the activated mast cells the mean intensity was found to be 1373 X 10^4 pixels/μm^2, which was the highest mean intensity value in oesophageal tumours. The mean intensity of keratin pearls of 220 X 10^4 pixels/μm^2 was the second lowest. As for TK in

the normal muscularis propria,the mean intensity value of 162 X 10^4 pixels/μm^2 represented non-specific labelling.

In the normal epithelium, the mean intensity TproK was found to be 768 X 10^4 pixels/μm^2. Although high intensity of labelling was seen in the periphery of the epithelium the mean intensity was not as high. The value was about half of that for TK. The mean intensity labelling of TproK in the tumour epithelium (320 X 10^4 pixels/μm^2) was also about half of that noted for TK (587 X 10^4 pixels/μm^2). Also, localisation of TproK was lost when the tumour epithelium was disintegrating. The mean intensity value of 1031 X 10^4 pixels/μm^2 for the giant tumour cells was almost equal to that of the TK mean intensity. The high mean intensity value of the giant cells confirmed the immunolabelling visualised in the ICC experiments. The high mean intensity value for the activated mast cells of 1225 X 10^4 pixels/μm^2 was however somewhat lower than that of the TK (1373 X 10^4 pixels/μm^2). The keratin pearls mean intensity (210 X 10^4 pixels/μm^2) was low but seemed to match that of the TK (220 X 10^4 pixels/μm^2).

4.3 Genes in carcinoma

Several genes have been implicated in the causation of oesophageal carcinoma: p53, Rb, APC, MCC and DCC (Stemmermann *et al.*, 1994). A recent and important discovery is the role of defects in DNA mismatch repair genes. The normal DNA mismatch repair genes (four have

been characterised: MSH 2, MLH 1, PMS 1 and PMS 2). Maintain the integrity of DNA by repairing errors that occur during DNA replication or incurred after exposure to genotoxic agents/events, for example chemicals or toxins. Defects in these repair genes lead to a genetic instability and this is therefore, thought to play a pivotal role in oncogenesis.

Scattered throughout the human genome are sort tandem repeat sequences which are referred to as microsatellites. These are non-transcribable and constitutes 10-15 percent of the mammalian DNA. In addition, these sequences are conserved and are stably inherited throughout the genome. Microsatellites were initially thought to play a functional role in the genome, either directly in gene regulation, or indirectly as hot spots for recombination, however, their exact role still remains elusive. Recently various studies have indicated that mutations occur in these microsatellites due to a mismatch repair errors termed replication errors (RERs). The mutations are due to the loss or gain of one or more of these repeats. This results from defective DNA repair producing different lengths of DNA in these regions. These mutations are referred to as microsatellie instability (MSI), which have been implicated in a host of human disorders, both hereditary (Keller *et al.,* 1995) and non hereditary disorders including tumours, for example oesophageal, colorectal and prostatic carcinomas. Therefore, replication errors in these simple repeat sequences serve as markers for mutational events occurring in key mismatch repair genes. Abnormalities in these genes may then increase the mutability of tumour DNA during the process of neoplasia. Microsatellite instability may be used as markers for the detection of tumours in histologically unremarkable specimens, loss of heterozygosity and also for gene mapping and linkage studies.

4.4 Expression of tissue kallikrein gene in oesophageal carcinoma

The circular Bluescript Stratagene plasmid shown diagrammatically in figure 37 was used to subclone HK1 cDNAs. Not-1 and Acc-1 were the restriction sites which flanked the HKLK1 cDNA insert. T7 polymerase was responsible for the synthesis of the antisense TK mRNA strand, and T3 was responsible for the synthesis of the sense TK mRNA strand. The linearised plasmid showing the antisense HK1 cDNA (A) and sense HK1 cDNA (B) is depicted in figure 38. The normal salivary gland labelled positively with an antisense TK mRNA probe. The striated ducts showed reactivity for TK mRNA, indicating synthesis of the protein in the ducts of the salivary gland. In other words the synthesis and localisation sites were the same in the salivary gland for TK. When the salivary gland was labelled with a sense mRNA probe, the tubules showed no positive reactivity for the TK mRNA; an expected finding with a sense probe. When the normal oesophageal epithelium was labelled with TK mRNA probe, the cells on the periphery of the normal epithelium showed immunoreactivity for the TK mRNA, and thereby confirming the results obtained by immunolabelling. Clearly, the normal oesophageal epithelium synthesises TK, probably by HKLK1 with a TK mRNA. When the oesophageal tumour epithelium was labelled with an antisense TK mRNA probe, the polyploid cells showed reactivity for TK mRNA, contrastingly there was no TK mRNA reactivity seen with a sense probe. Oesophageal carcinoma *in situ* labelled with a TK mRNA probe was largely infiltrated by inflammatory cells, probably neutrophils with some plasma cells which labelled for TK mRNA. Similarly activated mast cells in the oesophageal tumours labelled for TK

mRNA. The reactivity was very intense, indicating induction of the HKLK1 gene in the cells. In terms of control responses the normal oesophageal muscularis propria labelled neither with the antisense nor with the sense TK mRNA strands. Both showed also no immunoreactivity for the TK mRNA, confirming the immunolabelling results for TK in the muscularis propria.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): To confirm the expression of HKLK1 in oesophageal carcinoma, total mRNA was extracted from fresh normal and tumour resections and biopsy specimens of the oesophageal tissue. In general, the concentration of the total mRNA found in tumour oesophageal samples was higher than that of normal tissue. This finding was suggestive of increased expression of oncogenes and growth factors in oesophageal carcinomas (Wong *et al.,* 1994; Mario *e al.,* 1996; Yuko *et al.,* 1994; Yoshida *et al.,* 1993). The suggestion that the amplification or overexpression of oncogenes is associated with carcinogenic process of oesophageal carcinoma (Kanda *et al.,* 1994) might be associated with the increased total mRNA concentration found in this study. Epidermal growth factor (EGF) and α transforming growth facto (TGF_{α}) over-expression (Yoshida *et al.,* 1992), in oesophageal carcinomas together with tissue kallikrein acting as autocrine modulators may play a central role in invasion of adjacent tissue by the tumour and promote metastasis of the tumour cells.

4.5 Tissue kallikrein in tumourigenesis

Tumourigenesis is a complex multistage genetic process with hereditary predisposition involved in some cases. A complicated interaction of defects at many sites in the genome is likely to be needed to overpower the cells ability to regulate normal growth and replication, requiring the activation of positively acting, and inactivation of the negatively acting, growth regulators. The behaviour of the resulting tumour may be due to the array of genetic changes it has accumulated. Genetic profiling of tumours may therefore be of use clinically in determining tumour behaviour to aid in patient prognosis and possibly therapy, and also in terms of epidemiology to establish the important environmental factors in cancer. The genetic changes found in tumours may also be of use in tumour diagnosis and population screening in the future. Understanding the functions of the genes that are altered in cancers may aid in the development of new therapies to counteract or to correct such changes.

Recent studies have suggested the possibility of an increased expression of tissue kallikrein in cancer, arising from the induction of one or more of the kallikrein genes. TK has been localised in gastric carcinomas, Lewis lung tumours, ductal breast cancer cells and pituitary prolactin secreting adenomas. Embryologically these cells are derived from the entoderm, and share a common cellular ancestry with oesophageal carcinoma. In harmony with this concept is our localisation of TK and TproK in oesophageal carcinoma (Dlamini *et al.*, 1999). In addition, tissue kallikrein is expressed in prostate and endometrial cancers (Clements and Mukhtar, 1997). The other two kallikrein genes are expressed in the prostate, KLK2 and KLK3 (prostate specific antigen, PSA). It has been documented lately that KLK2 is expressed

in prostate cancer, and that the expression is incrementally increased from benign epithelium to high-grade prostatic intra-epithelial neoplasia (PIN). Expression of KLK2 indicates that this kallikrein antigen is both prostate localised and tumour associated (Darson *et al.*, 1997.

The concentration of PSA in serum is normally less than 4 μg/l, but elevated concentrations are found in a majority of patients with prostate cancer (CAP). Elevated levels of circulating tissue kallikrein may prove to be a marker for those carcinomas in which there is induction of the kallikrein gene. Clearly it would prove valuable as an additional marker in prostate carcinoma. Therefore, the components of the kallikrein-kinin cascade may have diagnostic relevance in oesophageal carcinoma.

It was recently reported that tissue kallikrein activates *in vitro* matrix degrading metalloproteinases present in carcinoma cells (Hermann *et al.*, 1995). Our study supports the thesis therefore that tissue kallikrein is implicated in the process of tumourogenesis, through the cell differentiating and growth action of bradykinin (Roberts & Gullick, 1989). Gene expression of tissue kallikrein (HKLK1), and the subsequent formation of vasoactive kinin peptides could stimulate proliferation of tumour cells and by increasing vascular permeability, enhance metastases. The localisation of the kinin B2 and B1 receptors in oesophageal carcinoma and the observation of increased kinin receptor expression due to oncogenic transformation (Roberts & Gullick, 1989) gives further importance to a mitogenic role for kinins in tumour tissue. There have been suggestions that transformation of cells *in vitro* by *ras* oncogene, an oncogene found mutated in a large number of human tumours (Lemaine,

1989), is associated with increased bradykinin receptor number (Pries *et al.*, 1987). Taken with the known release of bradykinin at sites of tissue injury and inflammation (Hargreaves *et al.*, 1988; Bhoola *et al.*, 1992), this implies a role for bradykinin in cell growth control during tumourogenesis.

4.6 Cellular and mitogenic actions of kinins in carcinoma

Bradykinin receptors mediate the majority of the diverse biological actions of kinins, and many of the biological roles attributed to endogenous kinins. The major advances in the area of bradykinin receptor pharmacology have resulted from the use of effective bradykinin receptor antagonists through molecular cloning, and producing colonies of knock-out mice with deletion of B1 and/or B2 receptors (Borkowski *et al.*, 1995). The two mammalian kinins, kallidin and bradykinin, influence the cardinal features of inflammation as well as a number of cellular functions, including blood pressure and local blood flow, electrolyte and glucose transport, and cell proliferation. The cellular actions of kinins are modified by their ability to stimulate the release of many second generation mediators, for example, platelet-activating factor, leukotrienes, prostaglandins, substance P (neurogenic inflammation), acetylcholine, and noradrenaline (sympathetic nerves). Kinins also stimulate the secretion of renin from the kidney, release vasopressin from the neurohypophysis, and secretion of catecholamines from the adrenal medulla (Bhoola *et al.*, 1992). Therefore, the homeostatic regulation of regional blood flow by kinins could be modulated in a manner that has been difficult to evaluate, except with selective antagonists. In addition, it would be necessary to establish the precise role of the secondary autacoids in the control of systemic or local blood flow. Kinins act as mitogens,

stimulating DNA synthesis and thereby promoting cell proliferation. The ability of kinins to induce cell division could enhance the spread of cancerous cells and increase proliferation of epidermal cells in disorders such as psoriasis. Kinins also appear to play an important role in a number of pathological states, namely, reduced sperm motility, allergic and viral rhinitis and asthma, postgastrectomy dumping syndrome, inflammatory bowel diseases, carcinoid, anaphylactic shock, and septic shock.

4.6 Kinin B1 and B2 receptors in oesophageal carcinoma

Most of the actions of bradykinin and kallidin are mediated through the constitutive B2 receptor, which essentially does not respond to desArg^9 -bradykinin or -kallidin, the kinin B1 receptor agonists. On most membranes the K_d of kinins for the B2 receptor is in the range 0.7 to 5 nm. Specific antagonists are essential to understanding the physiological role and cellular actions of endogenously active molecules. Ever since the description of the correct structure of bradykinin in 1960, many analogues have been synthesised.

Kinin B1 receptors in the anterior horn of the spinal cord (positive control) showed intense immunoreactivity in the neurons. When the kinin B1 receptor antibody was replaced with phosphate buffered saline or preabsorbed with an excess of antigen (method control) no labelling was detected for the kinin B1 receptor. Here again we notice intense labelling for the kinin B1 in the normal epithelium of the oesophagus. The immunoreactivity was more intense on the periphery of the mucosa, whereas the basal epithelium showed no immunoreactivity for the kinin B1 receptor. The disintegrating tumour epithelium showed some labelling for the

kinin B1 receptor, although the intensity seemed to be weaker than that seen in the normal epithelium. Very high intensity immunoreactivity was visualised in the activated mast cells. The degenerating giant tumour cells also showed high intensity of labelling for the kinin B1 receptor. The keratin pearls of the well differentiated, keratinising squamous cell carcinomas showed some moderately intense labelling for the kinin B1 receptor, unlike the submucosa which was negative.

