

The role of platelet-derived growth factor in radiation injury to the lower urinary tract

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The role of platelet-
derived growth factor
in radiation injury to
the lower urinary tract

Thesis for MD Res

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Abstract

Approximately half of all patients diagnosed with cancer will receive radiotherapy as a part of the management of their tumours. Radiotherapy allows organ sparing treatment but it is associated with a number of acute and late unwanted effects. Radiotherapy has a crucial role to play in the treatment of urothelial malignancies. However, the late non-tumoricidal consequences cause significant morbidity, particularly in relation to fibrosis of the pelvic structures. The unwanted effects of radiotherapy were originally thought to be due to the result of direct cell injury, cell death and subsequent fibroatrophic changes. It is now clear that radiation initiates a series of events in cells and tissues that is primarily based upon the release of cytokines, including growth factors, which lead to the development of late radiation injury. This study investigates the changes that occur in the lower urinary tract after radiotherapy and looks for a possible role for platelet derived growth factor (PDGF) in this process.

Fifty-six cystectomy specimens were retrieved from the archives of the Pathology Department of patients who had been treated with a similar schedule of radiotherapy for bladder cancer. Morphological changes were identified by routine light microscopy, focussing mainly on the bladder and prostate gland. The degree of fibrosis was measured using an image analysis system and this was related to time since irradiation. Immunohistochemistry for fibronectin, PDGFs and PDGF receptors was performed and the expression assessed by a semiquantitative scoring method. The results were compared to non-irradiated control bladders, either with or without tumours.

A range of histological changes were identified including inflammatory, epithelial, stromal, vascular and neural alterations. These were either more commonly seen in the irradiated group or the normal age/physiological changes usually encountered in these organs were exaggerated in this group after irradiation. Predictably, fibrosis and fibronectin production was more obvious in the irradiated group, and this increased with time since irradiation for period of study. PDGFs and their receptors were expressed after irradiation and the levels were higher than in the non-irradiated group.

The histological basis for the unwanted side effects of irradiation is described in this study. The fibrosis is progressive, with accumulation of connective tissue long after the radiation dose has been delivered. It is likely, from these results, that PDGF and its

receptors play a role in this process. These results pave the way for manipulation of growth factors and/or their receptors, including PDGF, in the future management of patients who are symptomatic with radiation injury.

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List of abbreviations

AAV	Avian adenovirus
ACE	Angiotensin converting enzyme
AML	Acute myeloid leukaemia
APEX	Aporicinic-apyrimidinic endonuclease
AT	Ataxia telangiectasia
BM	Basement membrane
BMP	Bone morphogenic protein
BRCA	Breast cancer gene
CCL	Chronic lymphocytis leukaemia/lymphoma
CCN2	Cyr61/CTGF/Nov family 2
CHK2	Csk-homoloogous kinase 2
COL	Collagen
CTC	Common toxicity criteria
CTGF	Connective tissue growth factor
CXCL	C-X-C ligand
CXCR	C-X-C receptor
DCRT	Dimension conformal radiation therapy
DFSP	Dermatofibrosarcoma protruberans
ECM	Extracellualr matrix
ED	Extra domain
EMT	Epithelial mesenchymal transition
FGF	Fibroblast growth factor
FN	Fibrinogen
GCSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumour
GMCSF	Granulocyte-monocyte colony stimulating factor

GTP	Guanidine triphosphate
HES	Hyperoesinophilic syndrome
HGF	Hepatocyte growth factor
ICAM	Intercellular adhesion molecule
IEG	Immediate early genes
IFN	Interferon
IFP	Interstitial fluid pressure
IL	Interleukin
IMRT	Intensity modulated radiotherapy
KLF	Kruppel-like factor
LAP	Latency associated protein
LET	Linear energy transfer
LMN	Lower motor neurone
LOH	Loss of heterozygosity
LP	Lamina propria
LRP	Lipoprotein related protein
M	Membrane
MAPK	Mitogen-activated protein kinase
MP	Muscularis propria
NCI	National Cancer Institute
NICE	National institute for clinical excellence
NO	Nitric oxide
PDGF (R)	Platelet derived growth factor (receptor)
PET	Positron emission tomography
PF	Pulmonary fibrosis
PH	Pulmonary hypertension
PI3	Phosphoinositide 3-kinase
PKC	Protein kinase c
PLC	Phospholipase c

PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RANTES	Regulated upon activation normal T cells expressed and secreted
RAS	Renin-angiotensin system
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTK (I)	Receptor tyrosine kinase (inhibitors)
SH2/3	Src homology 2/3
SMC	Smooth muscle cell
SNP	Single nucleotide pleomorphism
SOD	Superoxide dismutase
SSV	Simian sarcomavirus
TGF (R)	Transforming growth factor (receptor)
TNF	Tumour necrosis factor
tPA	tissue plasminogen activator
TURP	Transurethral resection of the prostate
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XP	Xeroderma pigmentosa
XRCC	X-ray repair cross complementing

Publications arising from this work

Papers

Sheaff MT, Baithun SI. Effects of radiation on the normal prostate gland. *Histopathology* 1997;30:539-549

Sheaff M, Baithun S. Pathological effects of ionizing radiation. *Current Diagnostic Pathology* 1997;4:106-115

Abstracts

Baithun S, Sheaff M, Badenoch D. Morphological changes due to radiation injury in non-neoplastic prostates. *Journal of Pathology* 1995;175S:133A

Sheaff M, Badenoch D, Baithun SI. Radiation-induced changes in thirty-nine cystectomy specimens. *Journal of Pathology* 1995;175S:134A

Sheaff M, Nicholls C, Hopster D, Martin J. Platelet-derived growth factor in radiation injury to the bladder. *Journal of Pathology* 1996;179S:39A

Sheaff M, Nickols CD, Martin JE. The expression of platelet-derived growth factor and receptors in pituitary adenomas. *Neuropathology and Applied Neurobiology* 1997;23:173A

Bulut I, Sheaff M. Quantification of post-radiation fibrosis in the human urinary bladder. *Journal of Pathology* 1999;187S:35A

Chapter 1. The effects of radiation on human tissues

Radiation can be defined as the passage of energy through matter or the transfer of energy from one object to another as it passes through matter. There are many different types of radiation which are classically separated into electromagnetic and particulate. The former is concerned with energy propagated as waves, the second by the movement of subatomic particles produced either by naturally decaying elements or artificially. Electromagnetic radiation comprises a spectrum of wavelengths that includes γ -rays, x-rays, radiowaves, ultraviolet (UV) and microwaves which are split into ionising (that which produces ions and ejects electrons as they pass through matter) and non-ionising. Their wavelength dictates the penetrating power. Gamma and x-rays are for all intents and purposes the same but the former are naturally produced and therefore the physical characteristics depend on the substance producing them, while x-rays are man-made and depend on the properties of the source. Although UV radiation is non-ionising it is still potentially harmful to humans and is associated with skin cancer. Particulate radiation is subclassified by the type of particle into alpha, beta, protons, neutrons, deuterons and mesons.

Radioactive materials are present universally either as naturally occurring or artificially produced molecules. They are present both inside and outside the body with the majority of external natural background radiation being from radon gas. Other background sources include radioactive elements naturally occurring in the earth and the body, and cosmic rays in the atmosphere. In addition to these, there are several artificial sources such as medical instruments concerned with diagnosis and treatment, fallout from atomic weapons and nuclear power installations. There are many less important sources such as “consumer products” (luminescent instruments etc) and “technologically enhanced” (radionuclides in fertilisers and building materials) of which

the majority of people are unaware. In consequence, nobody can escape radiation exposure and a large number of patients that seek the advice of a doctor also undergo radiological investigation as part of their medical assessment¹. In addition, half of all patients with cancer will have radiotherapy at some time during the treatment of their illness and in 1990 almost 500,000 patients with cancer in the USA were treated with this modality². Unfortunately all forms of radiation can potentially be harmful to humans under appropriate conditions and in some cases this can be lethal.

One of the earliest informal measures of the dose of radiation was the degree of skin erythema produced after exposure. The oldest unit of radiation introduced was the roentgen for x and gamma and is the unit of the charge produced when the rays ionise a given volume of air. Some time later the rad was introduced in order to try to compare with particulate radiation whereby 1 rad equals the dose of radiation that will result in the absorption of 100 ergs of energy per gram of absorbing substance. Energy of most tissues on exposure to 1 roentgen is 93 ergs or nearly 1 rad therefore some use the units interchangeably. The SI unit is the Gray which is equal to 100 rads. However these are not a measure of energy absorption only of exposure and a time unit required (usually per minute). However, 1 rad of particulate energy causes more damage than 1 rad of x or gamma rays and more recently the rem has been introduced. Loosely this represents the biological effect equivalent to 1 rad of x or gamma rays. The SI equivalent is the sievert which therefore equals 100 rem. The biological response of the same dose of different radiation types is different and so the terms linear energy and relative biological effect have also evolved.

1.1 Radiation and medicine

In 1895 Roentgen discovered a 'new ray' which was capable of producing an image on photographic film when directed from his ionising tube and shortly afterwards the Curies discovered radium and the ability of rays to produce images of human structures not visible by conventional means. It was only a matter of time before this became an important part of diagnostic and therapeutic medical practice. Now around half of the 25% of the population who will develop cancer will receive radiotherapy^{2,3}. It was soon appreciated however that radiation led to morphological changes in the tissue exposed. Since then the morphological and cancer induction properties of irradiation have been studied. From early observations and subsequent epidemiological studies it became clear that radiation could also cause non-morphological effects. These have been split into 4 main groups: genetic, fetal, aging effect and risk of cancer⁴.

The genetic effects are mostly discussed later with radiocarcinogenesis (section 1.8.1), but it should be noted that although it was previously thought that the effects of radiation exposure were restricted to the parent in humans and do not pass on to the offspring, this may not necessarily be the case according to recent mutagenesis studies⁵.

The fetal effects of radiation depend on the age of exposure with lethal consequences of very early exposure after conception. During the period of organogenesis exposure is more likely to result in a wide range of congenital malformations. Chromosomal aberrations may occur and radiation may be associated with Down's syndrome⁶. From 6-15/40 gestational stages exposure is associated with mental retardation, decreased IQ, decreased head size and seizures. Maternal treatment with radioiodine during pregnancy may result in cretinism due to passage across the placenta. The post-natal effects are discussed later with the specific organs.

It was predicted from animal experiments that non-specific ageing processes increased after irradiation and in the past it has even been held that 1 rad of exposure led to 10 days of life lost. However, when the data is interpreted correctly it seems there is no general life-shortening effect, and Doll suggests that with the current evidence this idea should be abandoned unless new evidence comes to light⁴. This view is not universally accepted however and some believe that radiation induces senescence mechanisms via telomere damage or loss⁸ or dysfunction without direct telomere DNA damage⁹.

Pioneer radiologists developed squamous and basal cell carcinomas of their hands and the carcinogenic risk of radiation exposure was noted early. By the 1950s this increased risk was confirmed by epidemiologists. However it became obvious that the increased risk also depended on many other factors such as sex, age and presence of underlying hereditary susceptibility, and that there were differences between tissues (the risk of developing leukaemia is greater than cervical carcinoma for example and the increased risk for bladder carcinoma develops later and persists longer). Although the risk of developing cancer was discovered early the mechanisms are still not elucidated. There continues to be debate about the risk of low dose exposure such as radon gas in the home. Several models (such as linear and linear-quadratic) have been employed to predict the risk of low dose exposure but their interpretation remains controversial¹. It is generally felt, however, that there is probably no risk with low dose exposure or at least the effects are so small as to be undetectable¹.

1.2 Radiobiology

1.2.1 Effects on the cell

Although radiation energy is capable of damaging many components of the cell, the main target of the cytotoxic effects is the nuclear DNA. The radiant energy delivered to

a tissue causes its effects by two main mechanisms, direct and indirect. The former is a consequence of direct interaction between the damaging waves and the nuclear chromatin. The second reflects the production of free radicals in the cytoplasm from ionisation of the cellular water (which constitutes around 80% of the cell). The ionisation results from electrons being ejected from cellular molecules as the radiant energy wave passes through. Free radicals are toxic oxygen metabolites that are able to cause local damage by oxidising and reducing structures in the local environment. The most important radicals implicated are hydroxyl groups. These lead to peroxides or cross-linkages with critical molecules such as DNA or RNA or attach to membranes to impair normal function. Therefore these two mechanisms both contribute to DNA damage causing inter and intra strand cross-linkages, strand breaks, mutations and result in impaired ability of DNA to act as template for new strand, and chromosomal aberrations such as translocations, deletions, fragments and rings. Radiation may break hydrogen bonds, damage nucleotides and disrupt the sugar-phosphate backbone of molecule. The damaged DNA is mostly repaired by efficient mechanisms and if the damage is limited then complete repair is possible⁹. This is thought to be the case with single-strand breaks and 90% of chromosomal breaks. However, with more significant injury, such as double strand breaks, there will be some which are partially repaired or irreparable and the critical reproductive integrity of the cell will be lost, leading to cell death. This reproductive cell death may occur either at the first or early subsequent mitotic phases. Incomplete repair of this damage may also lead to apoptosis, necrotic cell death (pyknosis or karyorrhexis), inhibition of mitotic division with giant cell formation and derangement of the mitotic process leading to abnormal mitotic figures. The effects on RNA synthesis are thought to be less radiosensitive than DNA and RNA may be more stable after irradiation. Proteins can also be directly damaged but larger

doses are necessary. Radiation can cause mitochondrial injury also, either direct injury to the mitochondrial structure or disruption of mitochondrial processes⁵.

1.2.2 Cell cycle changes

It has been known for a long time that after irradiation there is a cell division delay within exposed cells which may result from G1 block, G2 arrest or S phase delay. This is presumably important as a stop-gap for damage repair. Cell cycle regulation is controlled by several important regulatory proteins which include p53, GADD45, RAD-9, possibly bcl-2 and cyclin B. Most are increased after irradiation¹⁰ but cyclin B is transiently decreased¹¹. Cyclin B is required for the cell to proceed from G2 to M and when cyclin B levels are restored the delay is terminated¹¹. DNA double stranded breaks are easily detectable after radiation exposure but they are virtually completely repaired within 6 hours after administration of highly lethal doses. It has been suggested that the significant breaks are not repaired correctly leading to cell death in the next 1 or 2 mitoses¹³.

1.2.3 Other changes associated with cellular irradiation

After irradiation there is rapid induction of signal transduction pathways mediated by protein kinase c and tyrosine kinase with increased second messengers/inositol trisphosphate. In turn this leads to increased gene expression including the early response genes c-fos, c-jun and NF-kB. The activation of later responding genes including TNF, bFGF, TGF β is associated with subsequent protein production. This cascade of gene activation, gene transcription and protein synthesis is likely to promote key cellular functions that allow survival of the cell¹². Several other proteins, such as

tPA, are increased after irradiation and their presence and relevance to the radiation response is only just beginning to be unravelled¹⁴⁻¹⁶. These will be discussed in more detail later on in this chapter (section 1.4).