The B2 receptor labelling in the salivary gland gave a high mean intensity value, indicating that the salivary gland is rich in the B2 receptor. Such a result was not unexpected since the kinin B2 receptor is a physiological receptor for kinins released by tissue kallikrein. The discovery of kinin B2 receptors in such abundance in the human salivary gland is a new finding. High intensity of kinin B2 labelling was concentrated on the periphery of the mucosa, and week labelling was observed in the middle part of the epithelium. The mean intensity of 282 X 10^4 pixels/$\mu m^{2)}$ in the tumour epithelium was low, and this again was probably due to the disintegrating epithelium. As throughout this study the giant cells showed a considerably higher intensity, although it was not as high as that for TK, TproK and the kinin B1 receptor. Generally, the mast cells showed a very high intensity of labelling values throughout the many sections analysed. The keratin pearls gave a low mean intensity value for the kinin B2 receptor.

4.6. 1 Image analysis of immunolabelling of kinin B1 and B2 receptors

The mean intensity value for the normal submucosa of 198 X 10^4 pixels/μm^2 represented non-specific labelling. B1 receptor labelling in the spinal cord (positive control for B1) gave mean intensity of 516 X 10^4 pixels/μm^2; the method control showed no labelling. In the normal epithelium an intensity of 444 X 10^4 pixels/μm^2 was observed. Although the periphery of the normal epithelium showed intense labelling, the middle areas of the epithelium stained less intensely for the kinin B1 receptor. The mean intensity for tumour epithelium was found to be 419 X 10^4 pixels/μm^2, which was in the similar range as that of normal epithelium of 444 X 10^4 pixels/μm^2. On the giant tumour cells mean intensity (890 X 10^4 pixels/μm^2) gave a high value and this finding confirmed the immunolocalisation experiments. Once again the mast cells gave highest of 1344 X 10^4 pixels/μm^2. The keratin pearl mean intensity value for the kinin B1 receptor was found to be 862 X 10^4 pixels/μm^2. This value was higher than that for TK and TproK. The image analysis results gave quantitative confirmation of the level of grey scale grading visualised in the immuno-labelling experiments.

4.7 Epidemiology: Oesophageal cancer

Risk factors specific to South Africa

Numerous factors have been associated with oesophageal cancer in South Africa. The high rate in the Eastern Cape (former Transkei) has been attributed to the deficiencies of trace elements of Mo, Zn, Cu, Fe and Mg in the Beaufort sedimentary soils in which plants are

grown. It has also been postulated that the increase in oesophageal carcinoma was attributed to the importation of maize which replaced sorghum as the main staple diet. It is known that this cereal is deficient in nicotinamide and tryptophan. The consumption of mouldy maize contaminated with fungus, especially *Fusarium moniliforme,* and the resultant ingestion of mycotoxins has been implicated in the development of cancer. The association of smoking with the development of cancer of the oesophagus has been recorded especially in Zulu men. Alcohol consumption especially of home brewed beers has been found to be an important factor responsible for high incidence in the Eastern Cape. The beer is often made from mouldy maize that may result in the ingestion of mycotoxins. In the Coloured population the drinking of cheap wines may also play a role. Genetic susceptibility has been investigated as the Eastern Cape is predominantly inhabited by Xhosas.

4.7.1 Age distribution in three patient groups

The patient recruitment was sequential. The mean age of the oesophageal cancer patients was found to be 56.5 ±1.1, and this indicated that most people who develop oesophageal carcinoma were in their late fifties, and this type of tumour is commonly not seen in younger people. These observations are in line with the findings of Haffejee *et al.,* 1991. Although the mean age was 60 in their study was represented a marginally older population group, the epidemiological age data could be considered comparable. The mean age of the non-cancer patients was 43.6±1.9, and that meant that most people recruited in this study as non-cancer patients were not purposefully age matched. The mean age of non-oesophageal cancer patients was found to be 53.1±1.8. Although the mean age of the non-oesophageal cancer

group represented younger patients, it was reasonably close to oesophageal cancer patients. Because of the clear difference in age between the three groups, the holistic evaluation of the secretion of renal tissue kallikrein may have been skewed, particularly since the concentration of the enzyme in the urine gradually reduces with age.

4.7.2 Statistical analysis of the epidemiological data for the subgroups

The Pearson Chi-Square Test was used to test whether there was no association (null hypothesis) between the oesophageal carcinoma, non oesophageal carcinoma and non-cancer patients with regard to various modalities, namely family history, eyes water, smoking, smoking and alcohol, infected maize and infected maize subgroups

4.7.2 1 Family history

Ethnicity is a strong indicator of risk for this disease because ethnic groups tend to share similarities in diet and geography. There are, however, several reports that indicate an increased incidence among blood relatives (Pour *et al.,* 1974; Wu & Ran, 1979). A follow-up survey of 622 families with a history of oesophageal carcinoma was carried out in China, and the results showed that offspring from households where one or both parents died from oesophageal cancer showed a higher mortality from the same disease (Hu, 1990). During the

current investigation we found out that there was a significant difference in the number of cases with family history between oesophageal carcinoma and non-cancer patients. Oesophageal carcinoma had the largest number of cases with family history more than any patient group. This points to a strong familial tendency with the possible existence of genetically determined susceptibility.

There was a significance difference between the number of cases of with oesophageal carcinoma and those without cancer within the family history cohort. This was evident by the values for Fishers Exact Test (0.00882 and 0.01353), and the Pearson significance value of 0.01150. The large Chi-Square value of 6.38700 also confirmed the large difference. There was no significant difference in the number of cases with family history between the oesophageal carcinoma and non-oesophageal cancer patients, as indicated by the significance value of 0.19013. Also there was no significance difference in the number of cases with family history between non-oesophageal and non-cancer patients. The data are very suggestive of a strong familial association in the aetiology of oesophageal carcinoma.

4.7.2.2 Eyes watering

The smoke from fire wood used to cook meals in most of Black African house holds showed a close correlation with oesophageal carcinoma. As evident from the finding of a highly significant difference in the number of cases with eyes watering was observed between

oesophageal carcinoma and non-cancer patients (0.00001). This finding was also confirmed by the very large Pearson Chi-Square of 19.11872. There was a significant difference for the number of cases with eyes watering between oesophageal carcinoma and non-cancer patients (0.05261), and this finding was also confirmed by Fisher's Exact Test values of 0.03870 and 0.06174. There was again a significant difference of the number of cases with eyes watering between non-oesophageal cancer and non-cancer patients (0.01967).

4.7.2.3 Smoking

The role of tobacco abuse in the aetiology of oesophageal carcinoma appears to be well established (Graham *et al.*,1990). Results obtained from well-designed case-controlled studies report an association between cigarette smoking and risk of oesophageal carcinoma (Cheng *et al.*, 1992). The tumourogenic activity of tobacco smoke is contained either in the particulate matter or the tar fraction. The primary initiators are believed to be the polynuclear aromatic hydrocarbons, such as benzo(e)pyrene and volatile nitrosoamines (De Stefani *et al.*, 1990). Pipe and cigar smokers have a greater risk of cancer of the oral cavity, the oesophagus than cigarette smokers. It has been proposed that pipe tobacco residues are swallowed into the oesophagus, allowing close contact of the tobacco carcinogens with the oesophagus (De Stefani *et al.*, 1990). Hand-rolled cigarette smoking is associated also with a higher risk of oesophageal carcinoma, compared with the use of commercial cigarettes (De Stefani *et al.*, 1993). The risk increases with increasing number of cigarettes and duration of the smoking habit (De Stefani *et al.*, 1993). Our study confirms all these findings because we found a significant difference in

the number of cases of smokers between oesophageal carcinoma and non-cancer patients; between oesophageal carcinoma and non-oesophageal cancer patients. The largest number of cases who were smokers was in the oesophageal carcinoma group.

Statistically a highly significant difference was noted in the number of smokers between oesophageal carcinoma and non-cancer patients (0.00037). This large difference was shown also by the large Chi-Square number of 12.66081, and the Fisher's Exact Test values of 0.00030 and 0.00040. There was again a significant difference in the number of cases of smokers between oesophageal carcinoma and non-oesophageal cancer patients (0.00013). The Fisher's Exact Test values (0.00010 and 0.00018) also confirmed the large significant difference. There was no significant difference in the number of cases for smokers between the non-oesophageal cancer and non-cancer patients (0.79362).

4.7.2.4 Smoking and alcohol distribution in three patient groups

Alcohol and tobacco abuse in the aetiology of oesophageal carcinoma is well established (De Stefani *et al.*, 1993). It has been estimated that more than 80 per cent of cases in industrialised countries can be attributed to exposure to these two environmental factors, either singly or jointly (Tuyns, 1983). Differential risks for oesophageal carcinoma are observed also with different types of alcoholic drink (Yu *et al.*, 1988). For any given level of ethanol intake, the risk from spirits is usually more than twice that from beer; the risk from wine is intermediate

between that from spirits and that from beer. In our investigation we found that there was a highly significant difference in the number of cases who were smokers and drinkers between oesophageal carcinoma and non-cancer patients; between oesophageal carcinoma and non-oesophageal carcinoma patients and the largest number of cases was in the oesophageal carcinoma group. These findings support that of the previous researchers in oesophageal carcinoma

There was indeed a significant difference in the number of cases between oesophageal and non-cancer patients (0.00566). The large difference in the number of cases was shown also by the large Chi-Square value (7.65500), and the Fishers exact values (0.00440 and 0.0638). The large significance difference (0.00456) in the number of vases who smoke and drink was seen also between oesophageal carcinoma and non-oesophageal cancer patients. The Chi-Square value (8.04798) is large, and it explains the large difference of the number of cases between these groups. There is no significant difference in the number of cases between non-oesophageal cancer and non-cancer patients (0.95653). The very small Chi-Square (0.00297) explain this insignificance difference for the number of cases.

4.7.2.5 infected maize

Fungi have also been implicated as aetiological agents in the cancer of the oesophagus (Chang *et al.,* 1992). Studies in the high-risk areas for oesophageal carcinoma in China showed that some common species of fungus, belonging to the genera *Fusarium, Alternaria, Geotrichum,*

Aspergillus, Cladosporium and *Penicillium,* are frequently detected in grain (Yang, 1980). Of the various infections of the oesophagus, fungal infections, particularly those of *Candida* species, are by far the most common (Chang *et al.,* 1992). The presence of a high level of mycotoxins, such as Fumosin B1 (a cancer promoter), in the mouldy corn samples and the capability of producing nitrosoamines (carcinogens) by *Fusarium* may play an important role in oesophageal carcinogenesis in Black Africans (Dutton, 1996; Chu an Li, 1993; Gonzalo, 1994; Rheeder *et al.,* 1992). The findings in our study support previous reports. We found a highly significant difference in the number of cases who ate infected maize and drank beer made from infected maize between oesophageal carcinoma and non-cancer patients and between oesophageal carcinoma and non-oesophageal cancer patients.

A significant difference was noted in the number of cases between oesophageal carcinoma and non-cancer patients (0.00116). This was confirmed also by the significance values for the Fisher's Exact Test (0.00058 and 0.00070). The large Chi-Square value (10.55718) signifies the large difference in the number of cases. There was a significant difference in the number of cases between oesophageal carcinoma and non-oesophageal carcinoma patients (0.00104). The large Chi-Square (10.75583) explained also the large difference in the number of cases. There was no significant difference in the number of cases between non-oesophageal carcinoma and non-cancer patients (0.99060), and supported by the very small Chi-Square value (0.00014).

Patients who drank beer fermented from infected maize showed a highly significant difference between oesophageal carcinoma and non-cancer patients (0.00007). This significant

difference was supported by the large Chi-Square value (15.76393). The Fisher's Exact Test significance values (0.00005 and 0.00007) confirmed the significant difference. There is no significant difference in the number of cases between oesophageal carcinoma and non-oesophageal cancer patients (0.34022). There was a significant difference in the number of cases. There is a significant difference in the number of cases between the non-oesophageal cancer patients and non-cancer patients (0.00374) with regard to the drinking of beer prepared from infected maize. This large difference was shown also by the large Chi-Square value (8.40672). The significance values for the Fisher's Exact Test (0.00322 and 0.00544) confirms the large difference in the patient cases between these two groups.