Growth factors are not only stimulated by radiation but their presence also seems to affect the response of cells to irradiation and they may play a role in radioprotection¹⁷. For example IL-1, GM-CSF and G-CSF all modulate the effects of radiation on haematopoiesis. Similarly, bFGF increases the radioresistance of endothelial cells by stimulating damage repair¹⁷. Therefore growth factors are attractive as agents of radioprotection clinically (discussed later). On the other hand, other genes and proteins appear to confer radiosensitivity. This unlocks the possibility of gene therapy since it may then be possible to introduce target genes coupled with a radiation responsive element into cells prior to irradiation, or small molecule therapy for upregulation of specific protective genes.

1.2.4 Morphological changes in cells

Cellular morphological effects may occur in the nucleus, cytoplasm or membrane. The nucleus may appear swollen with clumped chromatin. It may have bizarre morphology due to aneuploidy/polyploidy and any mitoses present may also be abnormal. The cytoplasm is also swollen due to intracellular oedema. Ultrastructurally organelle damage is manifest as mitochondrial distortion with degeneration of the rough endoplasmic reticulum. Membrane defects and breaks may also be visible¹³.

1.2.5 General effects of irradiation on tissues after uncontrolled exposure

The effects of radiation on tissues depend on the dose delivered and the time since exposure¹. The results can either manifest soon after exposure or at some later time, especially if the dose of exposure is relatively low. It is unfortunate that the effects of uncontrolled large dose exposure to the entire human body are known, but following studies on nuclear fallout cases the information is all too readily available¹. The immediate and most rapidly lethal effects of high dose exposure are due to blast injury and heat. For those that survive the initial insult, acute radiation sickness may develop¹³. This is a syndrome with variable effects depending on the dose and therefore organs involved. Several thousand people died after the atom bomb explosions in Hiroshima and Nagasaki from acute radiation sickness. With lower doses of exposure, the effects can either be seen relatively early or some time after exposure. The most sensitive tissue is the bone marrow which will show effects after quite low doses of exposure. These develop over a period of weeks and result from pancytopenia and peripheral cytopenia. Death is unusual after exposure to lower doses but may occur after some time from haemorrhage or infection. The gastrointestinal tract is relatively more resistant, but will show evidence of damage at higher doses. These will manifest earlier than the effects on the bone marrow and will lead to earlier mortality if severe enough. The central nervous system is much more resistant but if exposure is large enough manifestations may be apparent within minutes. Symptoms reflect an increase in intracranial pressure associated with cerebral oedema and mortality is frequent and rapid¹.

Thankfully such high dose exposures are rarely, if ever, seen in medical practice. However, in contrast to chemotherapy, there is little prospective dose-escalation data to determine the maximum tolerated dose of radiation at any given site. It would obviously

be difficult to carry out such studies because sub-lethal radiation dose is usually limited by late normal tissue effects and not by acute effects. Consequently, the commonly accepted tolerable doses have largely been derived empirically during the history of radiation therapy, and are based on limited retrospective data and unpublished clinical observations and teachings. Although the radiation tolerance of most organs is not known, there are published guidelines which serve as reasonable estimates^{18,19}. A variety of factors can affect the usable dose and these will be discussed in more detail later. In many instances one crucial feature is the type of tissue exposed. In some tissues, a reasonable amount of radiation damage may be acceptable, especially if there is a realistic hope of tumour control. For example, a small amount of fibrosis in the lung is well tolerated and is commonly present after radiotherapy for lung cancer. On the other hand, in the central nervous system, the consequences of radiation injury can be severe and the dose must be modified to minimise the likelihood of serious brain injury. The symptoms may be mild or severe, self-limiting or progressive, gradual or sudden.

Often the most significant effects of irradiation are seen in rapidly proliferating cells which quickly leads to symptoms when functional cells are lost and they are not replaced by stem cells¹⁸. On the other hand, tissues with considerable stem cell pools and niches lead to better recovery – this is especially seen in the skin and gut. Radiation may also induce senescence and/or differentiation of cells rather than perishing and this may be particularly important in fibroblasts which then produce excess collagen and fibrosis. This will be discussed further later in section 1.5.

1.3 Tissue effects after controlled exposure

1.3.1 Overview

There appear to be quite predictable effects of radiation injury on human tissue. In many organs the manifestations follow a set pattern with early and late changes either before or after 12 months. These phases of post-radiation injury are frequently split further into three stages with acute changes occurring within 60 days, subacute changes progressing over the next year or so - due to persistence or evolution of the acute changes (sometimes called consequential changes), and late or delayed events developing after many months or years. Conventionally, three phases of radiation damage are described; pre-fibrotic inflammatory changes, followed by constitutive fibrotic cellular phase leading to a matrix densification but the processes behind these phases do not strictly begin and end during each stage, rather there is an overlap where one aspect of the process dominates over the others¹⁸.

Whereas the typical side effects of drug therapies are systemic, they are local or loco-regional after radiation therapy. Therefore, the side effects make themselves manifest in tissues and organs that have been irradiated. In most instances the pathological changes associated with radiation injury begin immediately after exposure, but the clinical and histological features may not become apparent for weeks, months, or even years after treatment. For example, radiation changes in the lung can be detected 6 weeks after irradiation but these are mild even after a high dose. However, by 6 months there is widespread fibrosis. As has been mentioned, radiation injury is commonly classified as acute, consequential, or late, depending on the time taken for symptoms to appear. Acute (early) effects are those that are observed during the course of treatment or within a few weeks after treatment. Consequential effects (sometimes called consequential late effects) appear later, and are caused by persistent acute damage. Late effects emerge

months to years after radiation exposure. The terms acute and late have been introduced in radiation therapy, but because the underlying molecular and cellular processes are complex and lead to a range of events, the definitions are rather loose and may be more operational than mechanistic. Early symptoms may not be apparent in some organs that develop late injury, such as the kidney, and trauma or surgery months or years after irradiation can precipitate acute breakdown of tissue that had been functioning normally²⁰.

The early or acute changes are seen most commonly in rapidly dividing cells such as gut epithelium and the early vascular changes reflect non-specific dilatation and increased permeability leading to oedema. The chronic or delayed effects are dominated by degeneration and repair which again probably result from vascular damage. It seems that other cells such as fibroblasts and smooth muscle cells may play important roles in the production of delayed effects²⁰.

In many ways, the acute and late manifestations may be consequences of the same underlying mechanisms. However, late effects tend to occur more in tissues with low cellular turnover than proliferation and therefore different parts of a tissue may be affected differently (such as the mucosa and muscularis propria of the bowel wall for instance). Therefore although there are overlaps between the mechanism of early and late injury, there are also important differences. Fibroatrophic processes dominate the late changes²¹. These tend to be local to the site of exposure, generally unavoidable and usually considered to be irreversible. The effects, together with their consequent functional effects can mimic or promote aging and/or growth retardation. They may also eventually be involved in the carcinogenetic process¹.

The late effects occur through complex interacting processes that are not completely understood especially with respect to cell death during proliferation. Cells exist in a

complex community where cells depend on each other for, and contribute individually to, the welfare of the whole tissue. Clearly some of these late manifestations and associations with vascular damage, endothelial cell injury and thrombosis can lead to downstream hypoxic cell death; additionally parenchymal cells may die and lead to atrophy and loss of vascular supply.

The consequential late features are produced by failure of acute lesions to heal or resolve and therefore the process persists into the later period. Consequential damage is most frequently seen in the urinary system, gastrointestinal tract and skin, and is more common after more intense therapies¹⁸.

The early or acute changes of radiation reflect a rapid molecular response to the insult including cytokine production leading to adaptive response in surrounding tissues and inflammation¹⁸. The process is very much the same as any acute inflammatory process with subsequent wound healing¹⁸. Early effects become manifest within a few weeks of the completion of a course of fractionated radiotherapy. These effects include skin erythema, dry or moist desquamation of the skin, mucositis, nausea and diarrhea, depending on the site of irradiation²². Late effects typically present after latent periods of months to years, and are generally related to radiation-induced fibrosis, atrophy, vascular damage, neural damage and a range of endocrine and growth-related effects. The pathophysiological and functional expression of this damage will be outlined in more detail later, but it depends very much on the tissue or organ affected. Of course, radiation-induced second malignancies are obviously important, especially in patients with a long life expectancy. In general the early effects are transient and they settle after a few weeks, but late effects tend to be irreversible and often even progressive in severity.

A short time after irradiation of any tissue there follows a combination of inflammatory and cellular (necrotic/apoptotic) events. In the small bowel for example, epithelial cells show evidence of cell death and, due to cell cycle arrest, there is a lack of repopulation from the crypts leading to erosion²³. An inflammatory cell infiltrate is seen and, although mild, is characterised by prominent numbers of eosinophils and mast cells. It is interesting that the latter are very radiosensitive and contain inflammatory mediators and fibrogenic proteins thereby suggesting a role for them in more chronic changes²⁴. Platelets are also packed with inflammatory mediators and their role in radiation injury may be significant, but there are few studies on this topic. With high enough doses any tissue may show necrosis. General epithelial changes include nuclear pleomorphism, giant cell formation and hypo/hyperplasia early on with atrophy, dysplasia, metaplasia and neoplasia developing later.

Early vascular changes include dilatation, increased permeability and endothelial cell swelling. More chronic lesions show endothelial cell atypia, intimal thickening, medial hypertrophy, luminal thrombosis and endarteritis obliterans with subsequent ischaemic consequences²⁵. A characteristic delayed consequence of irradiation is fibrosis with multinucleate fibroblasts and increased collagen deposition. This is probably a result of a combination of early repair followed by late ischaemia. The fibrosis appears to be a progressive feature. Other stromal changes include collagen degeneration and fat necrosis. Necrosis and ulceration may occur as a late complication following progressive vascular compromise and increasing ischaemia.

In general the tissue effects depend on the dose and type of exposure but many factors influence the response such as the age of the patient, the relative radiosensitivity of the cells, the capacity for repair and the architectural make up of the tissue. Effects in general may be stochastic or non-stochastic: the former vary with frequency but not

dose and have no threshold, the latter vary in severity but not frequency with dose and may exhibit a threshold¹.

1.4 Pathogenesis of radiation injury

For many years it was considered to be the direct cytotoxic effects of radiation on cells which produced the lesions that developed after tissue was irradiated. The prevailing 'target-cell' hypothesis in the late 1980s and 90s suggested that the main effect of ionizing radiation on tissues and organs was a direct consequence of cell killing, resulting in the depopulation of crucial cell populations and subsequent functional deficiency¹⁸. However, it became apparent from cellular radiobiology studies that cell-cell communication was vitally important in the processing of cellular radiation damage. Irradiating single cells in culture flasks with high-precision microbeams cells led to the death of nearby cells in the vicinity of an irradiated cell even though the neighbouring cells had not been irradiated themselves - the so-called 'bystander effect'²⁰. It is becoming clear now that the direct cellular effects of radiotherapy are not the main reason for subsequent tissue injury. It also became more obvious because local cells and progeny effects caused by signalling pathways also show changes after irradiation of neighbouring or parent cells. These bystander cells – cells not in direct contact - also show gene rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, mutations, amplifications and cell death. Many of the changes observed previously after whole cell irradiation could be induced in vitro by cytoplasmic irradiation alone. Therefore the theory was proposed that this was because of gap junction transmission by soluble factors especially reactive oxygen species and cytokines²⁶. Later, this was also recognised in vivo. For example, lungs irradiated with apical shielding showed similar changes in the apex to the rest of the lung

parenchyma²⁷; similar changes were observed in liver tissue protected from the radiation source²⁸.

Recent interest has concentrated on epigenetic changes such as methylation status and histone injury⁵. These areas, together with RNA associated silencing, have all become fashionable areas of interest. These are especially interesting as they provide additional clues as to how radiation induced changes may be passed onto cellular progeny and subsequent cells are consequently genetically unstable⁵.

When the focus of interest shifted away from direct cell injury during the 1990s, it became clear that the latent period between irradiation and the clinical expression of late normal-tissue injury is a highly active interlude. Soon after irradiation many cytokine cascades are activated, and these remain active throughout the phase of overt damage expression. Although radiation-induced cell killing might have a role as a triggering event, it is now clear that there is an orchestrated, active biological response brought about by the early release of cytokines¹⁷. This response is mediated by various cell types, including inflammatory, stromal, endothelial and parenchymal cells actively responding through the release or activation of downstream cytokines, growth factors or chemokines²⁰.

Direct cellular injury may be an important component of the early effects of radiotherapy, but studies of late side effects and, in particular, radiation-induced fibrosis indicate that this cannot be the only route for subsequent tissue remodelling. Clinically, radiation-induced fibrosis is characterized by reduced tissue flexibility, reduced compliance or strictures. Fibrosis is often associated with pain, neuropathy, reduced strength or restricted motion of joints and distal lymphoedema. The corresponding histopathological picture typically shows that the normal tissue is partly atrophic, partly replaced by mesenchymal cells, and that there is excessive collagen deposition²¹. It has

long been clear that fibroblasts are the main cell involved in the deposition of the extracellular matrix; but it was less obvious how the fibroblasts could also be the putative target cell for radiation-induced fibrosis. It seems counter-intuitive that killing fibroblasts would lead to increased collagen production. Other important observations came from clinical studies that showed that non-cytotoxic drugs such as tamoxifen could also increase the incidence of late radiation-induced fibrosis after radiation²⁹. Many studies have also shown that radiation induced changes may become manifest clinically many years after treatment (reviewed in 21).

1.5 Pathobiology of radiation fibrosis

Among the late effects of radiation therapy, radiation-induced fibrosis is probably the most extensively studied. This is partly due to the importance of this reaction after clinical radiotherapy and partly because a lot has been learned from molecular pathology studies of wound healing and of human diseases characterized by the excessive formation of fibrous tissue. Mechanistically, the early phases of fibrogenesis after irradiation can be seen as a wound-healing response characterized by an almost immediate upregulation of pro-inflammatory cytokines such as tumour-necrosis factor- α (TNF), interleukins 1 and 6 (IL1 and IL6) and many growth factors in the irradiated tissue³⁰ (Fig. 1). Chemokines are released and these recruit inflammatory cells from the surrounding tissue into the irradiated volume. The interactions between the many proteins involved in the fibrogenic process are still not completely understood. However, useful insights into the *in vivo* function of the more than 100 proteins involved in wound healing have been gathered from genetically modified mouse models, gene knockouts or mice that transiently or permanently overexpress one of these proteins³⁰.