4.8 Renal tissue kallikrein-a marker for holistic changes in carcinoma

The question whether holistic changes occur in patients with carcinoma was examined by determining the concentration of renal tissue kallikrein (enzyme-linked immunosorbent assay), and its enzymic [amidase and kininogenase (kinin formation)] activities in the urine of the three patient groups (oesophageal cancer, non-oesophageal cancer and non-cancer patients) (Jenzano *et al.*, 1986).

1. The means of TK ELISA values in the three groups showed a similar trend. There was no significant difference between the TK ELISA values of oesophageal carcinoma and non-cancer patients. This meant that the tissue kallikrein concentration was found to be more or

less the same between the two groups. This was shown also by the mean ranks (75.9 and 71.2) which were only very slight different. The small TK ELISA value for the chi-square (0.43) confirmed also this lack of significant difference. However, there was a significant difference in the ELISA values for oesophageal cancer patients, and non-oesophageal cancer patients and this was supported by the mean ranks (80.20 and 66.38) and the large chi-square (3.72). There was again a significant difference between the non-oesophageal cancer patients and the non-cancer patients. The larger difference between the mean ranks (55.66 and 66.43), and the large chi-square value (2.86) confirmed the significant difference of the TK ELISA values.

2. With regard to the amidase activity of TK there was a significant difference between the oesophageal carcinoma and non-cancer patients (0.095). This was shown by the mean ranks (978.41 and 68.93). The slightly bigger value of the chi-square (1.75) explained also the difference in the amidase activity values between the two groups. There was however no significant difference between the oesophageal cancer and non-oesophageal cancer patients (0,43). This was indicated by the mean ranks (71.70 and 77.34). The small chi-square value (0.62) confirmed the lack of significant difference between the amidase activity value of the two groups. There was a significant difference between the amidase activity values of the non-oesophageal cancer patients and the non-cancer patients (0.041); confirmed by the large difference between the mean ranks (54.56 and 67.55). The large chi-square value (4.15) explained also the large difference between the values of the two group.

3. There was no significant difference (0.630) between the basal kinin values for oesophageal carcinoma and non-cancer patients and this was shown also by the values of the mean ranks (75.39 and 71.99), and confirmed by small value for the chi-square (0.23). There was a significant difference in the basal kinin values between oesophageal carcinoma and non-oesophageal carcinoma patients (0.0066). The larger difference between the mean ranks of the basal values (82.52 and 63.07) indicated the difference between the kinin basal values; and the larger value for the chi-square also was an indication of a significant difference in the basal kinin values between the two groups. There was a significant difference (0.0353) on the basal kinin values between the non-oesophageal cancer and non-cancer patients. This was shown by the larger difference between their mean ranks (54.34 and 67.77); the big chi-square value (4.43) indicated a large difference.

4. There was no significant difference between the values for kinin generation (0.37) for oesophageal carcinoma and non-cancer patients. The values for the kinin generated mean ranks (71.39 and 77.78) showed also no significant difference; supported by the small value for the chi-square. There was no significant difference (0.2644) in the generated kinin values between the oesophageal cancer and non-oesophageal cancer groups; confirmed by the mean ranks (77.79 and 70.00) and the small chi-square value for the generated kinin value (1.246) indicated the very small difference. There was a significant difference (0.0462) in the generated values between the non-oesophageal cancer and non-cancer patients. This was supported also by the larger difference between the mean ranks (54.70 and 67.41); the larger chi-square value (3.97) confirmed this observation.

- No ready explanation comes to mind as to why the oesophageal cancer renal TK values did not differ from that of the non-cancer patients

- However it is necessary to take note of the fact that the excretion values of renal tissue kallikrein fall with age, and being similar to the younger group may have been to induction of TK synthesis and secretion by the kidney of the carcinoma patients.

CHAPTER 5: CONCLUSIONS

1. Oesophageal carcinoma is the sixth most common cancer in the world and one of the most lethal tumours. Cancer of the oesophagus is the second most important cancer in South African men and the most important cancer in South African Black men. Therefore, the current study was designed to examine the immunolabelling and expression of tissue kallikrein and kinin receptors, to visualise cancerous transformation of the squamous mucosal cells, and to determine holistic changes in kallikrein secretion. This research programme was unique to a primary ethnic group not studied previously in this manner.

2. In this study tissue kallikrein was localised in the normal oesophageal mucosal epithelial lining, excluding the basal epithelium. The high fluorescent intensity, quantitatively confirmed by image analysis, indicated that this enzyme occurs in the normal oesophageal mucosa.

3. The gene expression of the serine protease, tissue kallikrein, has been determined mainly in epithelial cells derived from the entoderm as evidenced by the induction of this enzyme in pituitary adenomas, and lung, gastric and colonic tumours. Embryologically, the mucosal epithelial cells of the oesophagus are derived also from the entoderm, and

therefore suggested the occurrence of tissue kallikrein in the oesophageal mucosa squamous cells.

4. This discovery of tissue kallikrein and the kinin receptors in the oesophageal tumour epithelium suggested that after transformation of the epithelial cells into tumour cells, the malignant cells continued to synthesise the enzyme and its proform. The presence of tissue kallikrein mRNA in the transformed squamous cells confirmed the gene induction of this enzyme in oesophageal carcinoma.

5. Tissue kallikrein is a serine protease, which in tumourigenesis may convert precursor proteinases and macromolecules into active molecules. In this regard, evidence suggests that tissue kallikrein is important in the cascade formation of prostate specific antigen (PSA). Gene expression of tissue kallikrein, and the subsequent formation of the kinin peptides which by its mitogenic action stimulate proliferation of tumour cells, and by its cellular action increases vascular permeability and thereby initiates and enhance metastatic migration of cancer cells. Therefore, it is considered that tissue kallikrein is implicated in the process of tumourigenesis both as a serine protease, and as well as through the mitogenic and cellular actions of bradykinin. The observation of increased kinin receptor expression due to oncogenic transformation gives further credence to a mitogenic role for kinins in tumour tissue.

6. Proteinases such as serine proteases are secreted by cancer cells and are responsible for the proteolytic cascade triggered during malignant cell invasion. However, hydrolysis of extracellular matrix is a necessary step for malignant cells to invade functionally normal adjacent tissue. Proteinases that show high activities in malignant tumour

homogenates may be related to the degradation of the surrounding cell matrix. However, a role for the tissue kallikrein in this regard has still to be researched.

7. Most of the oesophageal tumours in this study were moderately differentiated squamous cell carcinoma, with mean age distribution of 56.5± 1.1 (40 females and 47 males).

8. In general, the more differentiated the tumour was, the greater the concentration of TK in the tumour cells, as well as in the keratin pearls. Surprisingly, the mast cells were found to show the most intense labelling on tissue kallikrein and the kinin receptors. Mast cells are known to release substances during degranulation that are mitogenic and diapedesis of cancer cells. The expression of tissue kallikrein and TproK in mast cells, and the induction of the KLK1 gene in the tumour cells may be important in the infiltration and metastatic progression of oesophageal carcinoma.

9. The various modalities, namely family history, eyes water, smoking, smoking and drinking, infected maize and infected beer subgroups were tested for statistical significance between the three experimental groups. There was significance difference between the cases of oesophageal carcinoma and those without cancer within the family history cohort. The data are very suggestive of a strong familial association in the aetiology of oesophageal carcinoma. Our social and environmental study demonstrated clear significant differences between the oesophageal and the non-oesophageal cancer patients in the number of cases with eyes water, with smokers, with smokers and drinkers, with patients who ate maize and drank beer and with those who drank beer fermented with infected maize.

10. The question whether holistic changes occur in patients with carcinoma was examined by determining the concentration of renal tissue kallikrein (enzyme-linked immunosorbent assay), and its enzymic (amidase) and kininogenase (kinin formation) activities in the urine of three patient groups (oesophageal cancer, non-oesophageal cancer and non-cancer patients). The tissue kallikrein concentration of renal tissue kallikrein in urine samples as measured by ELISA showed no statistical difference in the values for oesophageal and non-cancer patients whereas there was a statistical difference between oesophageal and non-oesophageal cancer patients. The amidase activity showed that there was a significant difference between the values for oesophageal and non-cancer patients, but the difference between the values for oesophageal and non-oesophageal cancer patients showed no statistical significance. Generated kinin values indicating kininogenase activity for oesophageal and non-cancer patients showed no statistical significance difference, and also the values for oesophageal and non-oesophageal cancer patients did not show any significant difference statistically. The significance of these equivocal results on the holistic changes in the secretion of renal tissue kallikrein by carcinoma patients was difficult to interpret. However, firstly a larger number than 40 patients may have provided clearer trends. Because the measurements were made blind and retrospectively, the variation in the subgroup patient number could not be readily corrected, and thereby also may have limited significant differences.

11. These results are the first to demonstrate the expression of tissue kallikrein, kinin B1 and B2 receptors in oesophageal carcinoma. Inhibitors of tissue kallikrein will be of particular value in preventing the regulatory control that tissue kallikrein could exert

on the process of tumourigenesis. The identification of kinin receptors in oesophageal cancer may have therapeutic implications leading to possible use of the third generation kinin receptor antagonists which have recently been shown to be active against small cell carcinomas *in vivo,* and have been shown to be apoptotic to cancer cells *in vitro.*

APPENDIX B

Name of Interviewer

Date of Interview

Day | Month | Year

Patient general information

Name and address:

Telephone No: Home

Work

Test tube no.
(Print Consecutive Stickers - Prefix with a D)
D0001 - DXXXX

Date of Birth

Study No:

Sex *(please tick)* 1 M F 2

Day | Month | Year

Age Years

Hospital/Institution information

Name of Hospital/Institution:

File Number:

Diagnosis (Clinical)

Primary Site of Cancer *(If more than one primary site, list all)* ICD

Histological Type in Words *(if applicable)* M

Clinical Extent of Cancer at this admission *(Please tick)*

1 Local 2 Regional 3 Metastatic 4 Not applicable

5 Unknown

Most valid basis of diagnosis of this cance *(Please tick)*

Path. Lab. No.

1 Clinical only 2 Clinical investigation (X-ray, ultrasound etc.)

3 Exploratory surgery/autopsy 4 Specific biochemical/immunological tests

5 Cytology/Haematology 6 Histology of metastasis

7 Histology of primary 8 Autopsy with histology 9 Unknown

Date of first admission for this condition Day Month Year

Date of diagnosis of this condition Day Month Year

Classification on diagnosis (stage):

Family History of OC 1 Yes No 2

Relation:

Medical History

Did you have any swallowing complaints? 1 Yes No 2

When did the complaints for this condition first start? Year

Received/Receiving Medical Treatment: 1 Yes No 2

Date: Day Month Year

Surgery	
Radiation	
Drugs	
Type of drug	
Type of surgery	

Where were you born? . *(please tick)*

1 ☐ Soweto 2 ☐ Jhb 3 ☐ Gauteng 4 ☐ NP 5 ☐ MPUM 6 ☐ NWP

7 ☐ FS 8 ☐ ECape 9 ☐ WCape 10 ☐ NWCape 11 ☐ KWZNatal

12 Other _____ 1 ☐ Urban 2 ☐ Rural

How long have you lived there? ☐☐ Years

Where do you normally live? *(please tick)*

1 ☐ Soweto 2 ☐ Jhb 3 ☐ Gauteng 4 ☐ NP 5 ☐ MPUM 6 ☐ NWP

7 ☐ FS 8 ☐ ECape 9 ☐ WCape 10 ☐ NWCape 11 ☐ KWZNatal

12 Other ..... 1 ☐ Urban 2 ☐ Rural

How long have you lived here? ☐☐ Years

What are the walls of your home made of? *(please tick)*

1. Brick	2. Tin	3. Plastic	4. Wood	5. Mud

6. Other .....

Describe your occupation (e.g. driver, machine operator)

Current: .....

Previous 5 years: .....

Previous 10 years: .....

Describe what your usual workplace does or did (e.g. a bank, a chemical factory, a gold mine)

Location: .....

Current: .....

Previous 5 years: .....

Previous 10 years: .....