Normal wound healing is regulated by a complex balance between profibrotic proteins such as transforming growth factor- β (TGF)³¹ and its downstream effector connective tissue growth factor (CTGF, also known as CCN2)³² on the one hand and antifibrotic proteins such as TNF and interferon- γ (IFN) on the other³³. The pro-inflammatory TNF is expressed in macrophages during wound healing but downregulates the expression of matrix genes³⁴. In addition, IFN is a pro-inflammatory cytokine released by T cells after irradiation, that has been shown to downregulate TGF and suppress collagen synthesis³⁵⁻³⁷.

The molecular processes underlying rectal radiation injury have been investigated and inflammation-related proteins are clearly important. Reduced amounts of endothelial thrombomodulin have been observed in normal rectum and tumours after irradiation, which may lead to increased fibrin deposition and associated upregulation and release of inflammatory and fibrogenic cytokines³⁸. Increased concentrations of mRNA for TGF β 1 and TNF α have been found in colorectal tissues of fibrosis-susceptible and fibrosis-resistant strains of mice 6 months after irradiation³⁹. In the ileum of rats, increases in TGF β 1, TGF β 2, and TGF β 3 were observed 2 weeks after a radiation dose of 12 or 21 Gy, but after 26 weeks, only TGF β 1 remained high⁴⁰. TGF β 1 was particularly prominent in areas with chronic fibrosis, in smooth muscle, mesothelium, endothelium, and fibroblasts.

In the lung, the pathogenic link between pneumonitis early and the development of fibrosis later on is still being unravelled. Animal and human studies indicate that vascular injury and the coagulation cascade, cellular adhesion molecules, proinflammatory and profibrotic cytokines, and oxidative stress all seem to have vital roles in the development of radiation pneumonitis⁴¹. It is interesting to note that mice lacking the gene for the endothelial cell adhesion molecule ICAM1 do not develop

pneumonitis or show infiltration of inflammatory cells after irradiation⁴². The mice without ICAM still develop fibrosis, but only at high radiation doses, suggesting that an inflammatory response is not the only factor underlying the development of radiation fibrosis. Furthermore, individuals with high plasma concentrations of the proinflammatory cytokine interleukin 1, or the profibrotic cytokines interleukin 6 and TGF β , before or during radiotherapy have a higher risk of developing pneumonitis^{41,43} and patients with increased concentrations of TGF β at the end of their course of radiotherapy have a higher risk of symptomatic radiation-induced lung injury 6 months to 2 years after radiotherapy⁴⁴. In a fibrosis-prone strain of mice given a single dose of 20 Gy to the thorax, increases in interleukin 1 were observed during the early post-irradiation period and were associated with pneumonitis, whereas increases in TNF α , TGF β 1, and TGF β 2 occurred later and were associated with the development of fibrosis⁴⁵. Fibrosis-prone mice express more and different chemokines and chemokine receptors 6 months after irradiation than fibrosis-resistant mice⁴⁶. These results clearly point towards the recruitment and activation of monocytes, macrophages and lymphocytes in the development of fibrosis in irradiated lung tissue. Gene therapy with manganese superoxide dismutase (MnSOD) reduces fibrosis in these mice⁴⁷, suggesting a role for oxidative stress in the development of radiation injury as described previously. The antifibrotic action of the related compound copper and zinc-containing superoxide dismutase (Cu/ZnSOD) could well be mediated by a reduction in the expression of TGF β by myofibroblasts⁴⁸. The renin-angiotensin system also appears to be involved in the development of pulmonary injury after irradiation. However, although angiotensin-converting enzyme inhibitors or an angiotensin receptor blocker protected rat lungs from both pneumonitis and fibrosis after radiation exposure⁴⁹, patients taking these drugs for their hypertension did not have a reduced incidence of pneumonitis⁵⁰.

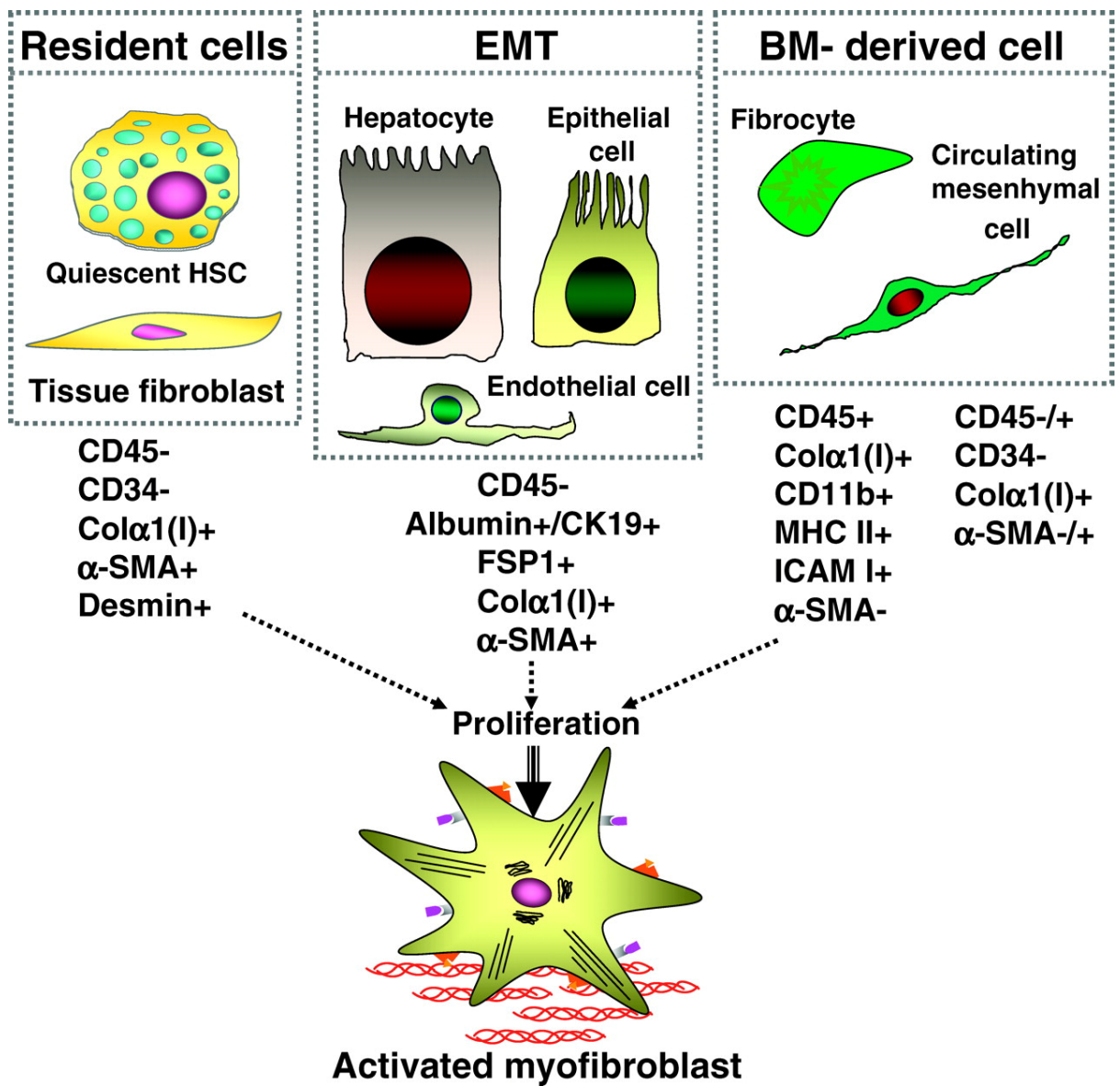


Figure 1. Mechanisms of fibrogenesis³⁰ (with permission)

In general, immediately after damage has occurred to the epithelial/endothelial barrier, there follows a release of TGF with the recruitment of inflammatory cells and the induction of reactive oxygen species. This leads to recruitment and activation of collagen producing cells, matrix activation of myofibroblasts and collagen deposition. The source of the fibrogenic cells is quite interesting. Some are indigenous fibroblasts (or local fibroblast like cells such as myofibroblasts). Others are produced by epithelial to mesenchymal transition of local epithelial cells³⁰. There is still further recruitment of fibrocytes from the bone marrow⁵¹.

Normal wound healing is a precisely orchestrated response to tissue injury, from the initial platelet response immediately after the trauma, to the final remodelling of the scar tissue more than a year later. Radiation activates wound-healing machinery, but in addition to these processes the unique nature of radiation damage initiates a series of processes that are distinct from those involved in normal wound healing. These processes span the whole timescale of normal wound healing, and it is probable that it is this continued interference with the normal control of wound healing that leads to the excessive deposition of extracellular matrix (ECM) and collagen that is characteristic of radiation fibrosis⁵¹.

In contrast to normal wound healing, the radiation fibrogenic process can be perpetuated over periods of many years. This has led to some labelling radiation fibrogenesis as a 'wound that does not heal'⁵¹. Understanding the homeostatic feedback control of normal wound healing — and why this is dysfunctional in radiation fibrogenesis — would clearly represent a breakthrough in the attempts to develop interventions to dampen, or possibly even reverse, radiation fibrosis.

1.6 Some of the key players in radiation fibrosis

There are numerous proteins and other components involved in the process of fibrosis which could be discussed at this point. The focus of this thesis is PDGF. Chapter 6 is devoted to this growth factor. Amongst the others, some of the crucial key players will be outlined here, in particular TGF(β).

1.6.1 Transforming growth factor β (TGF)

TGF is a multi-functional cytokine, a 25 kDa polypeptide that is strongly profibrotic. When TGF was discovered it was classified as a growth factor, a term that reflects its involvement in the proliferation and differentiation of cells³¹. At that time, the term cytokine was used to refer to signalling proteins in the haematopoietic and immune systems, but as cellular molecular biology evolved it was realized that the same kind of cell–cell signalling also has a fundamental role in solid tissues. TGF can usefully be classified as a cytokine and a growth factor. It has attracted much interest in fibrosis research, and there is a large and rapidly growing body of knowledge on this protein and its biological action. TGF belongs to a superfamily comprising over 60 proteins in multicellular organisms, with at least 29 of these encoded by the human genome and more than a dozen related molecules in invertebrates³³. These proteins regulate a wide range of processes, including embryonic development, homeostasis, cell-cycle control and wound healing³³. Dysfunction of the TGF system seems to be involved in various severe human diseases, including immunodeficiency, cancer, defective wound healing and a long list of fibrotic diseases in the kidney, liver and lung, as well as arteriosclerosis, rheumatoid arthritis and scleroderma. TGF has a dual role in tumour suppression and tumour promotion^{52,53}. TGF is a potent inhibitor of endothelial cell proliferation, in the mammary gland for example⁵⁴. It has been proposed that during

malignant progression breast cancer cells might become refractory to the growth-inhibitory effect of TGF, and that TGF, through its effect on the extracellular matrix, might promote invasion and metastasis in patients with advanced disease⁵⁴⁻⁵⁶.

TGF(β) exists in three isoforms (TGF β 1–3), and these show a high degree of homology between various species³³. TGF is secreted in latent form and requires dissociation of the active mature TGF from a latency associated peptide (LAP) to bind its receptor⁵⁷. This means that a large extracellular pool of latent TGF can be rapidly mobilized after a triggering event. Ionizing radiation is one of the few exogenous factors that have been shown to induce TGF activation, and this happens within an hour or less of giving doses as low as 0.1 Gy^{58,59}. The active TGF binds to pairs of two distinct transmembrane receptors, TGFR1 and TGFR2, and it has been shown that TGFR1 is unable to bind TGF in the absence of TGFR2, and conversely, that the binding of TGF to TGFR2 does not activate the signalling pathway in the absence of TGFR1⁶⁰. The biological advantage of this relatively complex — compared with other similar ligand–receptor systems — activation of the signalling pathway is not clear, but it probably contributes to the versatility of the transcriptional response to TGF. The signalling pathway itself is rather simple: the only established intracellular signalling effectors are the Smad proteins, of which there are five R-Smads (receptor-regulated Smads; SMAD1, 2, 3, 5 and 8) that are directly phosphorylated by the type I receptor, a co-Smad (SMAD4) that forms heteromeric complexes with the R-Smads and two inhibitory Smads (SMAD6 and 7) that antagonize TGF signalling⁶¹. The final transcriptional response of a specific target gene is determined by a number of DNA-binding transcription factors and various co-activators and co-repressors⁵¹.

Ionizing radiation directly activates TGF through the dissociation of the latency-associated peptide (LAP) from the active mature form of TGF. Furthermore, radiation

damages endothelial cells, which in turn initiate a cellular response that also leads to the release of pro-fibrotic cytokines, including TGF. A third main effect of radiation is that it perturbs the homeostatic control of the reactive oxygen and nitrogen species (ROS and RNS), which again leads to the activation of TGF and directly interferes with the Smad signalling pathway. These extracellular events activate the TGF signalling pathway, which in turn produces various transcriptional responses, all of which lead to increased extracellular matrix (ECM) and collagen deposition³³. The radiation-induced vascular damage and uncontrolled tissue remodelling can lead to tissue hypoxia, which could be one of the mechanisms perpetuating the fibrogenic response.

One of the many effects of TGF is that it promotes terminal differentiation along a lineage from proliferation-capable progenitor fibroblasts to postmitotic functional fibrocytes⁶²⁻⁶⁵ and it has been suggested that the ratio between the number of colony-forming late and early progenitor fibroblasts would be correlated with the incidence of clinical fibrosis after radiotherapy. Two studies found some support for this hypothesis^{66,67} but concluded that the association was too weak to be used as a potential predictive assay in the clinic.

One puzzling aspect until recently was where the fibroblasts originated. Epithelial–mesenchymal transition (EMT) of cells has long been recognized as an important part of embryonic development, but more recent data suggest that EMT occurs during wound healing and fibrogenesis in adult tissues, including radiation fibrosis⁶⁸. In the case of renal fibrosis it has been estimated that more than a third of all disease-related fibroblasts stem from tubular epithelia⁶⁹. It is probable that EMT has an important role in radiation fibrogenesis, and studies have shown that the loss of SMAD3 blocks EMT and reduces fibrogenesis⁷⁰. There is also a strong experimental case for the mobilization

of bone-marrow stem cells^{71,72} and human mesenchymal stem cells⁷³ as an important element in the processing of radiation injury.

Although some of the signalling pathways are known in detail, it is still not clear why these cytokine cascades are continually perpetuated. One suggestion is that endothelial cell killing, which leads to vascular damage and subsequent tissue hypoxia, could drive radiation fibrogenesis^{74,75}. This model is supported by experimental studies of fibrosis in the mouse lung showing that moderate hypoxia is present in the lung at 6 weeks after irradiation — long before pathological signs of restrictive lung injury become manifest⁷⁶. There is also evidence in other fibrotic diseases that hypoxia is involved in their progression at least in the final stage of disease⁷⁷⁻⁷⁹.