Are you working now? *(please tick)* 1 Yes | No 2

Are you on a pension/disability allowance? *(please tick)* 1 Yes | No 2

What is the highest standard you passed at school? *(please tick)*

1	2	3	4	5	6	7
None	Sub A	Sub B	Std 1	Std 2	Std 3	Std 4

8	9	10	11	12	13	14
Std 5	Std 6 Form 1	Std 7 Form 2	Std 8 Form 3 NTC 1	Std 9 Form 4 NTC 2	Std 10 Form 5 NTC 3	Univ Tech

Where do you normally cook your food in your house? 1 Inside | Outside 2

What type of fuel do you MOSTLY use in your home for cooking? *(please tick)*

1. Wood	2. Charcoal	3. Coal	4. Anthracite	5. Paraffin	6. Gas	7. Electricity

8. Other .....

What type of fuel do you MOSTLY use in your home for keeping warm? *(please tick)*

1. Wood	2. Charcoal	3. Coal	4. Anthracite	5. Paraffin	6. Gas	7. Electricity

8. Other .....

Where did you normally cook your food in your house **20 years ago?**

1 | Inside | Outside | 2

What type of fuel did you MOSTLY use in your home for cool **20 years ago?** *(please tick)*

1. Wood	2. Charcoal	3. Coal	4. Anthracite	5. Paraffin	6. Gas	7. Electricity

8. Other .....

What type of fuel did you MOSTLY use in your home for keeping warm **20 years ago?** *(please tick)*

1. Wood	2. Charcoal	3. Coal	4. Anthracite	5. Paraffin	6. Gas	7. Electricity

8. Other .....

In any of the houses you lived in, did the smoke from cooking or keeping warm ever make your eyes water? *(please tick)*

1 | Yes, more than 5 years | No | 2

Habits

Have you ever smoked cigarettes or a pipe regularly? *(please tick)*

1. Yes (now)	2. In the past	3. Never

If yes: In the past five to ten years, how many would you usually smoke in a day?

(number of)

1. Cigarettes	2. Hand rolled cigarettes	3. Pipes

How old were you when you first started smoking regularly? ☐☐ Years old ☐☐

If you have stopped smoking, how old were you when you stopped? ☐☐ Years old ☐☐

Have you ever used snuff? *(please tick)*

1. Yes (now)	2. In the past	3. Never

Age commenced ☐☐ Years ☐

In the past five to ten years, how often would you use snuff each day? ☐☐ times per day ☐☐

Have you ever used chewing tobacco? *(please tick)*

1. Yes (now)	2. In the past	3. Never

Age commenced ☐☐ Years ☐

Age stopped ☐☐ Years

Have you ever smoked and used alcohol together? *(please tick)*

1. Yes (now)	2. In the past	3. Never

Age commenced ☐☐ Years ☐

Before you became ill, how often did you drink? *(please tick)*

	1 Most days	2 More than once a week	3 Less than once a week	4. Never	5 Unknown
Maize beer					
Sorghum beer					
Other homemade beer					
Commercial beer					
Homemade spirits					
Commercial spirits					
Wine					
Other (specify)					

Dietary information ---- Interviewer to show photographs of mycotoxin infected maize

20 years ago, how often would you eat:

1. All year round,
2. When in season

	1 Most days	2. More than once a week	3. Less than once a week	4 Never	5 unknown	1	2		
Sorghum									
Maize									
Green, leafy vegetables									
Green pod vegetables									
Imifino (wild/veld greens)									
Orange/Yellow vegs.									
Beans in Umngqusho									
Fruit									
Milk									
Margarine or butter									
Pickled Food									
Meat (beef/mutton/pork/ chicken/mince/sausage)									
Fish									

1 Yes | No 2 For how long? Yrs

1 Yes | No 2 For how long? Yrs

Have you ever lived in a house which had a granary for:

Maize 1 Yes | No 2

Sorghum 1 Yes | No 2

Other grain 1 Yes | No 2

Did you ever eat mielies that looked like this? *(show picture)*

1 Yes | No 2 For how long? ☐☐ Yrs

Did your family ever make beer out of

Stored Maize 1 Yes | No 2

Stored Sorghum 1 Yes | No 2

Other 1 Yes | No 2

Did your family ever prepare beer from maize that looked like this? *(show picture)*

1 Yes | No 2 For how long? ☐☐ Yrs

Are you? *(please tick)*

1 ☐ Single (never been married) 2 ☐ Married (living as married)

3 ☐ Widowed 4 ☐ Separated

How many husbands/wives have you had? ☐☐

How many children (dead or alive) do you have? ☐☐

How many mothers/fathers do the children have? ☐☐

How old is your oldest child now? *(include dead and alive)* ☐☐ Years old

How old is your youngest child now? *(include dead and alive)* ☐☐ Years old

How many boyfriends/girlfriends have you had? ☐☐

FOR WOMEN ONLY:

How old were you when your periods began? ☐☐ Years old ☐☐

Have your periods ended? *(please tick)* 1 Yes | No 2 ☐

If yes, how old were you when they ended? ☐☐ Years old ☐☐

Have you ever taken oral contraceptives? *(please tick)* 1 Yes | No 2 ☐

If yes, how old were you when you started taking them? ☐☐ Years old ☐☐

How old were you when you stopped taking them? ☐☐ Years old ☐☐

If yes, for how long in total did you take them? ☐☐ Years old ☐☐

Have you ever used an injectable contraceptive? (e.g. depo-provera) *(please tick)* 1 Yes | No 2 ☐

If yes, how old were you when you started using it? ☐☐ Years old ☐☐

How old were you when you stopped using it? ☐☐ Years old ☐☐

If yes, for how long in total did you take them? ☐☐ Years old ☐☐

Have you ever been pregnant? *(please tick)* 1 Yes | No 2 ☐☐

If yes, how many times have you been pregnant? ☐☐ ☐☐

If yes, how many times have you had a miscarraige? ☐☐ ☐☐

If yes, how many of your children were born alive? ☐☐ ☐☐

APPENDIX C

CONSENT

1 I, (Name _____

Hereby certify that I give concent to the collection of samples of blood, urine and biopsy (X3) for the Cancer of the Oesophagus Research Project.

2 I have been informed _____

concerning the research project and understand that the specimen I donate is for research and may not be used for my treatment.

3 I give my concent freely and I also realise that I can recall my consent at any time, without prejudice

1 Mina (Igama) _____

ngiyavumelana nokuthi kuthathwe igazi, umchamo kanye nezicutshana ezintathu okuzosetshenziswa kuCwaningo Lomdlavuza Womqala.

2 Ngitsheliwe ngu _____

mayelana nalolucwaningo futhi ngiyaqonda ukuthi lokhu enginikeza imvume ukuthi kuthathwe kimi kuzosetshenziselwa ucwaningo hayi ukungelapha.

3 Nginikeza imvume ngokukhululeka futhi ngiyazi ukuthi ngingenqaba noma nini uma ngithanda futhi akekho oyongiphatha kabi ngokwenza kanjalo

SIGNED /YASAYINWA **DATE/USUKU**

_____ _____

PATIENT/ISIGULI

_____

PERSON WHO INFORMED PATIENT/UMUNTU OWAZISA ISIGULI

APPENDIX D

TABLE 4-BIOPSIES AND RESECTIONS -OESOPHAGEAL CARCINOMA

Patient Number	Hospital Number	Histological grading
1	1496/98	Infiltrating moderately differentiated squamous cell carcinoma
2	1094/98	Normal epithelium
3	138/98	Normal epithelium
4	1501/98	Invasive moderately differentiated squamous cell carcinoma
5	2006/98	Invasive moderately differentiated squamous cell carcinoma
6	1096/98	Invasive moderately differentiated squamous cell carcinoma
7	1733/98	Invasive moderately differentiated squamous cell carcinoma
8	501/98	Invasive moderately differentiated squamous cell carcinoma
9	1515/98	Infiltrating moderately differentiated squamous cell carcinoma
10	1546/98	Infiltrating moderately differentiated squamous cell carcinoma
11	1645/98	No tumour-ulcerated oesophageal mucosa
12	1997/98	Inflammation with oesophageal ulceration
13	2216/98	Poorly differentiated squamous cell carcinoma.
14	130/98	Invasive moderately differentiated squamous cell carcinoma
15	1746/98	Invasive moderately differentiated keratinising squamous cell carcinoma
16	1662/98	Invasive well differentiated squamous cell carcinoma
17	1838/98	Invasive moderately differentiated squamous cell carcinoma
18	2439/98	Infiltrating moderately differentiated squamous cell carcinoma
19	1898/98	Invasive moderately differentiated squamous cell carcinoma
20	248/98	Infiltrating moderately differentiated squamous cell carcinoma
21	732/98	Poorly differentiated squamous cell carcinoma.
22	359/98	Normal epithelium with inflammation
23	1659/98	Infiltrating moderately differentiated squamous cell carcinoma
24	1749/98	Invasive moderately differentiated squamous cell carcinoma

25	T394/98	Inflammation with oesophageal ulceration
26	1744/98	Invasive moderately differentiated keratinising squamous cell carcinoma
27	504/98	Infiltrating moderately differentiated squamous cell carcinoma
28	2026/98	Invasive moderately differentiated squamous cell carcinoma
29	98086903	Invasive moderately differentiated keratinising squamous cell carcinoma
30	1745/98	Infiltrating moderately differentiated squamous cell carcinoma
31	2068/98	Invasive moderately differentiated squamous cell carcinoma
32	135/98	Invasive moderately differentiated squamous cell carcinoma
33	3177/97	Invasive well differentiated squamous cellcarcinoma
34	249/98	Invasive moderately differentiated squamous cell carcinoma
35	247/98	Infiltrating moderately differentiated squamous cell carcinoma
36	4575/98	Invasive mooderately differentiated squamous cell carcinoma
37	401/98	Invasive moderately differentiated squamous cell carcinoma
38	706/98	Invasive well differentiated squamous cell carcinoma
39	5982954	Moderately differentiated infiltrating squamous cell carcinoma
40	5976278	Infiltrating moderately differentiated squamous cell carcinoma
41	5982794	Infiltrating moderately differentiated squamous cell carcinoma
42	5974836	Infiltrating moderately differentiated squamous cell carcinoma
43	960/99	Invasive moderately differentiated squamous cell carcinoma
44	962/99	Invasive moderately differentiated squamous cell carcinoma
45	98126013	Infiltrating moderately differentiated squamous cell carcinoma
46	875/99	Infiltrating moderately differentiated squamous cell carcinoma
47	780/99	Moderately differentiated infiltrating squamous cell carcinoma
48	821/99	Invasive moderately differentiated squamous cell carcinoma
49	98119859	Invasive moderately differentiated squamous cell carcinoma
50	98116341	Invasive moderately differentiated squamous cell carcinoma

APPENDIX E

Table .1-Fresh Biopsies

Patient Number	Sex	Age	Family history	Eyes water	Smoking	Smoking & Alcohol	Infected Maize	Infected Beer
1	F	63	875/99	5982954	960/99	5976278	960/99	5982954
2	M	53		5974836	962/99	960/99		5982794
3	F	57		960/99	875/99	875/99		5974836
4	F	78		98126013	780/99	821/99		960/99
5	M	70		875/99	98119859	98119859		98126013
6	M	62		780/99	98116341			780/99
7	F	63		98116341				98119859
8	M	48		98081863				98116341
9	M	62						
10	M	75						
11	M	36						
12	M	44						
13	F	55						

APPENDIX F

Table 1.1 OESOPHAGEAL CARCINOMA (URINE SAMPLES)

Patient Number	Hospital Number	Age	Sex	TK ELISA [TK]	TK Activity	Kinin Elisa [kinin] generated	basal
1	2172/98	68	M	266.86	1.3	16.4	4.1
2	2568/98	65	M	211.58	0.81	42.2	3
3	2556/98	54	M	224.92	0.43	39.6	7.2
4	2562/98	56	M	168.01	0.33	13.9	5.8
5	3009/98	53	M	260.19	0.54	19.4	5.7
6	2026/98	68	M	246.96	0.07	22.8	8.3
7	1997/98	42	M	196.81	0.13	18.6	6.6
8	2284/98	40	M	141.12	0.17	22.6	7.6
9	1501/98	69	M	195.86	0.09	23.1	7.7
10	1470/98	81	M	93.88	0.14	51.9	18.3
11	1746/98	52	M	284.73	1.54	33.7	7.5
12	1096/98	45	M	186.8	0.75	30.9	5.6
13	1515/98	58	M	265.43	0.99	21.5	6.4
14	2068/98	47	M	129.26	1.47	30.8	9.7
15	130/98	55	M	233.98	0.58	14.5	4.3
16	1733/98	63	M	226.95	0.81	7.1	3
17	1546/98	52	M	267.22	0.01	19.6	7.8
18	1094/98	55	M	210.87	0.7	19.2	8.4
19	1501/98	69	M	253.04	2.66	20.1	9.3
20	1893/98	57	M	257.57	0.15	30.2	11.1
21	2138/98	68	M	246.84	1.23	34.8	11.2
22	96/98	49	M	200.62	0.37	16.3	7.6
23	1496/98	49	M	246.61	1.53	25.8	6.4
24	2554/98	49	M	202.29	1.55	14.4	6.2
25	2574/98	46	M	165.24	1.08	15.6	6.3
26	980341019	35	M	218.73	0.85	29.7	13.6
27	980020395	57	F	112.7	0.76	28.1	11.4
28	980360722	36	F	245.3	0.13	41	10
29	980021758	66	F	181.92	1.76	27.3	7.6
30	980046432	49	F	160.71	2.17	21.8	7
31	980306167	51	F	237.79	0.76	23.7	6.3
32	980370132	50	M	206.1	0.14	5	2.9
33	980367301	54	F	216.47	0.11	44.8	17.6
34	98075204	57	F	214.32	1.03	19.4	6.2
35	98100811	63	F	177.51	0.82	24.7	5.8
36	98078447	41	M	146.77	1.57	16.7	4.1
37	98097950	43	M	207.65	1.12	28.2	2.7
38	98087317	55	M	282.82	0.81	16.3	5.4
39	7699/98	65	F	191.33	0.35	28.9	11.5

40	980333636	67	F	122.59	0.08	30.8	6.4
41	709/99	56	M	170.36	0.37	27.4	8.6
42	710/99	53	M	305.58	0.88	37.8	7
43	98117518	57	M	22.04	1.56	54.6	12.4
44	98103809	82	M	16.8	0.43	33.1	5.7
45	98114683	71	M	124.02	1.83	51.4	5.6
46	98119859	36	M	11.08	0.2	43.5	8.6
47	98118679	39	F	6.91	0.17	36.9	10.6
48	98111731	69	F	144.03	1.66	18.6	4.5
49	98105683	52	F	298.07	1.83	50.1	14.5
50	98106722	55	F	3.81	0.12	25.3	7
51	98105773	53	M	22.64	0.06	48.7	16.4
52	98116071	71	M	43.37	0.03	32.4	10.5
53	98112189	59	F	290.8	0.72	52.9	14.4
54	98105582	59	M	34.43	0.02	36.9	10.7
55	98115145	47	M	10.48	0.1	26.7	19.1
56	980369304	42	F	203.12	0.74	38	4.8
57	580/98	49	M	196.09	1.03	49.5	17.5
58	6761/98	66	F	232.91	0.59	29.2	6.1
59	98124834	60	F	29.55	1.06	36.8	6.6
60	98124436	60	F	309.03	0.1	37.7	15.7
61	98121611	58	M	128.31	1.95	27.6	6.1
62	98119768	62	M	261.86	0.35	38.1	4.8
63	98124704	58	F	91.5	0.41	27.9	7.4
64	821/99	75	M	6.79	0.94	28.8	5.1
65	98123135	68	F	23.35	0.22	33.6	21
66	98126013	63	F	15.48	1.69	42.2	11.2
67	98126978	51	M	10.6	0.46	53.8	28.1
68	780/99	62	M	13.82	0.04	34.9	24.3
69	98124742	57	M	302	0.84	46	10.7
70	869/99	70	M	21.8	0.04	46.3	17.3
71	43619	63	F	25.97	1.2	32.7	11.7
72	98134220	46	F	189.06	1.63	54	11.7
73	98136285	70	F	174.89	1.26	53.8	24.6
74	981140048	70	F	174.53	0.94	53.8	16
75	98130506	60	F	173.58	0.23	35.9	8.3
76	98129153	44	F	171.67	0.16	39.5	11.5
77	98034194	67	F	160.71	0.38	29.5	7.9
78	98133398	67	F	177.51	0.5	37.3	22.5
79	5982794	57	F	159.04	0.58	18.7	7.2
80	98129266	49	F	163.93	1.23	49.5	14.6
81	5982883	47	F	148.56	9.14	22.5	8.2
82	5982954	63	F	119.37	7.84	26.9	4.9
83	98134445	64	F	146.05	0.88	8.3	4.7

84	98134181	43	F	153.92	0.37	25.8	13.9
85	5928325	53	F	43.6	0.08	47	12.9
86	98117073	46	F	143.44	0.01	21.6	8.2
87	209/98	45	F	127	0.27	29.8	5.9

Table 1.1 1 FAMILY HISTORY-CA OESOPHAGUS (URINE SAMPLES)

Pat. No.	Hosp. No.	[TK] (ng TK/ml sample)	TK activity (ng TK/ug Protein	Kinin ELISA [Kinins] Generated	Basal
1	3009/98	260.19	0.54	19.4	5.7
2	1515/98	265.43	1	21.5	6.4
3	2068/98	129.26	1.47	30.8	9.7
4	130/98	233.98	0.58	14.5	4.3
5	1733/98	226.95	0.81	7.1	3
6	1546/98	267.21	0	19.6	7.8
7	96/98	200.62	0.37	16.3	7.6
8	980020395	112.7	0.76	28.1	11.4
9	980360722	245.3	0.13	41	10
10	980021758	181.92	1.76	27.3	7.6
11	980046432	160.71	2.17	21.8	7
12	980367301	216.47	0.12	44.8	17.6
13	7699/98	191.33	0.35	28.9	11.5
14	580/98	196.09	1.04	49.5	17.5
15	6761/98	242.91	0.59	29.2	6.1
16	98124834	29.55	1.06	36.8	6.6
17	98124436	309.03	0.1	37.7	15.7

Table 1.1.2 EYES WATER-CA OESOPHAGUS (URINE SAMPLES)

Pat.No.	Hosp.No	[TK] (ng TK/ml sample)	TK activity (ng TK/ug Protein)	Kinin ELISA [Kinins] Generated	Basal
1	2172/98	266.84	1.3	16.4	4.1
2	2568/98	211.58	0.81	42.2	3
3	2556/98	224.92	0.43	39.6	7.2
4	2562/98	168.1	0.33	13.9	5.8
5	3009/98	260.19	0.54	19.4	5.7
6	2026/98	246.96	0.07	22.8	8.3

7	1997/98		196.81			0.57		18.6	6.6
8	2284/98		141.17			0.17		22.6	7.6
9	1501/98		195.86			0.09		23.1	7.7
10	1470/98		93.88			0.14		51.9	18.3
11	1746/98		284.73			1.54		33.7	7.5
12	1096/98		186.8			0.75		30.9	5.6
13	1515/98		265.43			1		21.5	6.4
14	2068/98		129.26			1.47		30.8	9.7
15	130/98		233.98			0.58		14.5	4.3
16	1733/98		226.95			0.81		7.1	3
17	1546/98		267.21			0.001		19.6	7.8
18	1094/98		210.87			0.7		19.2	8.4
19	1501/98		253.04			2.66		20.1	9.3
20	1893/98		257.57			0.15		30.2	11.1
21	2138/98		246.84			1.8		34.8	11.2
22	96/98		200.62			0.37		16.3	7.6
23	1496/98		246.61			1.63		25.8	6.4
24	2554/98		202.29			1.55		14.4	6.2
25	2574/98		165.24			1.08		15.6	6.3
26	980341019		218.73			0.85		29.7	13.6
27	980360722		245.3			0.13		41	10
28	980046432		160.71			2.17		21.8	7
29	980306167		237.79			0.78		23.7	6.3
30	980370132		206.1			0.14		5	2.9
31	980367301		216.47			0.12		44.8	17.6
32	98078447		146.77			1.57		16.7	4.1
33	980333636		122.59			0.08		30.8	6.4
34	98123135		23.35			0.22		33.6	21
35	98126013		15.49			1.69		42.2	11.2
36	98126978		10.6			0.46		53.8	28.1
37	780/99		13.82			0.04		34.9	24.3
38	98124704		91.5			0.41		27.9	7.4
39	98105582		34.43			0.02		36.9	10.7
40	98115145		10.49			0.1		26.7	19.1
41	980369304		203.12			0.74		38	4.8
42	580/98		196.09			1.04		49.5	17.5
43	6761/98		242.91			0.59		29.2	6.1
44	98124834		29.55			1.06		36.8	6.6
45	98105773		22.64			0.06		48.7	16.4
46	98116071		43.37			0.03		32.4	10.5
47	98105683		298.07			1.83		50.1	14.5
48	98103809		16.8			0.43		33.1	5.7
49	98136285		174.89			1.26		53.8	24.6
50	981146048		174.53			0.94		53.8	16

51	98130506		173.58			0.24		35.9	8.3
52	98034194		160.71			0.38		29.5	7.9
53	98129266		163.93			1.23		49.5	14.6
54	5982883		148.56			9.1		22.5	8.2
55	5982954		119.37			7.84		26.9	4.9
56	98134181		153.92			0.37		25.8	13.9
57	5928325		43.6			0.08		47	12.9
58	209/98		127			0.27		29.8	5.9

Table 1.1.3 SMOKING -CA OESOPHAGUS (URINE SAMPLES)

Pat.No.	Hosp.No		[TK] (ng TK/ml sample)		TK activity (ng TK/ug Protein)		Kinin ELISA [Kinins] Generated	Basal
1	2172/98		266.84		1.3		16.4	4.1
2	2568/98		211.58		0.81		42.2	3
3	2556/98		224.92		0.43		39.6	7.2
4	3009/98		260.19		0.54		19.4	5.7
5	1997/98		196.81		0.57		18.6	6.6
6	2284/98		141.17		0.17		22.6	7.6
7	1501/98		195.86		0.09		23.1	7.7
8	1470/98		93.88		0.14		51.9	18.3
9	1746/98		284.73		1.54		23.7	7.5
10	1096/98		186.8		0.75		33.7	7.5
11	1515/98		265.43		1		30.9	5.5
12	2068/98		129.26		1.47		30.8	9.7
13	130/98		233.98		0.58		14.5	4.3
14	1733/98		226.95		0.81		7.1	3
15	1546/98		267.21		0		19.6	7.8
16	1501/98		253.04		2.66		20.1	9.3
17	1893/98		257.57		0.15		30.2	11.1
18	2138/98		246.84		1.8		34.8	11.2
19	1496/98		246.61		1.63		25.8	6.4
20	2554/98		202.29		1.55		14.4	6.2
21	2574/98		165.24		1.08		15.6	6.3
22	980021758		181.92		1.76		27.3	7.6
23	980370132		206.1		0.14		5	2.9
24	98078447		146.77		1.57		16.7	4.1
25	98097950		207.65		1.12		28.2	2.7
26	98087317		207.53		0.81		16.3	5.4
27	709/99		170.36		0.37		27.4	8.6

28	710/99		305.58		0.88	37.8	7
29	98117518		22.04		0.54	54.6	12.4
30	98105683		298.07		1.83	51.4	5.6
31	98103809		16.8		0.43	33.1	5.7
32	98119859		11.08		0.2	43.5	8.6
33	98105773		22.64		0.06	48.7	16.4
34	98116071		43.37		0.03	32.4	10.5
35	98105582		34.43		0.02	36.9	10.7
36	98115145		10.49		0.1	26.7	19.1
37	98121611		128.3		0.02	27.6	6.1
38	98224742		302		0.84	46	10.7
39	869/99		21.8		0.04	46.3	17.3
40	98126978		10.6		0.46	53.8	28.1
41	780/99		13.82		0.04	34.9	24.3
42	98114683		124.02		0.31	51.4	5.6
43	98119768		261.86		0.35	38.1	4.8
44	98130506		173.58		0.23	35.9	8.3
45	98129153		171.67		0.16	39.5	11.5
46	5982794		159.04		0.58	18.7	7.2
47	98134445		146.06		0.88	8.3	4.7
48	98134181		153.92		0.37	25.8	13.9
49	5928325		43.6		0.08	47	12.9

Table 1.1.4 SMOKING + ALCOHOL-CA OESOPHAGUS (URINE SAMPLES)

Pat.No	Hosp.No.		[TK] (ng TK/ml sample)		TK activity (ng TK/ug Protein)		Kinin ELISA [Kinins] Generated	Basal
1	2172/98		266.84		1.3		16.4	4.1
2	2562/98		168.1		0.33		42.2	3
3	3009/98		260.19		0.54		19.4	5.7
4	1997/98		196.81		0.57		18.6	6.6
5	1501/98		195.86		0.09		23.1	7.7
6	1470/98		93.88		0.14		51.9	18.3
7	1746/98		284.73		1.54		33.7	7.5
8	1515/98		265.43		1		21.5	6.4
9	2068/98		129.26		1.47		30.8	9.7
10	1733/98		226.95		0.81		7.1	3
11	1546/98		267.21		0		19.6	7.8
12	1501/98		253.04		2.66		20.1	9.3