1.6.2 Reactive oxygen and nitrogen species (ROS and RNS)

Another key element in fibrogenesis seems to be the homeostatic control of reactive oxygen and nitrogen species (ROS and RNS) in the cell. It has long been appreciated that ionizing radiation creates ROS in the cell¹, but after the discovery of the biological role of nitric oxide and its interaction with the superoxide radical O^{2-} , oxidative and nitrosative stress are now seen as two sides of the same coin. It has been proposed that O^{2-} is the initiator and NO and its derivatives the effectors of the activation of cytoplasmic signalling pathways²⁰. However, it has been shown that the total concentration of ROS is dwarfed by the contribution from cell metabolism and other sources: a 1 Gy radiation dose produces less O^{2-} averaged over the cell volume than a human cell produces in 20 seconds^{80,81}. Therefore, it would seem that it is the perturbation of the homeostatic control of ROS and RNS rather than the radical species themselves that drives the active biological response to oxidative and nitrosative stress. Superoxide dismutase (SOD) is the most important antioxidant enzyme, effectively

catalysing the conversion of O^{2-} to H_2O_2 and thereby controlling the concentration of superoxide⁸⁰. There is direct evidence that the SOD enzymes have an important role in the molecular pathology of fibrosis^{74,82-84}. The ROS and RNS pathways as interventional targets in fibrotic disorders is described below.

1.6.3 The renin–angiotensin system

The renin–angiotensin system (RAS) regulates blood volume, arterial blood pressure and cardiovascular function. Although research into hypertension has traditionally focused on haemodynamic regulation by the RAS, more recent research points to the role of angiotensin II in vascular remodelling and fibrosis as a pathogenic factor in this disease^{85,86}. The RAS has been known for some time to be implicated in kidney injury after irradiation, and studies in the early 1990s showed that the angiotensin-converting enzyme (ACE) inhibitor captopril reduced the level of kidney damage²⁰. Captopril contains a thiol group, and could therefore potentially function as a free-radical scavenger, but it has become clear that ACE inhibitors without a thiol group also work²⁰. Furthermore, it has been shown that these drugs are effective at doses that do not lower blood pressure and that other blood-pressure lowering drugs have no effect on radiation-induced kidney damage. Recently, Robbins and Diz⁸⁷ have reviewed the evidence that indicates that RAS might contribute to the development of radiation effects in other organs, for example the lung and the brain. As ACE inhibitors seem to decrease the risk of radiation effects irrespective of any systemic effects, it has been proposed that they act on locally generated angiotensin II, perhaps generated in response to oxidative stress. Angiotensin II has several functions, including an ability to upregulate TGF, and could therefore contribute to the fibrogenic response in irradiated tissue.

1.7 Summary of relationship between mechanism and effects of radiation injury

Acute radiation damage most commonly involves tissues with rapidly proliferating cells, such as in epithelial surfaces of the skin or alimentary tract. Symptoms develop after functional cells are lost as part of normal tissue turnover and are not replaced. This is because of impairment to the stem-cell compartment. In tissues such as the skin and gut, there is compensatory proliferation in the stem cell population, often involving the stem cell niche, which appear to be more tolerant to radiation than other types of cells. Subsequent replacement of functional cells leads to a degree of recovery. Symptoms tend to subside thereafter, usually during the course of radiotherapy.

The ionisation events and free radicals produced by radiation cause damage to various components of the cell, including vital cellular structures and processes. DNA damage from radiation commonly leads to cell death in the first cell division after irradiation or certainly within the first few divisions. Cell death during mitosis (mitotic death) is generally caused by unrepaired or improperly repaired chromosomal damage. Cell death may also occur by apoptosis. In some cell types (especially lymphocytes, spermatogonia, and salivary gland serous cells) undergo apoptosis during interphase after irradiation. This type of death is rapid. Cells may also leave the reproductive pool by differentiation rather than proliferation. This senescence may be a particularly important response of fibroblasts, resulting in excess collagen deposition and fibrosis.

Radiation also activates various cellular signalling pathways that lead to expression and activation of pro-inflammatory and profibrotic cytokines⁸⁸⁻⁹⁰ vascular injury⁹¹, and activation of the coagulation cascade⁹². These changes are involved in the development of oedema, inflammatory responses, vasculopathic processes and the initiation of wound-healing. Sometimes the acute reaction fails to resolve and it may persist for

some time. The resulting lesions, often called consequential late effects can contribute to the overall damage⁹³.

The late effects of irradiation develop many months or years after treatment. The symptoms may be mild or severe, self-limiting, or progressive, and may develop gradually or suddenly. In some instances there may be progression of the late effects for 20–34 years after therapy^{94,95}. Late effects tend to occur in tissues with a slow turnover of cells, such as subcutaneous tissue, fatty tissue, muscle, brain, kidney and the liver, including neighbouring sites of slow turnover within tissues that contain rapidly-proliferating cells, such as the wall of the intestine. The resulting lesions can be quite variable, but usually include fibrosis, necrosis, atrophy, and vascular damage.

Clinical and experimental animal data show that the fibrogenic pathway is heavily involved in the development and expression of many types of late side effects of radiation therapy, and it does seem to be a universal response to irradiation. Under the target-cell paradigm, the most important pathogenic mechanism was thought to be parenchymal cell depletion, and it is probable that this does have a role not only in early but also in late side effects of radiotherapy. However, in many cases this will be accompanied by a fibrotic–atrophic response, and it might not be possible to separate the relative contributions of the two in a given setting.

Late effects develop through complex interacting processes that appear to include cell death during proliferation. Cells co-exist in a complex community and all members depend on each other for the welfare of the whole tissue. Irradiation of tissue activates a rapid molecular response. Part of this response is the production of cytokines, which leads to an adaptive response in the surrounding tissue and cellular infiltration. Direct vascular damage and indirect release of vasoactive cytokines enables fibrin to leak into the tissues, thereby promoting local collagen deposition. In many ways, the response

has the features of general wound healing; except that the process often persists due to waves of cytokine production^{89,96}. Leukocyte adhesion to endothelial cells and thrombi can block local vessels, as can endothelial-cell aggregates during vascular regeneration, which can lead to down-stream loss of cells dependent on those vessels⁹⁷⁻⁹⁹. Additionally, death of parenchymal cells can lead to atrophy of the vessels supplying the tissue they make up¹⁰⁰. The response is often perpetuated by cell loss, dysregulated cell-cell interactions and hypoxia¹⁰¹. In some tissues accelerated senescence of stromal cells and their infiltration into sites of damage results in further fibrotic consolidation whilst in others it is necrosis that is the most serious complication. This is almost certainly a result of the reparative capacity of the tissue involved and the cell type injured – tissues composed predominantly of permanent cells most affected by necrosis whereas labile or stabile cell populations either regenerate well or may be replaced by fibrous tissue.

1.8 Non-fibrotic late effects

1.8.1 Radiocarcinogenesis

Carcinogenesis is an important consequence of radiation exposure, which is considered here. As mentioned previously epidemiological studies have documented a definite increase in a range of cancers after irradiation exposure, particularly at higher doses. The higher the linear energy transfer (LET) the higher the rate of tumours¹. Important clues to the effects of radiation and radiation-related carcinogenesis have come from patients who appear to lack the normal DNA repair apparatus alluded to above. Patients with ataxia telangiectasia (AT), Fanconi's anaemia and xeroderma pigmentosa (XP) and similar inherited disorders fall into this category and develop increased numbers of tumours after exposure¹³. Also mutant mammalian cell lines defective in repair of

double strand breaks are more radiosensitive than parental cell lines from which they were derived. The dose required for tumour formation appears to be important as extremely high doses seem to cause cell death and very low doses seem to have limited effects (we will come back to this later). At intermediate doses there is a peak incidence for tumour formation and several theoretical models have been developed in order to estimate the risk and discover whether low dose exposure is associated with an increased risk. The present models do not always reflect the epidemiological evidence and the negative studies do not rule out the possibility that cancer is induced by low doses as the effect may be so small that at present it is undetectable¹⁰². Radiation has been described as a universal carcinogen as it may produce tumours in virtually all tissues of all species at all ages. The mechanisms however are poorly understood, but with the obvious role of DNA/chromosome damage and alteration following radiation it seems likely that these structures are significant. In addition the production of proto-oncogenes and other tumour promoting proteins plus the downregulation or loss of tumour suppressor genes/proteins probably all have a role. For example it has been shown that p53 mutations may occur after irradiation although this protein does not seem to play a general role in radiocarcinogenesis⁸. Other possible influences include the inability to repair DNA damage due to lack of the relevant proteins, and autocrine effects of growth factors. There is also evidence that there is prolonged genetic instability and long term mutagenic events after irradiation thereby linking the well-known phenomenon of latency with DNA damage¹³. Whatever the mechanism, it seems likely that it follows at least some of the pathways now established for non-radiation induced cancers¹⁰³.

1.9 Factors affecting response

These are often separated into the physical, biological and chemical factors of each radiation episode. The physical characteristics of the radiation, total amount delivered and time in which it is given are important determinants of the response. The type of cell is also important as indeed is the tissue make-up and architectural arrangement. The tolerance of normal tissue depends upon its functional reserve and its structural organisation. For example, the lung is able to tolerate a high dose in a small volume, but is less able to tolerate a low dose to the whole lung¹⁸. Conversely, a high dose to a small volume in the spinal cord could be hazardous, but a low dose to a large area may be innocuous¹⁸.

There are several chemical factors that can either sensitise tissue to irradiation damage or actually protect it. Oxygen serves as a substrate for the production of free radicals and therefore increases damage, utilized in hyperbaric enhanced therapy. In fact this is the main reason for using hyperbaric therapy. Halogenated pyrimidines (DNA base analogues) are also tissue sensitizers. Protective chemical substances include sulphhydryl amines, cysteine and cystamine which act as free radical scavengers and therefore limit damage. Growth factors are not only stimulated by irradiation, but their presence has also been shown to affect the response to irradiation, and they play a role in radioprotection. This radioprotective role is established for haematological growth factors such as GM-CSF and G-CSF and it has been shown that bFGF increases the radioresistance of endothelial cells by stimulating damage repair¹⁷.

Biological factors include the phase in the cell cycle, with cells being more susceptible in G2 and mitosis than G1 and S phase. This may be because the repair proteins are present in the more resistant phases. Rapidly dividing cells are therefore more

radiosensitive than slowly dividing ones (the Bergondie/Tribondeau Law) and slowly proliferating tissues show their signs of damage months or years after exposure¹³.

The incidence and the severity or grade of a specific side effect depends on the details of how therapy is delivered but shows large variability among patients, even after delivery of identical treatment. Recently, the importance of this topic has been highlighted by the flurry of new radiation-treatment strategies in various stages of pre-clinical or clinical development. Experimental radiotherapies are not always better than the standard therapy — but they are often more toxic as they typically represent attempts to intensify therapy. Some divide the factors that influence the outcome of the radiation therapy into those related to the treatment, those associated with the patient and those related to the tumour.

1.9.1 Treatment-related factors

The effects of irradiation seem to depend on the dose and type of radiation (ionising versus UV radiation) and also the stage in cell cycle when irradiation is encountered. As noted above, cells in G2 and mitosis are more susceptible to the damaging effects of radiation. Treatment related factors are usually a reflection of the total and fractional dose and schedule; late changes appear to be associated more with changes in fraction size and less with changes in overall treatment time than early changes. Chemotherapy can exacerbate reactions, whether given at the same time or metachronously. Other drugs or compounds which alter cell dynamics can also increase radiosensitivity if given appropriately, and this is one mechanism being explored for enhancing the effects of radiotherapy for the treatment of tumours. The volume of tissue irradiated is also important. Larger volumes carry a higher risk of subsequent organ-function impairment.

Appreciation and manipulation of the dose-response curves for radiotherapy, together with the limited capacity of tumour cells for repair has led to the development of alternative models for radiation delivery, apart from single large doses. Methods include accelerated (standard fractions, but several in one day) and hyper-fractionation (lots of mini doses). Both can deliver the same overall dose but they allow one to focus a higher dose on the tumour rather than the surrounding normal tissues. Conformal radiotherapy uses the same radiotherapy machine as the normal treatment (this is called a linear accelerator) but uses metal blocks placed in the path of the x-ray beam to alter the shape of the beam¹⁰⁴. This means that the metal blocks can be carefully arranged for each patient to shape the treated area more precisely. More recently a device called a multi-leaf collimator has been produced as an alternative to the metal blocks. This consists of a number of layers of metal sheets, which are fixed to the linear accelerator. Each layer can be adjusted to a different position and so alters the shape and intensity of the beam of x-rays reaching the patient. A further development has been the ability to move the layers of the multi-leaf collimator whilst the actual treatment is in progress. It has been argued that this can give even more precise shaping of the treatment areas to the contours of the tumour that is being treated. This method of moving the collimator during the radiotherapy treatment is called intensity modulated therapy. There is evidence that conformal treatments reduce the number of side effects. NICE (the National Institute for Clinical Excellence) produced a manual in September 2002 ('Improving outcomes in urological cancers') which recommends conformal radiotherapy as the best type of external radiotherapy for prostate cancer¹⁰⁵. At present it is not known how intensity modulated therapy compares with standard conformal treatment.

In addition to dose and method of delivery, an appreciation of the effects of time fractionation has allowed radiation schedules to try to discriminate between tumour and

normal tissue. It has been found that around 6 hours is ideal for normal tissue to recover after a dose of radiation. However, some tumours repopulate extremely quickly and tumours are said to become tolerant. The current practice of fractionating radiotherapy treatments arose from observations that late effects were less severe and better local tumour control rates could be achieved with multiple, small radiation fractions than with one or a few large fractions.

1.9.2 Patient-related factors

Patients vary in their response to a specific course of radiotherapy¹⁰⁶. There are many reasons for this, as everyone is unique in terms of their individual anatomy, tissue and cellular composition. In fact, tissue type is an important determinant in the outcome of radiation delivery. As discussed previously, cells and tissue have different integral tolerances; in some areas of the body small volume high dose delivery is better tolerated than low dose to a wider area. The severity of response depends very much on the radiosensitivity of the target cell(s) and the architectural arrangement (functional subunits) of the tissue being irradiated.

There are also important general factors which influence the response to irradiation, in addition to the make up of the tissue. These include local and systemic features such as the presence of recent trauma, diabetes mellitus, vascular disease and medication (including anti-inflammatories and steroids) which will be familiar determinants of all wound healing processes. Age can also be considered an influencing factor, but age by itself must not be considered a reason for avoiding the use of a curative regimen.

As late effects of irradiation can be associated with significant human suffering and direct health-related costs, even costly treatment modifications would be justified in patients at high risk of developing late toxicity if they could be reliably identified by an

assay. At the same time, the identification of predictive markers could point to new interventional targets for ameliorating late effects. In addition, any break-through could be broadened to include the early detection and repair of cellular damage and the tissue-remodelling response in general, not only that caused by irradiation.

Genetic susceptibility has been mentioned before. This genetic influence can be readily appreciated with conditions which interfere with DNA damage repair such as AT. These patients develop severe radiation reactions because of defects in the repair of DNA after radiation damage. Furthermore, LOH studies have shown that many other genetic alterations which involve proteins that impact on inflammatory and fibrotic cytokine production and function could also influence the ability of an individual to tolerate their radiotherapy. Such alterations have been observed in patients with lung cancer and in tissues adjacent to breast and bladder tumours. This has fuelled research into phenotypic or genotypic predictive assays with the perspective of modifying therapy in radiosensitive individuals and perhaps intensifying therapy in relatively resistant cases.

It is clear that genetic variations have a role in determining radiosensitivity as observed in the hyper-radiosensitivity associated with some rare genetic syndromes such as Nijmegen breakage syndrome, Fanconi anaemia and ataxia telangiectasia (AT). Is it possible that anything could be learned from detailed study of these conditions which could be extrapolated to the general population? AT is a rare autosomal recessive disorder characterized by progressive neuronal degeneration, immunological deficiency and an increased incidence of cancer. The gene mutated in AT (ATM) encodes a kinase that amplifies the DNA-damage signal induced by DNA double-strand breaks. ATM and its downstream kinase CHK2 phosphorylate several targets that regulate DNA repair, cell-cycle checkpoints and apoptosis. The *in vitro* radiosensitivity of skin fibroblasts from AT homozygous patients is typically threefold higher than that of

normal human fibroblasts¹⁰⁷, and clinical case studies show that these patients have an extreme normal-tissue reaction to radiotherapy and might achieve local tumour control at a fraction of the radiation dose used normal¹⁰⁸⁻¹¹⁰. However, protein-truncating ATM mutations do not seem to be more prevalent among patients with breast cancer who have a pronounced reaction to radiotherapy than among patients who have a normal reaction¹¹¹⁻¹¹³. Among the downstream targets of ATM and CHK2 is the tumour-suppressor gene BRCA1^{114,115}. One might speculate that germline mutations in BRCA1 might lead to increased risk of early or late radiotherapy toxicity in the cases with mutated BRCA, but this does not appear to be the case.

Radiogenomics is the study of genetic differences in the response to radiation¹¹⁶. This is an emerging field of research in which attempts at investigating a possible genetic background for variations in clinical radio-responsiveness have concentrated on single nucleotide polymorphisms (SNPs) in selected candidate genes and the screening of multiple genes using gene-expression arrays either by genome-wide or candidate-gene studies¹¹⁷⁻¹¹⁹.

In a series of 41 patients that received postoperative radiotherapy for breast cancer, Andreassen et al. assessed 17 specific SNPs in TGF1, SOD2, XRCC1, XRCC3 and APEX, and found that 7 of these were associated with a significantly increased risk of developing severe subcutaneous fibrosis¹²⁰. However, applying a Bonferroni correction to the published data in their paper shows that only one of these (XRCC3 codon 241 Thr/Met) remains significant after the correction. ICAM deficient mice do not appear to develop fibrosis after pulmonary irradiation and patients with high TGFbeta, IL1 and IL6 levels have all been found to be at a higher risk of developing pneumonitis than those without¹²⁰. The early experience in this research field is sufficiently positive to

warrant further studies to try to arrive at true-positive SNPs associated with radiotherapy side effects.

The transcriptional response of normal cells and tissues to radiation has been the subject of a few studies using cDNA microarrays¹²¹⁻¹²³. Although these are also powerful discovery tools in radiation research¹²⁴ there is still a long way to go before we have a gene signature of value in clinical response prediction.

In summary, determination of the radiosensitivity of cells isolated from individual patients has not yet proved to be a reliable predictor for clinical use, except in rare cases of extreme radiosensitivity. Studies of the comparison of early and late responses in individual patients have shown that patients who have severe acute responses do not necessarily or predictably develop significant late reactions. This finding may be a reflection of differences in underlying mechanisms involved in the development of these types of injury.

1.9.3 The role of the tumour

In addition to the contribution of radiation itself, the presence of the tumour may predispose the surrounding normal tissue to injury. Tumours can alter or influence their surroundings in a number of ways. The presence of the tumour can physically distort normal tissue architecture resulting in defects that can add to damage produced by therapy. Tumours also release proteolytic enzymes that facilitate invasion and metastasis and which may alter the 'normal' radiation response¹. Tumour vessels are leaky. They allow fibrinogen to escape into the surrounding tissues, which is converted to fibrin, and which results in collagen deposition and fibrosis³⁰.

1.10 Clinical and tissue manifestations of radiation injury

Radiation injury varies from organ to organ, as described above. However, radiation is usually delivered to a localised area with consequent injury and symptoms. These are primarily the thorax (lung and breast tumours), head and neck, and pelvis (prostate and cervical tumours)¹⁸. In the following sections the general changes associated with locality irradiation will be outlined, followed by a more in depth description of organ-specific changes.

1.10.1 Radiation to the thorax

The lung is one of the most radiosensitive organs, yet one or both lungs are frequently irradiated as part of treatment programmes for cancers of the lung, oesophagus, breast and haematopoietic system. The early effects of radiation to the thorax become clinically apparent soon after radiotherapy (about 1–3 months) with congestion, cough, dyspnoea, fever and chest pain caused by pneumonitis (pneumopathy). The histological changes in the lung at this time include reactive changes and hyperplasia of type II pneumocytes with a decrease in parenchymal cells and surfactant secretion together with oedema and inflammatory cell infiltration and hyaline membranes⁹⁷. Radiographic studies show changes which reflect these findings with an infiltrate within the irradiated field. The same changes can also be caused by systemic or inhaled toxins, various drugs, infections, and tumour recurrence and these need to be differentiated clinically as the pathological and radiological features can be identical, whatever the underlying cause⁹⁷. The dyspnoea is variable and rather unpredictable, it can be mild or severe. In severe cases, hypoxaemia and signs of right-sided heart failure may be present. Partial lung irradiation occasionally induces a bilateral, immunologically-mediated pneumonitis that generally resolves without treatment¹²⁵.

The inflammation induced by radiation generally subsides after a few weeks. In many cases it is followed by a phase of chronic inflammation and fibrosis that develops months or years after irradiation, and which can progress for a considerable time afterwards. In this phase, vascular damage and collagen deposition become more obvious^{97,126,127}. If the volume affected is small, the patient may be asymptomatic and the scarring may be detected as an incidental finding radiographically. If a larger volume has been irradiated, the patient may develop symptoms due to a reduction in the diffusion capacity and respiratory volume caused by pulmonary fibrosis and scarring with surrounding tissue retraction¹²⁷. A positron emission tomography (PET) scan may help to distinguish radiation injury from tumour recurrence because the latter may be more metabolically active.

Current treatment approaches for severe acute radiation pneumonitis (assuming there is not an alternative cause for their acute respiratory distress) include the use of systemic corticosteroids¹²⁷. Supplemental oxygen, and even mechanical ventilation, may occasionally also be necessary. In the future it may be possible to predict patients at high or low risk by measuring profibrotic or proinflammatory cytokines (or both) in the circulation, thereby enabling individualisation of treatment fields and dose on the basis of a risk profile for normal tissue injury¹²⁸. Overall, strategies to prevent radiation injury to the lung are likely to be more effective than subsequent treatment of the problem when it arises. Recent improvements in imaging and computer technology have contributed to the development of 3-dimensional conformal radiotherapy (3DCRT) and intensity-modulated radiotherapy (IMRT), which enable more precise delivery of the dose distribution to the tumour, with deliberate avoidance of sensitive normal tissues¹²⁹. The normal tissue within the treatment field should be able to tolerate these higher doses because of the smaller volumes involved. In the case of thoracic tumours, gating techniques are being used to minimise or accommodate tumour movement during

breathing¹³⁰. However, these methods are relatively new and the long-term consequences are not yet known.

1.10.2 Radiation to the head and neck

The skin, mucosa, subcutaneous tissues, bone and salivary glands are often affected when radiotherapy is used in the treatment of head and neck cancers. In the skin, the early responses include erythema and dry or moist desquamation, the latter resulting from loss of proliferating cells with subsequent failure to replace functional cells¹³¹. The reaction is commonly accompanied by itching, hypersensitivity and/or local pain. Symptoms usually develop during a course of treatment and often subside before the end of the treatment, but dermatitis may not resolve until weeks later.

Mucositis is caused by a similar process as dermatitis, resulting from loss of functional cells with a temporary lack of replacement from the pool of rapidly proliferating cells¹³². If mucositis is severe, the patient may have difficulty eating and a feeding tube may be necessary to provide adequate nutrition. This is especially common with very intensive combined modality treatment or multiple daily fractions. A temporary interruption in treatment may be required.

Similar microscopic changes are seen in the skin and mucosa with typical inflammatory features such as vascular congestion, vasodilation, and plasma leakage, with denudation of the epithelium. Later effects in the skin include alopecia, pigmentary alterations, telangiectasia, atrophy, fibrosis and ulceration. Although acute effects occur mostly in rapidly proliferating cells of the epidermis and mucosa, these later changes largely reflect damage to the vascular and connective tissues. Microscopically there may be atrophy, atypical epithelial cells, vascular lesions and fibrin exudates leading to collagen deposition⁹⁷. Again there may be individual differences between patients with high

concentrations of salivary epidermal growth factor before and during radiotherapy apparently be associated with less oral mucositis¹³³.

The salivary glands, particularly the parotid glands, are frequently irradiated during treatment of tumours of the head and neck. The parotid glands contain serous cells that are radiosensitive and die by apoptosis¹³⁴. The more resistant submandibular and sublingual glands contain mucous and serous cells. Damage occurs primarily in the parenchyma of the salivary gland, rather than in the ducts, but inflammation, vascular changes and oedema all contribute to the damage¹³⁵. Functional impairment correlates well with the volume exposed and the radiation dose¹³⁶. Clinically, swelling and tenderness often develop after the first treatment, but they generally subside within a few days. Xerostomia is the most common and distressing symptom because saliva becomes scant, sticky and viscous during a course of radiotherapy. It makes eating, speech and wearing dentures difficult. It may take months or years for this to improve and return to normal, if it occurs at all¹³⁵.

Xerostomia can cause the patient to become more susceptible to fulminant dental caries. Carious teeth can result in infection of the underlying bone leading to osteoradionecrosis. This complication is more common in the jaw bone than in the upper jaw bone, because of the relatively poorer blood supply of the former¹³⁷. Osteoradionecrosis may be prevented in most patients by removing unsalvageable teeth before treatment and initiating a programme of aggressive prophylactic dental care, including daily fluoride rinses. Antibiotic therapy and resection of devitalised bone may be necessary. Xerostomia can be treated by saliva substitutes, sialogogues water, and sugarfree sweets and gum. The radioprotector amifostine, given before each fraction of radiotherapy, reduces the incidence of xerostomia¹³⁸. Treatment plans using 3DCRT and

IMRT can be designed to reduce the dose of radiation to the salivary glands, particularly the volume exposed to high doses (conformal avoidance)¹²⁹.

1.10.3 Radiation to the pelvis

The rectum is the area most often affected by pelvic irradiation for treatment of prostate and cervical cancer. Acutely, diarrhoea results from loss of integrity of the epithelium and increased secretion of mucus. The tissues develop oedema and hyperaemia. The most common late effects include increased stool frequency, urgency, spotting of blood and partial incontinence. Less commonly mucosal ulceration, severe bleeding, pain, strictures, severe incontinence and fistulae may develop¹³⁹. Fibrosis and ischaemia in the submucosa and muscularis, accompanied by telangiectasia and other vascular abnormalities lead to collagen deposition and abnormal fibroblasts which causes the mural changes described above¹⁴⁰. The risk of complications depends chiefly on the total dose and the amount of rectum in the treatment field. Brachytherapy (radioactive implants) techniques, 3DCRT and IMRT, have been used so that an escalation of dose to the tumour can be accomplished without an increase in normal tissue injury^{141,142}.

Treatment of rectal complications includes oral anti-inflammatory agents, pain management, stool softeners, intrarectal steroids, blood transfusions (for bleeding) and dilatation of strictures. For serious or refractory complications, hyperbaric oxygen or surgical intervention with temporary or permanent colostomy may be required.

1.11 Specific tissue effects of radiotherapy¹³

It will become clear that many of the following represent a combination of the general effects outlined above and specific tissue effects, together with

functional/symptomatic effects that may not correlate with tissue changes. Functional disturbances may also occur but they will not be documented in any depth here.

1.11.1 Nervous system

Radiation damage occurs to the brain, spinal cord and peripheral nerves. Neurons are very radioresistant but the supporting cells are less so. The acute changes manifest as increased vascular permeability with vasogenic oedema and a rise in intracranial pressure. If severe, areas of necrosis that are either focal or diffuse may be produced predominantly affecting the white matter, but with some extension into the deeper layers of the grey matter (a feature said to be specific for radiation injury¹⁴³). These areas are surrounded by demyelination (possibly due to oligodendrocyte damage), macrophage infiltration (but fewer than in ordinary cerebral ischaemia) and astrocyte changes. In fact radiation may accelerate demyelination in multiple sclerosis and irradiation may produce transient disruption of myelin synthesis. The necrotic areas may lead to cystic degeneration and subsequent calcification. Later changes also include reactive astrocytosis and gliosis, astrocyte atypia with nuclear vacuolation and hyperchromasia and radiation vasculopathy (Fig. 2). The latter comprises endothelial cell proliferation, luminal narrowing, fibrinoid necrosis, medial hyaline thickening and luminal thrombosis. In children mineralising microangiopathy may be seen¹⁴³. These vascular changes often result in coagulative necrosis. Cerebral radionecrosis may present as a space occupying lesion which can mimic post therapy relapse due to its latency¹⁴⁴. It should be remembered that radiation injury to the brain may not only produce morphological disturbances but a combination of

leukoencephalopathy and cerebral atrophy will lead to intellectual and functional problems.