13	2138/98		246.84		1.8		34.8	11.2
14	1496/98		246.61		1.63		25.8	6.4
15	2554/98		202.29		1.55		14.4	6.2
16	2574/98		165.24		1.08		15.6	6.3
17	980021758		181.92		1.76		27.3	7.6
18	980370132		206.1		0.14		5	2.9
19	98078447		146.77		1.57		16.7	4.1
20	98097950		207.65		1.12		28.2	2.7
21	98087317		207.53		0.81		16.3	5.4
22	980333636		122.59		0.08		30.8	6.4
23	98114683		124.02		0.31		51.4	5.6
24	869/99		21.8		0.04		46.3	17.3
25	98224742		302		0.84		46	10.7
26	98115145		10.49		0.1		26.7	19.1
27	98105582		34.43		0.02		36.9	10.7
28	98105773		22.64		0.06		48.7	16.4
29	98116071		43.37		0.03		32.4	10.5
30	98119859		11.08		0.2		43.5	8.6
31	98105683		298.07		1.83		51.4	5.6
32	709/99		170.36		0.37		27.4	8.6
33	710/99		305.58		0.88		37.8	7
34	98117518		22.04		1.56		54.6	12.4
35	980360722		245.3		0.13		25.3	7
36	98124704		91.5		0.41		27.9	7.4
37	98124436		309.03		0.1		37.7	15.7
38	98130506		173.58		0.24		35.9	8.3
39	98129153		171.67		0.16		39.5	11.5
40	5982794		159.04		0.58		18.7	7.2
41	98134445		146.06		0.88		8.3	4.7
42	98134181		153.92		0.37		25.8	13.9
43	5928325		43.6		0.08		47	12.9

Table 1.1.5 INFECTED MAIZE-CA OESOPHAGUS (URINE SAMPLES)

Pat.No.	Hosp.No	[TK] (ng TK/ml sample)	TK activity (ng TK/ug Protein)	Kinin ELISA [Kinins] Generated	Basal
1	3009/98	260.19	0.54	19.4	5.7
2	1501/98	195.86	0.09	23.1	7.7
3	1746/98	284.73	1.54	33.7	7.5
4	1096/98	186.8	0.75	30.9	5.6
5	1515/98	265.43	1	21.5	6.4
6	130/98	233.98	0.58	14.5	4.3
7	1733/98	226.95	0.81	7.1	3
8	1546/98	267.21	0	19.6	7.8
9	1094/98	210.87	0.7	19.2	8.4
10	1501/98	253.04	2.66	20.1	9.3
11	980020395	112.7	0.76	28.1	11.4
12	980021758	181.92	1.76	27.3	7.6
13	980046432	160.71	2.17	21.8	7
14	7699/98	191.33	0.35	28.9	11.5
15	980360722	245.3	0.13	41	10
16	580/98	196.09	1.04	49.5	17.5
17	6761/98	242.91	0.59	29.2	6.1

Table 1.1.6 INFECTED BEER-CA OESOPHAGUS (URINE SAMPLES)

Pat.No.	Hosp.No.	[TK] (ng TK/ml sample)	TK activity (ng TK/ug Protein)	Kinin ELISA [Kinins] Generated	Basal
1	2172/98	266.84	1.3	16.4	4.1
2	3009/98	260.19	0.54	19.4	5.7
3	2026/98	246.96	0.07	22.8	8.3
4	1997/98	196.81	0.57	18.6	6.6
5	1501/98	195.86	0.09	23.1	7.7
6	1096/98	186.8	0.75	30.9	5.6
7	1733/98	226.95	0.81	7.1	3
8	1546/98	267.21	0	19.6	7.8
9	1094/98	210.87	0.7	19.2	8.4
10	1501/98	253.04	2.66	20.1	9.3

11	2138/98		246.84		1.8	34.8	11.2
12	96/98		200.62		0.37	16.3	7.6
13	1496/98		246.61		1.63	25.8	6.4
14	980341019		218.73		0.85	29.7	13.6
15	980360722		245.3		0.13	41	10
16	980046432		160.71		2.17	21.8	7
17	980370132		206.1		0.14	23.7	6.3
18	980367301		216.47		0.12	44.8	17.6
19	98078447		146.77		1.57	16.7	4.1
20	7699/98		191.33		0.35	28.9	11.5
21	980333636		122.59		0.08	30.8	6.4
22	98124834		29.55		1.06	36.8	6.6
23	98105773		22.64		0.06	48.7	16.4
24	98116071		43.37		0.03	32.4	10.5
25	98105683		298.07		1.83	50.1	14.5
26	98103809		16.8		0.43	33.1	5.7
27	98105582		34.43		0.02	36.9	10.7
28	98115145		10.49		0.1	26.7	19.1
29	980369304		203.12		0.74	38	4.8
30	780/99		13.82		0.04	34.9	24.3
31	980360722		245.3		0.13	25.3	7
32	98119859		11.08		0.2	43.5	8.6
33	580/98		196.09		1.04	49.5	17.5
34	98123135		23.35		0.22	33.6	21
35	98126013		15.49		1.69	42.2	11.2
36	98111731		144.03		1.66	18.6	4.5
37	98118679		6.91		0.17	36.9	10.6
38	6761/98		242.91		0.59	29.2	6.1
39	98136285		174.89		1.26	53.8	24.6
40	98034194		160.71		0.38	29.5	7.9
41	98129266		163.93		1.23	49.5	14.6
42	5982883		148.56		9.14	22.5	8.2
43	5982954		119.37		7.84	26.9	4.9
44	5928325		43.6		0.08	47	12.9

TABLE 1.1.7 MALES AND AGE

Pat.No.	Hosp.No.	Age	[TK] (ng TK/ml sample)	TK activity (ng TK/ug Protein)	Kinin ELISA [Kinins] Generated	Basal
1	2172/98	68	266.86	1.3	16.4	4.1
2	2568/98	65	211.58	0.81	42.2	3
3	2556/98	54	224.92	0.43	39.6	7.2
4	2562/98	56	168.01	0.33	13.9	5.8
5	3009/98	53	260.19	0.54	19.4	5.7
6	2026/98	68	246.96	0.07	22.8	8.3
7	1997/98	42	196.81	0.57	18.6	6.6
8	2284/98	40	141.12	0.17	22.6	7.6
9	1501/98	69	195.86	0.09	23.1	7.7
10	1470/98	81	93.88	0.14	51.9	18.3
11	1746/98	52	284.73	1.54	33.7	7.5
12	1096/98	45	186.8	0.75	30.9	5.6
13	1515/98	58	265.43	0.99	21.5	6.4
14	2068/98	47	129.26	1.47	30.8	9.7
15	130/98	55	233.98	0.58	14.5	4.3
16	1733/98	63	226.95	0.81	7.1	3
17	1546/98	52	267.22	0.01	19.6	7.8
18	1094/98	55	210.87	0.7	19.2	8.4
19	1501/98	69	253.04	2.66	20.1	9.3
20	1893/98	57	257.57	0.15	30.2	11.1
21	2138/98	68	246.84	1.8	34.8	11.2
22	96/98	49	200.62	0.37	16.3	7.6
23	1496/98	49	246.61	1.53	25.8	6.4
24	2554/98	49	202.29	1.55	14.4	6.2
25	2574/98	46	165.24	1.08	15.6	6.3
26	980370132	50	206.1	0.14	5	2.9
27	98078447	41	146.77	1.57	16.7	4.1
28	709/99	56	170.36	0.37	27.4	8.6
29	710/99	53	305.58	0.88	37.8	7
30	98117518	57	22.04	1.56	54.6	12.4
31	98103809	82	16.8	0.43	33.1	5.7
32	98114683	71	124.02	1.83	51.4	5.6
33	98119859	36	11.08	0.2	43.5	8.6
34	98105773	53	22.64	0.06	48.7	16.4
35	98116071	71	43.37	0.03	32.4	10.5
36	98105582	59	34.43	0.02	36.9	10.7
37	98115145	47	10.48	0.1	26.7	19.1
38	580/98	49	196.09	1.03	49.5	17.5
39	98121611	58	128.31	0.02	27.6	6.1

40	98119768	62	261.86		0.35		38.1	4.8
41	821/99	75	6.79		0.94		28.8	5.1
42	98126978	51	10.6		0.46		53.8	28.1
43	780/99	62	13.82		0.04		34.9	24.3
44	98124742	57	302		0.84		46	10.7
45	869/99	70	21.8		0.04		46.3	17.3
46	98097950	43	207.65		1.12		28.2	2.7
47	98087317	55	282.82		0.81		16.3	5.4

TABLE 1.1.8 FEMALES AND AGE

Pat.No.	Hosp.No.	Age	[TK] (ng TK/ml sample)		TK activity (ng TK/ug Protein)		Kinin ELISA [Kinins] Generated	Basal
1	980360722	36	245.3		0.13		41	10
2	980021758	66	181.92		1.76		27.3	7.6
3	980046432	49	160.71		2.17		21.8	7
4	980306167	51	237.79		0.76		23.7	6.3
5	980367301	54	216.47		0.11		44.8	17.6
6	98075204	57	214.32		1.03		19.4	6.2
7	98100811	63	177.51		0.82		24.7	5.8
8	980341019	35	218.73		0.85		29.7	13.6
9	980020395	57	112.7		0.76		28.1	11.4
10	7699/98	65	191.33		0.35		28.9	11.5
11	980333636	67	122.59		0.08		30.8	6.4
12	98118679	39	6.91		0.17		36.9	10.6
13	98111731	69	144.03		1.66		18.6	4.5
14	98105683	52	298.07		1.83		50.1	14.5
15	98106722	55	3.81		0.12		38	4.8
16	98112189	59	290.8		0.72		52.9	14.4
17	980369304	42	203.12		0.74		38	4.8
18	6761/98	66	232.91		0.59		29.2	6.1
19	98124834	60	29.55		1.06		36.8	6.6
20	98124436	60	309.03		0.1		37.7	15.7
21	98124704	58	91.5		0.41		27.9	7.4
22	98123135	68	23.35		0.22		33.6	21
23	98126013	63	15.48		1.69		42.2	11.2
24	43619	63	25.97		1.2		32.7	11.7
25	98134220	46	189.06		1.63		54	11.7
26	98136285	70	174.89		1.26		53.8	24.6
27	981140048	70	174.53		0.94		53.8	16
28	98130506	60	173.58		0.23		35.9	8.3

29	98129153	44	171.67		0.16		39.5	11.6
30	98034194	67	160.71		0.38		29.5	7.9
31	98133398	67	177.51		0.5		37.3	22.5
32	5982794	57	159.04		0.58		18.7	7.2
33	98129266	49	163.93		1.23		49.5	14.6
34	5982883	47	148.56		9.14		22.5	8.2
35	5982954	63	119.37		7.84		26.9	4.9
36	98134445	64	146.05		0.88		8.3	4.7
37	98134181	43	153.92		0.37		25.8	13.9
38	5928325	53	43.6		0.08		47	12.9
39	98117073	46	143.44		0.01		21.6	8.2
40	209/98	45	127		0.27		29.8	5.9

Table 2.1 NON - CANCER (URINE SAMPLES)

Patient No	Hospital No	Age	Sex	TK ELISA [TK]	TK Activity	Kinin Elisa [kinin] Generated	basal
1	97009618	33	M	237.08	1.81	20.2	4.4
2	98090507	30	M	91.38	0.29	34.9	4.9
3	98102528	20	F	154.76	0.02	24.6	9
4	85082583	36	F	191.69	0.53	17.1	5
5	98076789	37	F	231.36	5.77	23	4.4
6	98102828	27	M	220.99	0.16	22.1	5
7	98098151	46	M	292.59	0.64	26.3	7.4
8	98101121	27	F	256.62	1.06	38	4.8
9	98104668	50	F	213.49	0.49	49.5	17.5
10	98105212	30	F	263.64	2.14	29.2	6.1
11	88032233	53	F	112.22	0.17	36.8	6.6
12	98108397	51	F	255.3	0.47	37.7	15.7
13	98076119	25	M	156.18	1.09	27.6	6.1
14	98036019	71	M	64.1	0.75	38.1	4.8
15	98075791	51	F	168.69	0.46	27.9	7.4
16	98089103	31	F	200.03	1.48	28.8	5.1
17	98116367	59	F	229.21	0.5	33.6	21
18	98036440	64	F	229.21	0.5	42.2	11.2
19	98106253	65	F	213.37	0.3	53.8	28.1
20	98116296	35	F	205.86	1.25	34.9	24.3
21	980359147	51	M	234.1	0.15	46	10.7
22	98043135	18	F	171.08	0.14	46.3	17.3
23	980332460	42	M	224.33	5.91	32.7	11.7
24	98036872	33	F	230.76	1.16	54	11.7
25	98033802	61	F	200.03	4.27	53.8	24.6
26	89028533	40	F	223,26	0.14	53.8	16
27	960521295	44	F	241.25	3.27	35.9	8.3
28	980338026	61	F	200.03	4.27	39.5	11.5
29	980344158	59	F	127.12	1.55	29.5	7.9
30	98046363	38	F	252.09	8.49	37.3	22.5
31	980092810	71	M	47.42	0.01	18.7	7.2
32	98041574	46	F	100.31	0.48	49.5	14.6
33	98124486	46	M	179.77	1.67	22.5	8.2
34	98123682	27	M	89.35	0.63	26.9	4.9