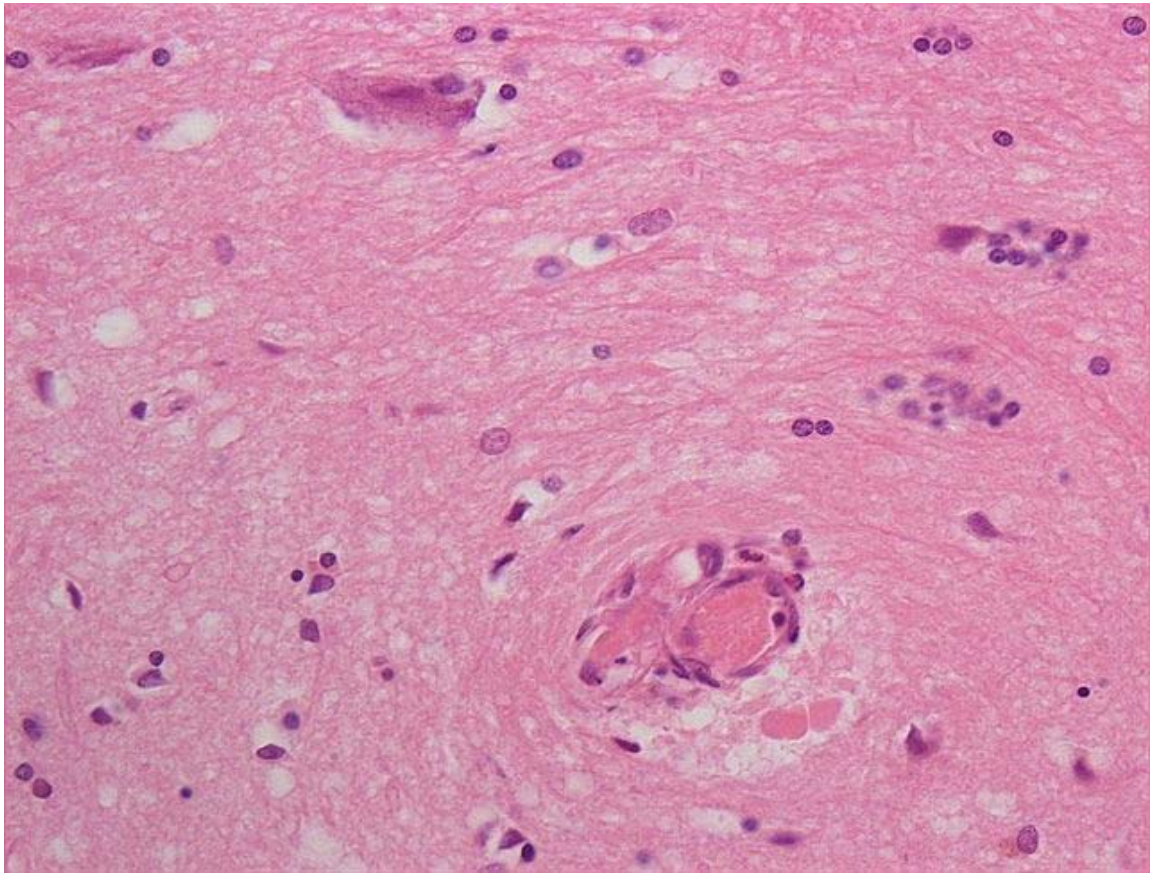


Figure 2. Vasculopathic changes in the brain after irradiation. Picture of MS

The changes in the spinal cord are similar but they manifest earlier due to shorter latent period¹⁴⁵. Areas of necrosis with surrounding demyelination represent acute transient myelitis which occurs 2-4 months after exposure. This is probably due to transient demyelination of ascending sensory neurons and it usually recovers. However delayed myelitis tends to be more permanent, occurs at around 20-30 months and affects the lumbar and thoracic areas predominantly. The lower motor neurons (LMN) may also be secondarily affected by vascular damage, but the subsequent atrophy of these cells may just reflect their very slow dividing times and hence manifestation take some time to appear.

Peripheral nerves are one of the most resistant structures to irradiation but a peripheral neuropathy may occur. It is difficult to establish the direct role of radiation in this, and to exclude other factors such as surgery or drugs. Perineural inflammation and thickening has been described around the peripheral nerves in the pelvis after irradiation with ganglionitis²⁴.

The association of radiation with intracranial tumour formation has long been known with gliomas and meningiomas both being more common after radiation.

1.11.2 Cardiovascular system

There is an extensive literature describing the effects of radiation on the heart as the heart is frequently included in the radiation field of thoracic and breast tumours. Irradiation of mediastinal tumours appears to have the greatest effects. It is said to affect 2-9% of patients who receive mediastinal irradiation and 3-4% for breast cancer¹⁴⁶. In addition to the heart itself changes in peripheral vessels have been appreciated for a long time and it is now clear that the changes are not only a

morphological marker of irradiation, but they are a vital component in the mechanism of delayed injury²⁵. The most consistent acute event after cardiac irradiation is the production of a pericardial effusion (rarely producing tamponade). This is associated with a fibrinous pericarditis which if protracted may organise and form fibrous adhesions. In fact radiation is now one of the most common causes of chronic constrictive pericarditis in the USA¹⁴⁶. Early myocardial functional changes are also observed with transient decrease in left ventricular function. Myocardial fibrosis results from a combination of myocyte necrosis and repair, diffuse capillary damage with secondary fibrosis and ischaemic fibrosis from coronary artery narrowing. This fibrosis is pericellular and perivascular and a moderate/severe degree was found in 50% of hearts examined in one study¹⁴⁶. The administration of cardiotoxic chemotherapeutic drugs may exaggerate these myocardial changes. The cardiac myocytes may be hypertrophic. As there is virtually no inflammatory cell infiltrate it has been suggested that radiation-induced cardiomyopathy is a better term for this than myocarditis¹⁴⁶. Later consequences in the endocardium are thickening that commonly involves the valves. The left sided valves show fibrosis and calcification more often than the right and functional regurgitation may occur. It is not clear why pulmonary valve changes are seldom seen although this valve is the most anterior. ECG changes may reflect conducting abnormalities which may be secondary to conducting system fibrosis. Radiation induced changes in the coronary arteries are well established with intimal thickening, foam cell accumulation and medial fibrosis resembling atheromatous plaques. It is not clear whether these changes reflect potentiation of atherosclerosis, but similar changes may be seen in medium sized arteries at any irradiated site. A helpful differentiating feature may be the less frequent presence of cholesterol clefts²⁵.

Obviously similar changes elsewhere lead to vascular occlusion and delayed ischaemia.

Small peripheral arteries seldom show significant acute changes but endothelial swelling and hyperchromasia may be seen. In the longer term there may be intimal fibroplasia and hyaline change to the media similar to systemic hypertension with possible fibrinoid necrosis, thrombosis, subendothelial fibrosis, accumulation of foam cells, adventitial fibrosis and even rupture. In medium sized arteries again delayed changes predominate and are similar to those in smaller vessels although intimal thickening may be more prominent and late medial fibrosis is likely to reflect earlier inflammatory changes. Obviously the earlier changes need to be differentiated from systemic vasculitis. Large arteries are the least affected probably because the vessel wall provides strong support for the damaged endothelium and the lumen is larger thereby preventing occlusion. Although uncommon with severe injury the consequences may be the most dramatic with occasional rupture which is usually fatal. This is a result of transmural necrosis of the wall and may reflect vasa vasorum injury. The capillaries are the most sensitive vessels to acute injury and may show postirradiation endothelial cell swelling with narrowing or occlusion of the lumen. Subsequent platelet and fibrin thrombi may be seen and possible rupture of capillary wall occurs with microhaemorrhage. There may be compensatory proliferation but vascularisation remains reduced and is an important factor in radiation heart disease, pneumonitis and nephropathy. Later on a consistent feature of these small vessels is telangiectasia.

Veins appear more resistant to radiation damage than arteries but fibro-obliterative changes may occur and are an important feature in hepatic radiation damage²⁵ (see liver section).

1.11.3 Respiratory system

Irradiation of head and neck tumours and upper respiratory lesions may lead to laryngeal complications in as many as 23% of patients¹. One of the most common features of laryngeal or tracheal irradiation is early transient oedema which may be followed by fibrin deposition in the stroma. Later evidence of irradiation manifests as telangiectasia, subepithelial fibrosis, epithelial cell atypia and the vessel changes described above. Other changes include vocal cord fibrosis, ulceration and the presence of granulation tissue with bizarre fibroblasts. Glandular atrophy occurs late occasionally with a histological appearance similar to necrotising sialadenopathy¹⁴⁷. The latter is thought to result from ischaemic compromise. A fairly specific change in this region is aseptic necrosis of the cartilage.

Radiation damage to the lung and pleura commonly follows irradiation of lung, mediastinal or breast tumours. The effects depend on any coexisting lung disease such as COPD. The lungs are very vascular and therefore very susceptible to irradiation injury. Again the effects depend on the dose and volume of lung exposed. If the whole lung is irradiated then 10-15% will develop radiation pneumonitis defined as decreased lung function, dyspnoea and dry cough appearing 3-16/12 after irradiation with X-ray changes in the field exposed¹⁸. Histologically the early changes comprise interstitial and intra-alveolar oedema (due to increased capillary permeability), endothelial swelling, scanty

inflammation with foamy macrophages. Later changes are dominated by fibrosis (Fig.3).

Epithelial cell damage occurs some time after with necrosis of type I pneumocytes followed by hypertrophy and hyperplasia of type II pneumocytes. In more severe cases hyaline membranes may form and the histological picture is that of ARDS. After around 8-15/12 there is delayed fibrosis with atelectasis and occasionally bronchiolitis obliterans. Vessel changes are once again present with myointimal proliferation, intimal foam cells and with fibrosis leads to increased pulmonary artery pressure. There may be coexisting infection. Pleural effusions and similar changes to those seen in the pericardium may be seen in the pleura. Radiation is associated with lung cancer and the latent period is over 9 years. The most common type in uranium miners is small cell carcinoma. Smoking has at least an additive effect.

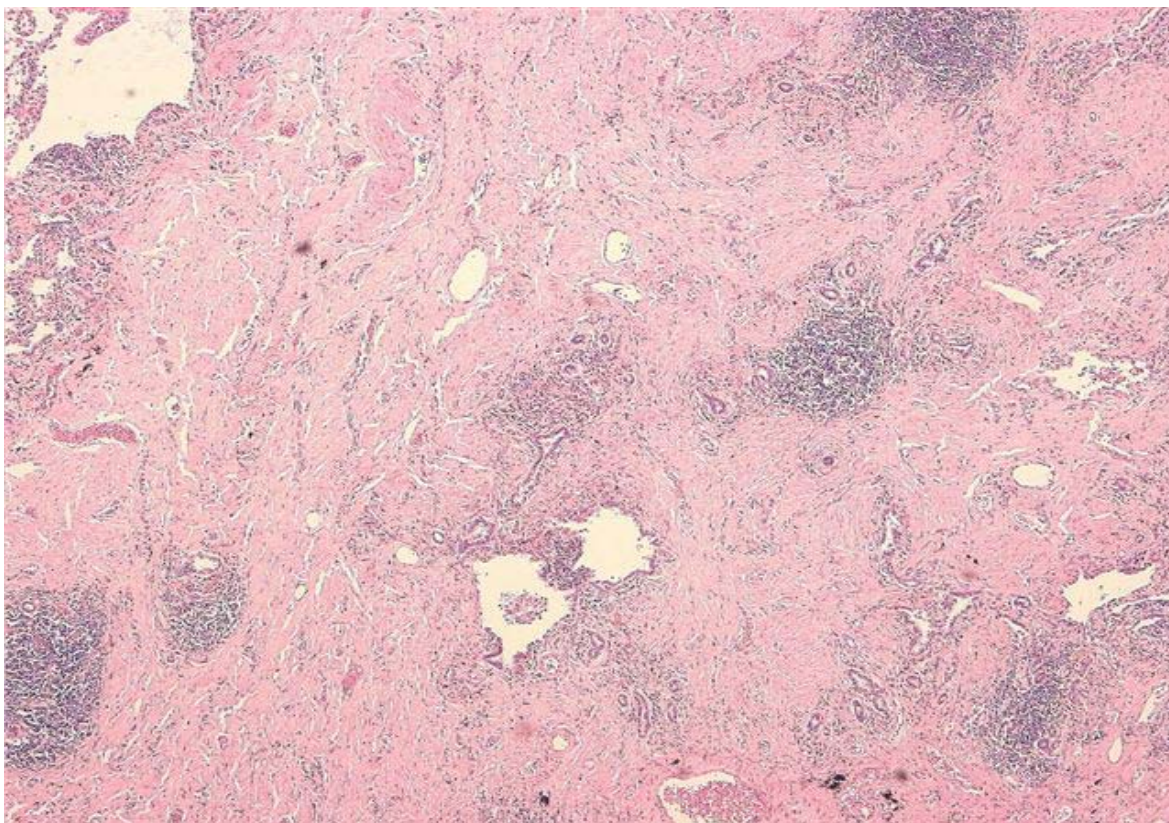


Figure 3. Fibrosis and chronic inflammation in the lung many years after thoracic irradiation. Picture of MS

1.11.4 Gastrointestinal system

The changes throughout the gastrointestinal tract follow a similar pattern, but the severity and timing of the effects vary depending on the site and the mobility of the particular segment involved, and additional factors such as recent surgery or coexisting diabetes mellitus, hypertension or vasculitis. The small bowel and stomach are more sensitive than oesophagus and rectum; predominantly a reflection of rate of cell turnover. Post-radiation carcinomas have been described at all sites in the body.

The early changes seen in the oropharynx are epithelial depletion with erosive mucositis and vascular dilatation. Later this may progress to chronic ulceration and submucosal fibrosis. Delayed vessel changes may be conspicuous and lingual necrosis has been observed¹⁴⁸. A picture resembling necrotising sialadenopathy is occasionally seen. Salivary gland changes have been well studied with early oedema and swelling, epithelial necrosis and desquamation accompanied by mild inflammation. The latter includes many eosinophils. Serum amylase is usually raised. Delayed manifestations include interstitial fibrosis with telangiectasia, squamous metaplasia, atrophy and residual inflammation. Arteriosclerosis is once again a feature.

The oesophagus shows similar mucosal injury with early erosive mucositis followed by fibrosis, ulceration, stricture formation, fistulae, perforation and either atrophic or thickened epithelium. The latter may show radiation-induced cellular atypia. Functional disturbances may result with abnormal motility and dysphagia. Candidal infection may be superimposed.

Radiation therapy used to be employed to treat gastric ulcers. However it is now recognised that irradiation is associated with chronic inflammation and epithelial

degeneration leading to ulcer formation. In the long term, gland atrophy and chronic atrophic gastritis may occur with any of the other complications of vascular compromise seen elsewhere in the gastrointestinal tract.