35	98107406	31	F	94.59	0.02	8.3	4.7
36	98086635	36	M	82.68	0.36	25.8	13.9
37	98046345	20	F	61.95	2.18	47	12.9
38	98121765	31	F	70.89	0.3	21.6	8.2
39	98098506	27	F	142.96	1.83	29.8	5.9
40	98062254	70	M	68.26	0.34	35.5	3.5
41	98105731	35	M	118.18	0.11	19.3	6.1
42	98093276	55	F	94.24	1.21	38	4.3
43	98069569	19	F	122.95	0.71	40.9	20.4
44	980431344	58	F	249.35	0.21	18.3	2.7
45	98130746	49	M	120.44	0.52	27.7	8.5
46	98130008	40	F	153.44	0.75	10.8	1.6
47	98129367	48	F	179.06	1.21	29.5	15.3
48	98121267	26	F	161.9	1.55	37.6	11.2
49	98135426	41	F	112.7	0.12	38.7	4.8
50	98124133	25	F	156.42	0.07	25.4	15.8
51	98135945	59	M	159.28	0.43	39.7	8.1
52	96053651	66	F	148.44	1.81	23.5	3.8
53	98140161	75	F	71.84	0.05	33.1	5.7
54	98139114	46	M	170	0.87	31.8	6.3
55	98139317	49	F	88.76	0.68	33.3	5.6
56	98132541	48	F	4.29	0.52	33.7	7.5
57	98094456	41	M	75.41	3	30.9	5.6
58	98042539	44	F	21	0.3	21.5	6.4
59	98078607	46	M	151.6	0.67	30.8	9.7
60	98114286	51	M	98.32	0.47	34.8	11.2

Table 2.1 1 FAMILY HISTORY-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No		[TK] (ugTK/ml sample)		TK Activity (ugTK/ugProtein)		Kinin ELISA [Kinins] Generated	Basal
1	98104668		213		0.49		49.5	17.5
2	98046345		62		2.18		47	12.9
3	98130746		120		0.52		27.7	8.5

TABLE 2.1.2 EYES WATER-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No	[TK] (ugTK/ml sample)	TK Activity (ugTK/ugProtein)	Kinin ELISA [Kinins] Generated	Basal
1	98102828	221	0.16	22.1	5
2	88032233	112	0.17	36.8	6.6
3	98108397	255	0.47	37.7	15.7
4	98075791	169	0.46	27.9	7.4
5	98106253	213	0.3	53.8	28.1
6	98043135	171	0.14	46.3	17.3
7	89028533	223	0.14	53.8	16
8	98046363	252	8.49	37.3	22.5
9	980092810	47.4	0.01	18.7	7.2
10	98123682	89.4	0.63	26.9	4.9
11	98105731	118	0.11	19.3	6.1
12	98098506	143	1.83	29.8	5.9
13	98130746	120	0.52	27.7	8.5
14	98135426	113	0.12	38.7	4.8
15	98124133	156	0.07	25.4	15.8
16	98139114	170	0.87	31.8	6.3
17	98094456	75.4	3	30.9	5.6
18	98114286	21	0.32	34.8	11.2

Table 2.13 SMOKING -NON-CANCER (URINE SAMPLES)

Patient No	Hospital No	[TK] (ugTK/ml sample)	TK Activity (ugTK/ugProtein)	Kinin ELISA [Kinins] Generated	Basal
1	97009618	237	1.81	20.2	4.4
2	98090507	91.4	0.29	34.9	4.9
3	98098151	293	0.64	26.3	7.4
4	98104668	213	0.49	49.5	17.5
5	98076119	156	1.09	27.6	6.1

6	980359147		234		0.15		46	10.7
7	980332460		224		5.91		32.7	11.7
8	98076789		231		5.77		23	4.4
9	98124486		180		1.67		22.5	8.2
10	98123682		89.4		0.63		26.9	4.9
11	98062254		68.3		0.34		35.5	3.5
12	98105731		118		0.11		19.3	6.1
13	98130746		120		0.52		27.7	8.5
14	98139114		170		0.87		31.8	6.3
15	98139317		88.8		0.68		33.3	5.6
16	98094456		75.4		0.55		30.9	5.6

Table 2.1.4 SMOKING+ALCOHOL-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No		[TK] (ugTK/ml sample)		TK Activity (ugTK/ugProtein)		Kinin ELISA [Kinins] Generated	Basal
1	97009618		237		1.81		20.2	4.4
2	98090507		91.4		0.29		34.9	4.9
3	98098151		293		0.64		26.3	7.4
4	98102828		221		0.16		22.1	5
5	98076119		156		1.09		27.6	6.1
6	98108397		255		0.47		37.7	15.7
7	980332460		224		5.91		32.7	11.7
8	98075791		169		0.46		27.9	7.4
9	980092810		47.4		0.01		18.7	7.2
10	98124486		180		1.67		22.5	8.2
11	98105731		118		0.11		19.3	6.1
12	98123682		89.4		0.63		26.9	4.9
13	98130746		120		0.52		27.7	8.5
14	98135945		159		0.43		39.7	8.1
15	98139114		170		0.87		31.8	6.3
16	98139317		88.8		0.68		33.3	5.6

Table 2.1.5 INFECTED MAIZE-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No	[TK] (ugTK/ml sample)	TK Activity (ugTK/ugProtein)	Kinin ELISA [Kinins] Generated	Basal
1	98106253	213	0.3	53.8	28.1

Table 2.1.6 INFECTED BEER-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No	[TK] (ugTK/ml sample)	TK Activity (ugTK/ugProtein)	Kinin ELISA [Kinins] Generated	Basal
1	98106253	213	0.3	53.8	28.1
2	980332460	224	5.91	32.7	11.7
3	98046363	252	8.49	37.3	22.5
4	980092810	47.4	0.01	18.7	7.2
5	98123682	47.4	0.01	26.9	4.9
6	98086635	82.7	0.36	25.8	13.9
7	98130746	120	0.52	27.7	8.5
8	98124133	156	0.07	25.4	15.8
9	98139114	170	0.87	31.8	6.3
10	98094456	75.4	0.55	30.9	5.6

Table 2.1.7 MALES AND AGE-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No	Age	[TK] (ugTK/ml sample)	TK Activity (ugTK/ugProtein)	Kinin ELISA [Kinins] Generated	Basal
1	97009618	33	237.08	1.81	20.2	4.4
2	98090507	30	91.38	0.29	34.9	4.9
3	980332460	42	224.33	5.91	32.7	11.7
4	980359147	51	234.1	0.15	46	10.7
5	98102828	27	220.99	0.16	22.1	5
6	98036019	71	64.1	0.75	38.1	4.8
7	98098151	46	292.59	0.64	26.3	7.4
8	98076119	25	156.18	1.09	27.6	6.1
9	980092810	71	47.42	0.01	18.7	7.2
10	98124486	46	179.77	1.67	22.5	8.2
11	98123682	27	47.42	0.01	26.9	4.9
12	98086635	36	82.68	0.36	25.8	13.9
13	98062254	70	68.26	0.34	35.5	3.5
14	98105731	35	118.18	0.11	19.3	6.1
15	98130746	49	120.44	0.52	27.7	8.5
16	98135945	59	159.28	0.43	39.7	8.1
17	98139114	46	170	0.87	31.8	6.3
18	98094456	41	75.41	551.96	30.9	5.6
19	98078607	46	151.6	0.67	30.8	9.7
20	98114286	51	21	0.32	34.8	11.2

Table 2.1.8 FEMALES AND AGE-NON-CANCER (URINE SAMPLES)

Patient No	Hospital No	Age	[TK] (ugTK/ml sample)	TK Activity (ugTK/ugProtein)	Kinin ELISA [Kinins] Generated	Basal
1	98102528	20	154.76	0.02	24.6	9
2	85082583	36	191.69	0.53	17.1	5
3	98076789	37	231.36	5.77	23	4.4
4	98101121	27	256.62	1.06	38	4.8
5	98104668	50	213.49	0.49	49.5	17.5
6	98105212	30	263.64	2.14	29.2	6.1
7	88032233	53	112.22	0.17	36.8	6.6
8	98108397	51	255.3	0.47	37.7	15.7
9	98075791	51	168.69	0.46	27.9	7.4
10	98089103	31	200.03	1.48	28.8	5.1
11	98116367	59	229.21	0.5	33.6	21
12	98036440	64	229.21	0.5	42.2	11.2
13	98106253	65	213.37	0.3	53.8	28.1

14	98116296	35		205.86		1.25	34.9	24.3
15	98043135	18		171.08		0.14	46.3	17.3
16	98036872	33		230.76		1.16	54	11.7
17	98033802	61		200.03		4.27	53.8	24.6
18	89028533	40		223.26		0.14	53.8	16
19	960521295	44		241.25		3.27	35.9	8.3
20	980338026	61		200.03		4.27	39.5	11.5
21	980344158	59		127.12		1.55	29.5	7.9
22	98046363	38		252.09		8.49	37.3	22.5
23	98041574	46		100.31		0.48	49.5	14.6
24	98107406	31		94.59		0.02	8.3	4.7
25	98046345	20		61.95		2.18	47	12.9
26	98121765	31		70.89		0.3	21.6	8.2
27	98098506	27		142.96		1.83	29.8	5.9
28	98093276	55		94.24		1.21	38	4.3
29	98069569	19		122.95		0.71	40.9	20.4
30	980431344	58		249.35		0.21	18.3	2.7
31	98130008	40		153.44		0.75	10.8	1.6
32	98129367	48		179.06		1.21	29.5	15.3
33	98121267	26		161.9		1.55	37.6	11.2
34	98135426	41		112.7		0.12	38.7	4.8
35	98124133	25		156.42		0.07	25.4	15.8
36	96053651	66		148.44		1.81	23.5	3.8
37	98140161	75		71.84		0.05	33.1	5.7
38	98139317	49		88.76		0.68	33.3	5.6
39	98132541	48		4.29		0.52	33.7	7.5
40	98042539	44		21		0.3	21.5	6.4

Table 3.1 NON -OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No	Age	Sex	TK ELISA [TK]	TK Activity	Kinin Elisa [kinin] generated	basal
1	98101114	47	F	203.48	0.73	36.7	11
2	98101624	51	F	210.75	0.13	24.9	16.1
3	98096179	49	M	248.16	0.1	22.2	6.8
4	98015321	53	M	165.72	0.18	15.1	5.5
5	98108553	51	M	249.47	0.3	31.5	5.7
6	98104968	53	F	119.97	0.43	31	8
7	98101723	48	F	219.8	0.13	26.7	3.5
8	98114308	60	F	214.08	0.2	14.9	2.9
9	98108595	41	F	228.86	0.25	49.1	9.6
10	98115956	65	F	258.76	0.32	23	4.3
11	98103449	63	F	199.79	0.3	37.5	8.6
12	98112231	72	F	219.68	0.3	37.2	5.3
13	98111969	76	F	267.81	0.62	51.2	7.7
14	980419000	52	F	209.79	0.41	23.3	6.6
15	980333580	40	F	299.74	0.53	28.4	9.5
16	98049708	54	F	246.37	1.67	17.1	6.9
17	98033886	35	F	93.64	0.47	27.4	9.9
18	980422947	59	F	265.67	1.57	45.1	4.8
19	980339812	41	F	87.68	0.31	34.4	4.3
20	98046040	59	F	279.96	0.12	23.8	3.8
21	9802680	61	F	267.1	0.08	19.8	4.5
22	980434074	44	F	188.47	12.82	14.8	2.8
23	980424925	39	F	209.56	0.16	23.6	6.2
24	98012270	40	F	109.37	1.35	48.1	5.4
25	980293839	29	F	274.48	0.12	33.9	2.8
26	98050426	28	F	112.94	0.87	23.8	5.6
27	98117208	41	F	9.06	0.46	48.3	9.4
28	98112172	50	F	3.46	0.4	37.7	3.1
29	98127001	73	F	14.1	0.35	6.6	3.4
30	98119510	42	F	17.99	0.55	15.4	3.8
31	98123793	35	F	15.96	0.11	21.2	3.5
32	97018815	50	F	9.53	1.91	20.8	12.6
33	98119606	59	F	292.71	0.18	20	3.7
34	98101216	65	F	13.58	0.91	25.3	4.5
35	98074069	26	F	64.1	0.62	18.2	5.4
36	98103508	58	M	20.02	2.19	18.7	3.4
37	98116069	59	F	43.12	2	19.8	3.1
38	98111044	66	F	3.34	0.25	41.4	11.1