The small intestine is the most sensitive section of the tract. The crypt cells proliferate and move to the tips of the villi to replace effete epithelial cells at the surface. Radiation impairs this process and leads to surface erosion which allows bacterial colonisation and fluid loss. Villous atrophy follows and malabsorption may occur. In the lamina propria there is a variable increase in fibrous tissue with inflammation and telangiectasia. There may be subepithelial collagen deposition mimicking collagenous colitis/sprue. In the submucosa vascular changes predominate. Progressive damage leads on later to chronic ulceration, stenosis, haemorrhage and adhesions. Other complications include fistulae, mucosal atrophy and vascular intimal thickening. A recent study indicated that the incidence of chronic irradiation enteropathy is significant with 18-30% of patients receiving between 50-60Gy having manifestations. This appears to be increasing¹⁴⁹. These authors found moderate to severe submucosal oedema in 86% of cases, intramural or serosal fibrosis in 76%, vascular injury (including foam cell plaques) in 57%, mucosal degeneration in 81%, atypical fibroblasts in 81%, subepithelial collagen in 10% and enteritis cystica profunda in 38%. Epithelial cell atypia was seen in 33% of cases¹⁴⁹. Ulceration was significantly more common in the long term group. Vascular changes alone do not account for many long term effects such as ulceration seen in the gut and the histology is distinct from ischaemic colitis. Therefore it is proposed that chronic injury results from a combination of vascular compromise together with other tissue components such as fibroblasts, smooth muscle cells or epithelial cells.

In the colon, sigmoid and rectum an early feature is inflammation with prominent eosinophils and eosinophilic abscesses¹⁵⁰. The epithelial cells may show atypia. There may be peritoneal fibrin deposition with subsequent organisation and fibrous adhesions. Mucosal ulceration may occur late with the formation of strictures or fistulae. Chronically there is shortening and fibrosis of colon with mucosal atrophy. Colitis cystica profunda may be seen. Vessel changes resemble those at other sites within the tract. The development of adenocarcinoma following atypia of the epithelium has been described many years after radiation exposure.

The liver has an intermediate sensitivity to radiation. The effects are more pronounced in children and in the regenerating liver. There may be direct hepatocyte necrosis, but as the liver has a large regenerative capacity it is usually the vascular effects that predominate. Early after irradiation one can see a mild hepatitis with sinusoid congestion, hyperaemia, central haemorrhage, fibrin deposition and intravascular coagulation leading to secondary necrosis and atrophy of central hepatocytes. There is central vein dilatation with progressive liver cell plate atrophy and decreased reticulo-endothelial cell function. In the subacute period, at about 3-6 months, fibrosis begins around the small central veins, lobular or sublobular veins and sinusoids forming a coalescent net leading to hepatic veno-occlusive disease. Later, after six months the liver is markedly less congested but atrophy is worse with increased collagen and parenchymal replacement. There is little information on the ductal epithelial cell changes. Thorotrast (thorium dioxide - an alpha emitter) was introduced for diagnostic radiology in the 1950s. This localised to phagocytes and was associated with several benign and malignant conditions. In the liver storage in Kupffer cells led to peliosis, cirrhosis, hepatocellular carcinoma, cholangiocarcinoma and

angiosarcoma. The latent period was long (15-40years). There is very little information about radiation changes in the gallbladder, but it is likely that the changes can be predicted from other mucosal sites.

In the pancreas there may be evidence of necrosis and lymphocytic infiltration followed by atrophy of the exocrine pancreas, fibrosis and vessel changes. The degree of fibrosis appears to be less in the islets although the endocrine cells are more sensitive than the exocrine.

1.11.5 Skin

Acute post-irradiation changes include epidermal intracellular oedema, appendage degeneration, dermal inflammation, erythema and oedema of the dermis due to capillary dilatation and increased vascular permeability with red blood cell extravasation. Epidermal necrosis may be seen with ulceration extending into the upper dermis. Less severe damage results in bulla formation which may heal or may lead to wet desquamation and pigmentation. Epilation may occur due to follicular necrosis with dry desquamation following sebaceous unit necrosis. Vascular injury may be seen in the subacute phases.

Chronic radiodermatitis shows epidermal atrophy with variable hyperkeratosis which is dry and shows hyper and hypo-pigmentation. The former results from increased melanocyte enzyme activity. Keratinocytes may show atypical nuclear features and individual cell keratinisation. The dermis becomes less cellular and develops homogeneous and hyaline collagen fibrosis with scattered plump fibroblasts. The superficial vessels are dilated, but the deeper ones show thickening of the vessel walls with luminal narrowing with possible occlusion by

thrombus. Secondary ulceration may develop. This damaged skin is susceptible to traumatic injury with poor healing and a propensity to dysplasia or malignancy. The finger nails may show ridging.

As early as 1902 an x-ray tube worker developed a squamous cell carcinoma on his hand and since then several studies have shown a link between irradiation and cutaneous neoplasia¹⁵¹. One unfortunate example is the development of basal cell carcinomas on the scalp of children treated with radiotherapy for tinea capitis¹⁵². As is the case with radiation-induced tumours there was a significant latent period of around 20 years. It appears that the tumorigenic effects are enhanced by prior or subsequent UV exposure.

1.11.6 Bone marrow and lymphoid tissue

Following irradiation of the bone marrow there is a decrease in the nucleated cells (myeloid more than erythroid) with necrosis and panhypoplasia resulting in cytopenia. Repopulation occurs early and in fact there may be compensatory hyperplasia in the early stages. If repopulation is not complete (for example with high doses, large fields of exposure or coexisting marrow disease) there may be fatty replacement with an increase in loose fibrous tissue. A relative increase in plasma cells may be observed due to hypocellularity of the other cell lines (Fig. 4).

In 1911 the leukaemogenic effects of irradiation were first noted and now it has become established that haematological malignancies are the most common radiation-induced malignancies with a latency of at least 3 years¹⁵³. All types of leukaemia have been described including multiple myeloma except for CLL and many cases followed spinal x-rays for ankylosing spondylitis.

Lymphocytes are extremely radiosensitive and are unusual in that they die in interphase. In addition to cell death, functional disturbances in homing and recirculation have been described. Any lymphoid aggregate including the thymus suffers the same fate of lymphocyte necrosis and subsequent atrophy, although subpopulations of lymphocytes appear to have differing sensitivities. In the lymph node there is cortical depletion with capsular and trabecular fibrosis, calcification, atrophy and fatty replacement. Obliterative endarteritis may be seen. With metastatic disease, there may be tumour necrosis or subsequent hyalinisation of a necrotic area. Radiation may be associated with lymphoid neoplasia due to immune surveillance damage as there were increased lymphomas in pioneer radiologists. However this association has not been identified in atom bomb survivors.

In the spleen early features include large atypical cells, prominent plasma cells and eosinophils and focal haemorrhage. This is followed by parenchymal collapse, atrophy, red pulp fibrosis and capsular thickening. The almost universal vascular changes may be seen here also.

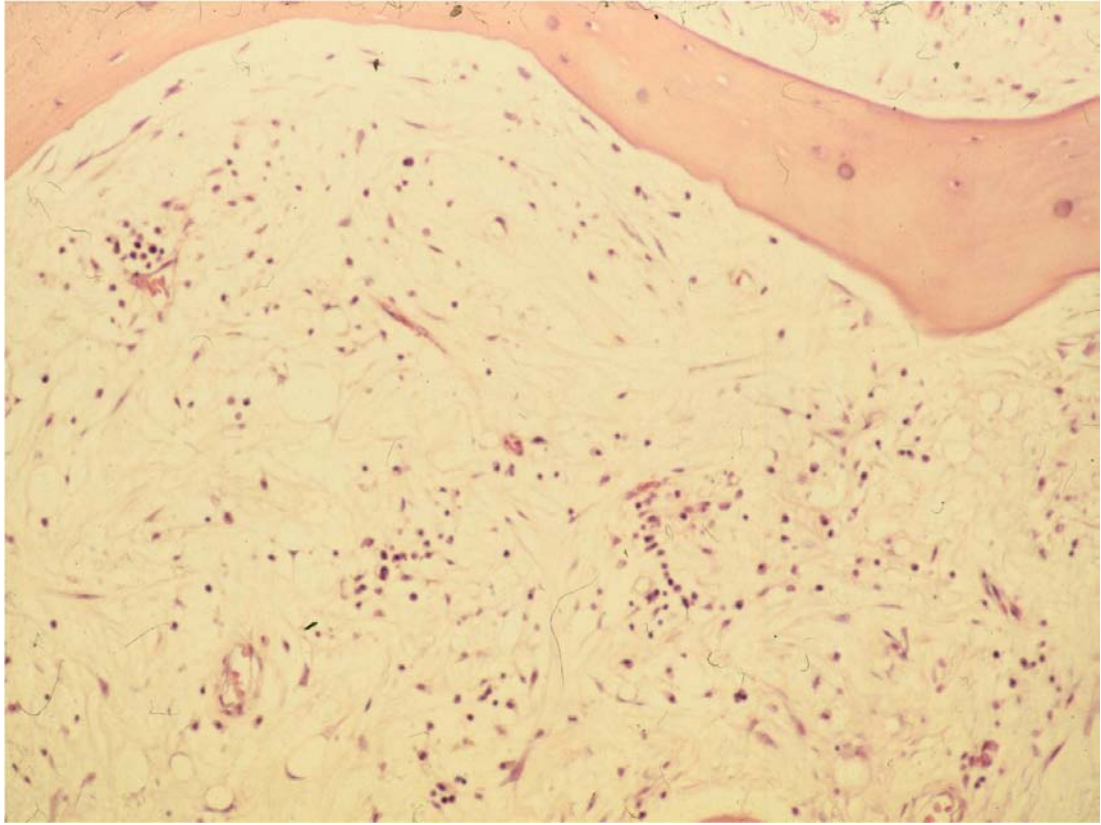


Figure 4. Bone marrow after irradiation with loss of haematopoietic components and occasional plasma cells remaining. Picture of MS

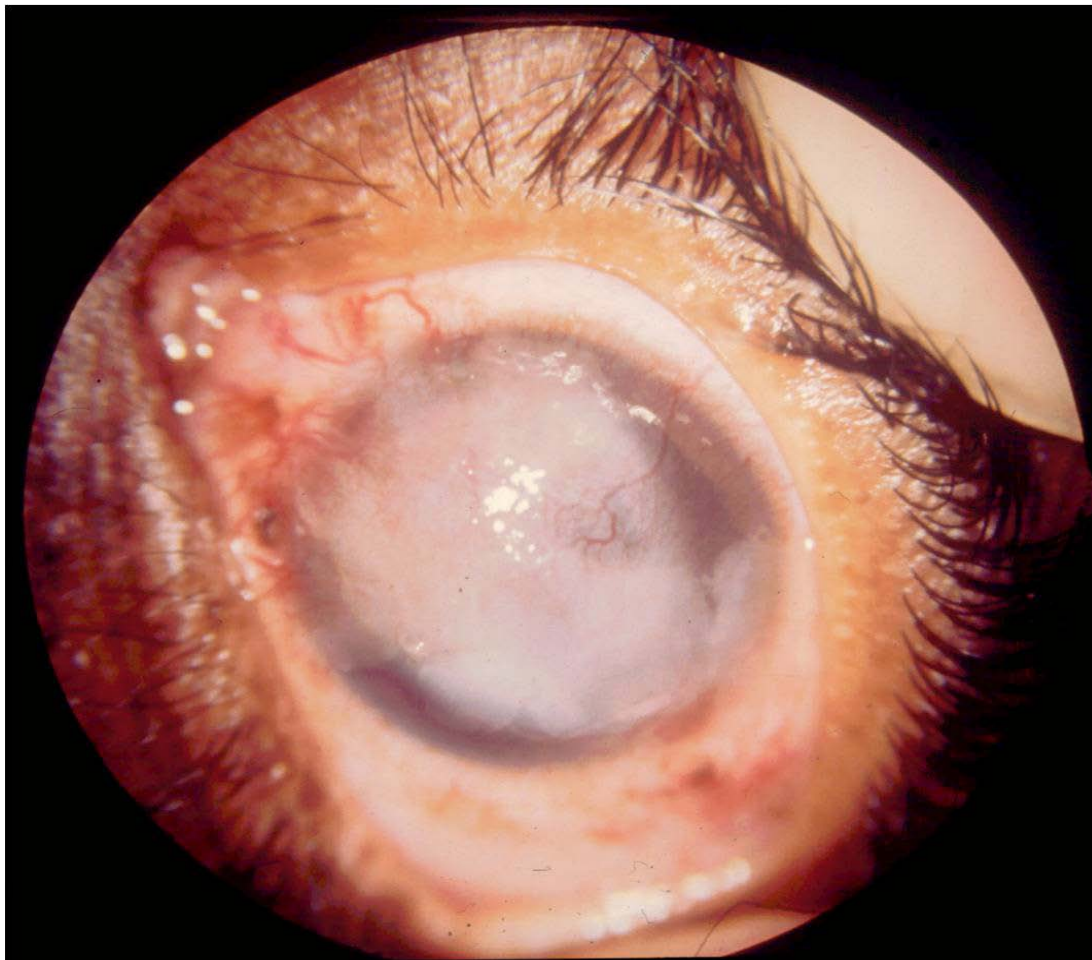
1.11.7 Locomotor system

Changes in the bone and cartilage are obviously very dependent upon age. Asymmetrical growth is produced by radiation to the growing skeleton. In the adult skeleton severe radiation damage results in radionecrosis of bone or cartilage with osteoblast and osteocyte necrosis¹⁵⁴. The maxilla, femur and pelvic bones are the most commonly involved. This is usually accompanied by the marrow changes described above and vessel changes leading to ischaemia. Radiation induced fractures have been described. With large doses chondrocalcinosis, skeletal muscle oedema, necrosis, atrophy and fibrosis have also been described. Radiation is associated with the development of several skeletal tumours including osteochondromas and osteosarcomas along with soft tissue tumours such as malignant fibrous histiocytoma and angiosarcoma¹⁵⁵.

1.11.8 Eye and ear

The eye of a child is more susceptible to radiation damage than that of an adult and exposure in utero (especially first trimester) is associated with significant ophthalmic complications. Damage may occur to the retina, lens or neurovascular supply. Fetal exposure may lead to microphthalmia, retinal pigmentation and cataract formation¹⁵⁶. Lens opacities also follow irradiation of the eye after birth with degeneration of lens fibres beginning posteriorly and leading to cataract (Fig. 5). Other structures damaged include optic neuropathy and retinal and ciliary artery damage. The epithelial changes resemble those seen elsewhere with keratitis and iritis and possible scarring. Retinal damage reflects vascular damage

and manifests as haemorrhages, exudates and microaneurysms with subsequent atrophy. Diabetes mellitus and chemotherapy add to these effects.



**Figure 5. A cataract which had developed after radiotherapy to the eye region.
(Courtesy of Professor C Berry)**

Acute hearing loss with tinnitus has been described after irradiation with the predictable capillary hyperaemia, exudate, vascular damage and sequelae, and otitis media. Ossicle necrosis due to vascular damage is also sometimes seen¹⁵⁷.