39	98122403	53	M	255.66	1.49	38.8	8.7
40	98118757	52	F	59.74	0.14	30.5	12.8
41	98104938	36	F	105.55	1.18	54.3	10.4
42	98137471	36	F	99.36	0.48	17.2	10.6
43	98111931	70	F	97.81	0.27	23.9	12.8
44	98132365	65	F	107.22	0.53	23.1	13.7
45	98128641	51	F	84.23	0.84	49.1	14
46	98130910	55	F	106.03	0.51	43.7	10.4
47	98140801	60	F	101.62	0.46	22.1	4.4
48	98139812	72	F	100.19	0.02	36.2	12.2
49	98140908	51	F	123.9	0.48	20.2	3
50	98130745	70	F	106.63	0.59	10.6	10.5
51	98122944	40	F	82.92	0.36	20.2	8.6
52	98078606	59	F	34.67	1.24	29.3	8.5
53	98140307	56	F	78.51	0.2	33.9	10.5
54	98130065	72	F	82.68	0.58	19	3
55	98135845	64	F	88.04	0.25	51.4	18.9
56	98140167	67	F	97.09	0.11	22.1	5
57	98132452	76	F	102.46	0.31	26.3	7.4
58	98132877	50	F	61	0.35	38	4.8
59	98136241	42	M	70.41	0.9	49.5	17.5
60	98117605	82	M	77.44	0.17	29.2	6.1
61	98048301	57	F	75.17	0.68	36.8	6.6

Table 3.1.1 FAMILY HISTORY-NON-OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No		[TK] (ngTK/ml sample)		TK Activity (ngTK/ugProtein)		Kinin Elisa [kinin] Generated	basal
1	98101723		219.8		0.13		26.7	3.5
2	98112231		219.68		0.3		37.2	5.3
3	98050426		274.48		0.12		23.8	5.6
4	98117208		112.94		0.87		48.3	9.4
5	98117208		9.06		0.46		48.3	9.4
6	98132877		61		0.35		38	4.8
7	98117605		77.44		0.17		29.2	6.1

Table 3.1.2 EYES WATER- NON-OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No		[TK] (ngTK/ml sample)	TK Activity (ngTK/ugProtein)	Kinin Elisa [kinin] Generated	basal
1	98096179		248.16	0.1	22.2	6.8
2	98015321		165.72	0.18	15.1	5.5
3	98114308		214.08	0.2	14.9	2.9
4	98108595		228.86	0.25	49.1	9.6
5	98115956		258.76	0.32	23	4.3
6	980333580		299.74	0.53	28.4	9.5
7	98049708		246.37	1.67	17.1	6.9
8	98033886		93.64	0.47	27.4	9.9
9	980422947		265.67	1.57	45.1	4.8
10	98046040		279.96	0.12	23.8	3.8
11	9802680		267.1	0.08	19.8	4.5
12	980434074		188.47	12.82	14.8	2.8
13	980424925		209.56	0.16	23.6	6.2
14	980293839		274.48	0.12	33.9	2.8
15	98127001		14.1	0.35	6.6	3.4
16	98123793		15.96	0.11	21.2	3.5
17	97018815		9.53	1.91	20.8	12.6
18	98074069		64.1	0.62	18.2	5.4
19	98103508		20.02	2.19	18.7	3.4
20	98118757		59.74	0.14	30.5	12.8
21	98130910		106.03	0.51	43.7	10.4
22	98140801		101.62	0.46	22.1	4.4
23	98140908		123.9	0.48	20.2	3
24	98122944		82.92	0.36	20.2	8.6
22	98140307		78.51	0.2	33.9	10.5
23	98130065		82.68	0.58	19	3
24	98135845		88.04	0.25	51.4	18.9
25	98140167		97.09	0.11	22.1	5
26	98136241		70.41	0.9	49.5	17.5
27	98117605		77.44	0.17	29.2	6.1
28	98048301		75.17	0.68	36.8	6.6

Table 3.1.3 SMOKING-NON-OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No	[TK] (ngTK/ml sample)	TK Activity (ngTK/ugProtein)	Kinin Elisa [kinin] Generated	basal
1	98096179	248.16	0.1	22.2	6.8
2	98015321	165.72	0.18	15.1	5.5
3	98108553	249.47	0.3	31.5	5.7
4	9810339812	87.68	0.31	34.4	4.3
5	98127001	14.1	0.35	6.6	3.4
6	98119606	292.71	0.18	20	3.7
7	98103508	20.02	2.19	18.7	3.4
8	98111044	3.34	0.25	41.4	11.1
9	98122403	255.66	1.49	38.8	8.7
10	98140801	101.62	0.46	22.1	4.4
11	98122944	82.92	0.36	20.2	8.6
12	98078606	34.67	1.24	29.3	8.5
13	98132877	61	0.35	38	4.8
14	98136241	70.41	0.9	49.5	17.5
15	98048301	75.17	0.68	36.8	6.6

Table 3.1.4 SMOKING +ALCOHOL- NON-OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No	[TK] (ngTK/ml sample)	TK Activity (ngTK/ugProtein)	Kinin Elisa [kinin] Generated	Basal
1	98096179	248.16	0.1	22.2	6.8
2	98015321	165.72	0.18	15.1	5.5
3	98108553	249.47	0.3	31.5	5.7
4	9810339812	87.68	0.31	34.4	4.3
5	98101723	219.8	0.13	26.7	3.5
6	98117208	9.06	0.46	48.3	9.4
7	98119606	292.71	0.18	20	3.7
8	98103508	20.02	2.19	18.7	3.4
9	98122403	255.66	1.49	38.8	8.7
10	98132365	107.22	0.53	23.1	13.7
11	98140801	101.62	0.46	22.1	4.4
12	98139812	100.19	0.02	36.2	12.2
13	98078606	34.67	1.24	29.3	8.5
14	98140307	78.51	0.2	33.9	10.5
15	98136241	70.41	0.9	49.5	17.5
16	98048301	75.17	0.68	36.8	6.6

Table 3.1..5 INFECTED MAIZE- NON-OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No		[TK] (ngTK/ml sample)		TK Activity (ngTK/ugProtein)		Kinin Elisa [kinin] generated	Basal
1	980419000		209.79		0.41		23.3	6.6

Table 3.1.6 INFECTED BEER- NON-OESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No		[TK] (ngTK/ml sample)	TK Activity (ngTK/ugProtein)	Kinin Elisa [kinin] generated	Basal
1	98114308		214.08	0.2	14.9	2.9
2	98108595		228.86	0.25	49.1	9.6
3	98115956		258.76	0.32	23	4.3
4	980419000		209.79	0.41	23.3	6.6
5	980333580		299.74	0.53	28.4	9.5
6	98049708		246.37	1.67	17.1	6.9
7	98033886		93.64	0.47	27.4	9.9
8	98046040		279.96	0.12	23.8	3.8
9	9802680		267.1	0.08	19.8	4.5
10	980293839		274.48	0.12	33.9	2.8
11	98074069		64.1	0.62	18.2	5.4
12	98103508		20.02	2.19	18.7	3.4
13	98122403		255.66	1.49	38.8	8.7
14	98118757		59.74	0.14	30.5	12.8
15	98140801		101.62	0.46	22.1	4.4
16	98140908		123.9	0.48	20.2	3
17	98122944		82.92	0.36	20.2	8.6
18	98140307		78.51	0.2	33.9	10.5
19	98130065		82.68	0.58	19	3
20	98135845		88.04	0.25	51.4	18.9
21	98140167		97.09	0.11	22.1	5
22	98132877		61	0.35	38	4.8
23	98136241		70.41	0.9	49.5	17.5
24	98117605		77.44	0.17	29.2	6.1
25	98048301		75.17	0.68	36.8	6.6

Table 3.1.7 FEMALES AND AGE-NON-0ESOPHAGEAL CANCER PATIENTS (URINE SAMPLES)

Patient No	Hospital No	Age	[TK] (ngTK/ml sample)	TK Activity (ngTK/ugProtein)	Kinin Elisa [kinin] Generated	basal
1	98101114	47	203.48	0.73	36.7	11
2	98101624	51	210.75	0.13	24.9	16.9
3	98104968	53	119.97	0.43	31	8
4	98101723	48	219.8	0.13	26.7	3.5
5	98114308	60	214.08	0.2	14.9	2.9
6	98108595	41	228.86	0.25	49.1	9.6
7	98115956	65	258.76	0.32	23	4.3
8	98103449	63	199.79	0.3	37.5	8.6
9	98112231	72	219.68	0.3	37.2	5.3
10	98111969	76	267.81	0.62	51.2	7.7
11	980419000	52	209.79	0.41	23.3	6.6
12	980333580	40	299.74	0.53	28.4	9.5
13	98049708	54	246.37	1.67	17.1	6.9
14	98033886	35	93.64	0.47	27.4	9.9
15	980422947	59	265.67	1.57	45.1	4.8
16	9810339812	41	87.68	0.31	34.4	4.3
17	98046040	59	279.96	0.12	23.8	3.8
18	9802680	61	267.1	0.08	19.8	4.5
19	980434074	44	188.47	12.82	14.8	2.8
20	980424925	39	209.56	0.16	23.6	6.2
21	98012270	40	109.37	1.35	48.1	5.4
22	980293839	29	274.48	0.12	33.9	2.8
23	98050426	28	112.94	0.87	23.8	5.6
24	98117208	41	9.06	0.46	48.3	9.4
25	98112172	50	3.46	0.4	37.7	3.1
26	98127001	73	14.1	0.35	6.6	3.4
27	98119510	42	17.99	0.55	15.4	3.8
28	98123793	35	15.96	0.11	21.2	3.5
29	97018815	50	9.53	1.91	20.8	12.6
30	98119606	59	292.71	0.18	20	3.7
31	98101216	65	13.58	0.91	25.3	4.5
32	98074069	26	64.1	0.62	18.2	5.4
33	98116069	59	43.12	2	19.8	3.1
34	98111044	66	3.34	0.25	41.4	11.1
35	98118757	52	59.74	0.14	30.5	12.8
36	98104938	36	105.55	1.18	54.3	10.4

37	98137471	36		99.36	0.48	17.2	10.6
38	98111931	70		97.81	0.27	23.9	12.8
39	98132365	65		107.22	0.53	23.1	13.7
40	98128641	51		84.23	0.84	49.1	14
41	98130910	55		106.03	0.51	43.7	10.4
42	98140801	60		101.62	0.46	22.1	4.4
43	98139812	72		100.19	0.02	36.2	12.2
44	98140908	51		123.9	0.48	20.2	3
45	98130745	70		106.63	0.59	10.6	10.5
46	98122944	40		82.92	0.36	20.2	8.6
47	98078606	59		34.67	1.24	29.3	8.5
48	98140307	56		78.51	0.2	33.9	10.5
49	98130065	72		82.68	0.58	19	3
50	98135845	64		88.04	0.25	51.4	18.9
51	98140167	67		97.09	0.11	22.1	5
52	98132452	76		102.46	0.31	26.3	7.4
53	98132877	50		61	0.35	38	4.8
54	98048301	57		75.17	0.68	36.8	6.6

Table 3.1..8 MALES AND AGE-NON-0ESOPHAGEAL CANCER PATIENTS (URINE SAMPLES

Patient No	Hospital No	Age		[TK] (ngTK/ml sample)	TK Activity (ngTK/ugProtein)	Kinin Elisa [kinin] Generated	basal
1	98096179	49		248.16	0.1	22.2	6.8
2	98015321	53		165.72	0.18	15.1	5.5
3	98108553	51		249.47	0.3	31.5	5.7
4	98136241	42		70.41	0.9	49.5	17.5
5	98117605	82		77.44	0.17	29.2	6.1
6	98122403	53		255.66	1.49	38.8	8.7
7	98103508	58		20.02	2.19	18.7	3.4

CHAPTER 6: REFERENCES

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