1.11.9 Endocrine system

The endocrine organs are remarkably radioresistant when one considers how vascular they are. This obviously reflects the variable sensitivity of endothelial cells in different sites²⁵. In general the changes in the endocrine glands are early degeneration with secondary vascular changes and necrosis or atrophy. Although the glands are relatively radioresistant, endocrine abnormalities are commonly documented after irradiation¹⁵⁸. This probably reflects functional disturbances that may not be associated with morphological abnormality. The parathyroid is said to show hyperfunction after irradiation and atrophy if it is embedded in the thyroid gland¹⁵⁹. The adrenals show a limited biological response to stress after irradiation and most effects are secondary to vascular damage. Damage to the pituitary gland results in necrosis (growth hormone producing cells most sensitive), fibrosis and cystic atrophy. Functional hypopituitarism may take years to manifest but secondary effects on target organs should be predictable. Pituitary hypofunction may also result from hypothalamic damage (the hypothalamus is the most radiosensitive area in the brain; even more than the surrounding white matter) and in children growth retardation may result. A late complication of pituitary irradiation is tumour formation with most documented cases being astrocytomas. Other tumours include meningiomas, oligodendrogliomas, osteosarcomas and fibrosarcomas.

Within 2 weeks of irradiation of the thyroid follicular destruction can be seen with epithelial cells shed into the lumen. Multinucleated cells may be present and vessel changes comprising thrombosis and haemorrhage are often seen. There is interstitial oedema and a lymphocytic infiltrate. Later changes again result from vascular sclerosis with ischaemic fibrosis and atrophy, especially at the centre of the gland. Irradiation of young children is associated with a range of thyroid disease such as solitary or diffuse nodular formation with goitre, thyrotoxicosis (including thyroid storm), hypothyroidism, chronic lymphocytic thyroiditis, adenomas and carcinomas (especially papillary)¹⁶⁰.

1.11.10 Breast and female genital tract

In the acute phases after irradiation of the breast, skin changes predominate. These have been outlined in the skin section and will not be repeated here. In the deeper tissue there may be evidence of fat necrosis and the common early events described for many other tissues above. Later changes include glandular atrophy, epithelial cell atypia, intralobular and interlobular fibrosis, telangiectasia and other vascular changes. It is the atypical epithelial cell changes that make the differentiation between radiation effect and recurrent tumour so difficult on occasion. It should be noted however that these radiation-induced changes are restricted to the terminal duct lobular unit and that atypical epithelium in the larger ducts should be viewed with considerable suspicion¹⁶¹. Other features of late irradiation injury to the breast include acinar loss and architectural disarray, a dense collagenised stroma and occasional abnormal mitoses (although the latter may be misleading in isolation). A range of benign and malignant breast disease

may be seen after radiotherapy with carcinomas developing after at least 10 years, peaking at around 15-20 years¹⁶². The age at exposure is an important factor.

The uterus is extremely radioresistant, but with high dose exposure endometrial gland necrosis may be seen in the early post-irradiation period, with areas of haemorrhage and fibrin deposition. Longer term changes include endometrial atrophy and epithelial cell atypia. There may be ulceration and scarring with scattered lipid laden macrophages and telangiectatic vessels. Rather surprisingly the myometrium does not appear to develop significant fibrosis or atrophy. Similar blood vessel changes to those described elsewhere may be seen. In the cervix early changes again include oedema and an inflammatory exudate. Ulceration with scarring (including atypical fibroblasts) occurs later with atrophic changes, and epithelial cell atypia may present many years after radiation exposure¹⁶³. The changes in the vagina are like those of other mucosae.



Figure 6. Wrinkling and altered pigmentation of the breast after radiotherapy to a breast cancer. (Courtesy of Professor C Berry)

1.11.11 Gonads

The germinal epithelium is extremely sensitive, particularly in the young. After irradiation of both testis and ovary, there is early suppression of meiosis followed by germ cell necrosis. Spermatogonia B are more sensitive than spermatogonia A, spermatocytes and spermatids. All may show nuclear vacuolation and subsequent apoptosis. Delayed effects on the testis include seminiferous tubular sclerosis, basement membrane thickening and stromal hyalinization (Fig. 7). Radiation vascular changes may be prominent. The Sertoli and Leydig cells are relatively more resistant, but with severe damage Sertoli cell loss and Leydig cell dysfunction or loss may be seen. Radiation to the ovary leads to an increase in atretic follicles within a few days. This follows follicular and corpora luteal degeneration. The granulosa cells are particularly sensitive. A temporary fertile period is seen followed by temporary sterility. Some follicles are spared however and may mature normally. Chronic damage manifests as atrophy with few maturing and the ubiquitous vessel changes. The latter may be more exaggerated in the ovary than elsewhere¹²⁶. Again radiation may play a role in the aetiology of some cases of ovarian cancer.

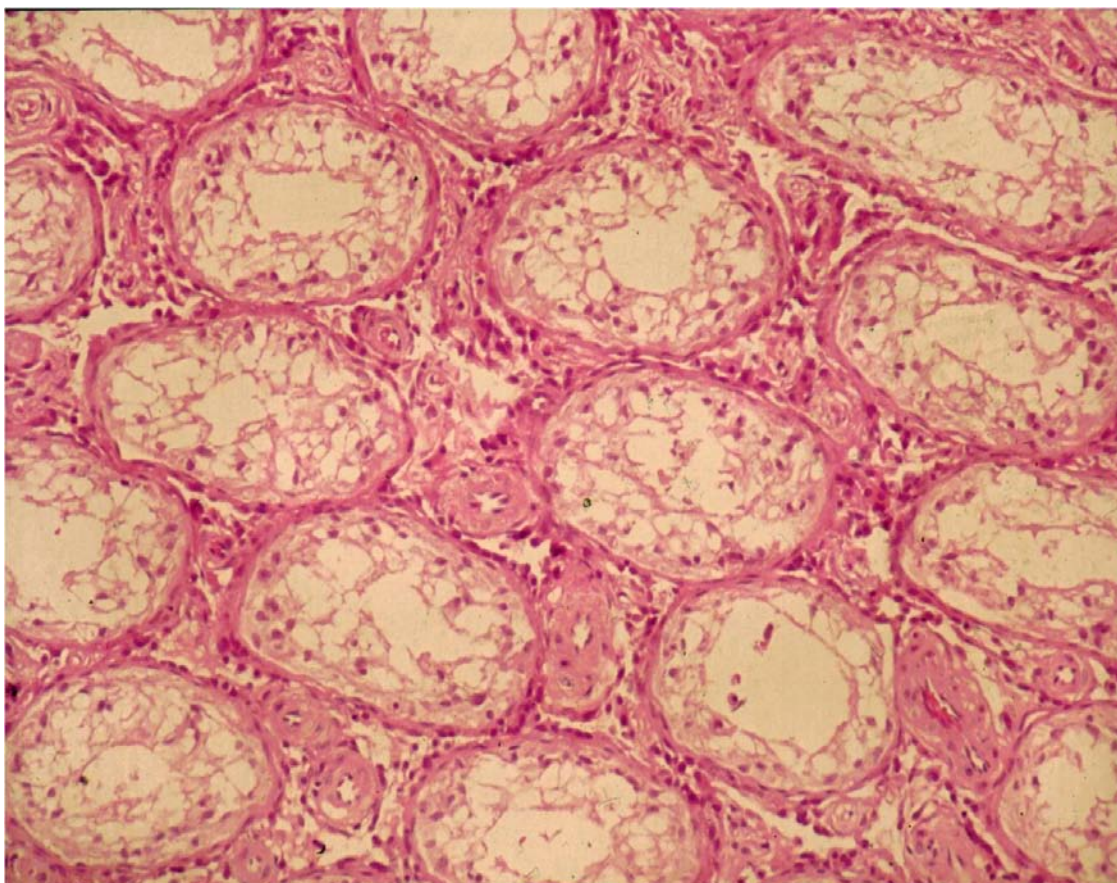


Figure 7. Testicular atrophy after irradiation of the testis. There is no spermatogenesis in the tubules. (Courtesy of Professor C Berry)

1.11.12 Genitourinary system

The kidney is moderately radiosensitive and once again the effects of irradiation depend on the administered dose and patients' age. In addition the amount of perinephric tissue, any preexisting renal disease and individual susceptibility all affect outcome. Frequently there are no discernible changes in the kidney but macroscopically the cortical atrophy and irregular subcapsular surface may look like any end stage kidney.

Microscopically, in the acute phase one may see a combination of interstitial oedema and small vessel endothelial damage¹⁶⁴. After 6-12 months the changes in the glomeruli consist of segmental necrosis with occasional small crescents and 'soft swellings' followed by segmental sclerosis or global scarring. The capillary walls are thickened and increased mesangium may extend into the peripheral loops producing a double contour appearance. The tubules are atrophic and many are lost. Tubular basement membrane thickening may be seen due to a direct radiation effect as well as a result of vascular ischaemia. Interstitial fibrosis may be accompanied by a lymphocytic infiltrate. The smaller arterioles and interlobular arteries show generalised radiation features already mentioned including dilatation, intimal thickening, fibrinoid necrosis, foam cell accumulation and thrombosis (Fig. 8). There is no distinctive immunohistochemical or fluorescence pattern. Electron microscopy reveals effacement of the epithelial cell foot processes and subendothelial zone thickening. New basement membrane material is deposited with swollen mesangial cells extending into the capillary loops. Clinically, early proteinuria and increased blood pressure may lead to progressive decrease in function,

anaemia and malignant hypertension (in fact the microscopic features may resemble any form of thrombotic microangiopathy).

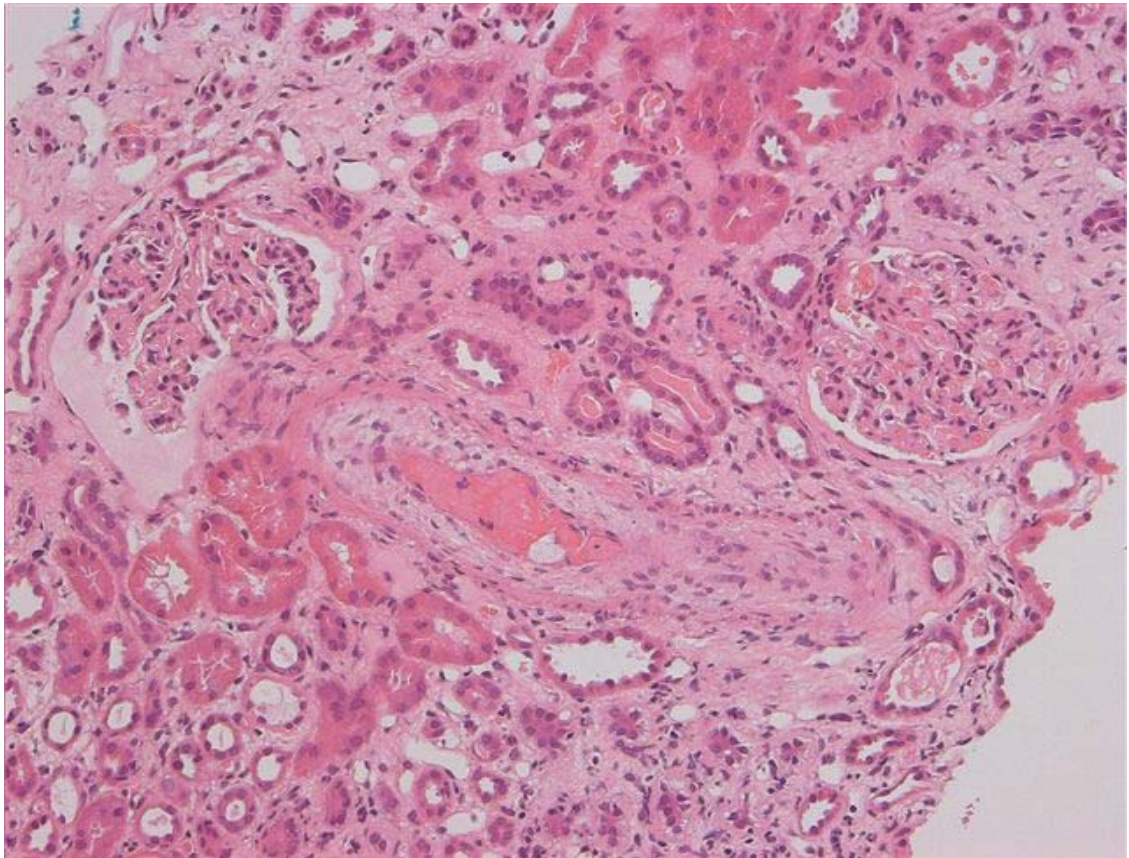


Figure 8. Thrombotic microangiopathy is a common feature of radiation injury to the kidney. Picture of MS

The bladder is highly susceptible to acute radiation injury with patients developing acute cystitis. The changes are usually transient and comprise epithelial cell loss, mucosal oedema, vascular dilatation and a variable inflammatory infiltrate. Ureteritis and urethritis may also develop. The inflammatory cell infiltrate contains numerous eosinophils and mast cells and commonly lies perineurally¹⁶⁵. Surface erosion may follow with submucosal fibrosis, continued inflammation and vascular damage. The latter is thought to lead to the later complications of ulceration, bleeding, fistulae, stricture formation, smooth muscle fibrosis and contraction. Coexisting infection may also be present. An important long term effect is the role of radiation in urothelial carcinogenesis. There will be a more detailed discussion of the effects on the bladder (and prostate gland) in the following chapters.

1.11.13 Prostate gland

The prostate is commonly irradiated either specifically to treat prostatic adenocarcinoma or indirectly in the treatment of transitional cell carcinoma of the bladder. The usual changes seen in such glands include exaggeration of the well-known features of benign prostatic hyperplasia²⁴. In addition giant cell reaction to ruptured glands and corpora amylacea, mild chronic inflammation, squamous metaplasia, atrophic glands with cytological atypia and stromal fibrosis with plump fibroblasts are commonly present. Vascular alterations already described are frequently identified. The possibility of prostatic carcinoma being radiation-induced is controversial.

1.11.14 Implications for pathological practice

The main implications for the pathologist are obvious. The effects of radiation on the architecture and nuclear appearance of many epithelia such as breast and prostate may mimic neoplasia extremely closely and the pathologist should be on his/her guard to get all the relevant info before confidently diagnosing malignancy in these situations. A search for further evidence of radiation injury is essential in such cases with particular note of radiation fibroblasts and the characteristic but non-specific vessel changes¹²⁶. Mimicry of neoplasia need not only apply to routine histological specimens, but is equally relevant to cytological specimens particularly those from FNA such as thyroid, breast¹⁶⁶, serous effusion¹⁶⁷ and urine, as well as exfoliative cytology¹⁶⁸. There are several publications highlighting the difficulties in interpretation of cytological material after irradiation which document that nuclear enlargement, hyperchromasia and atypia are frequently seen (Fig.9). Helpful points in the differentiation from recurrent tumour include degenerative features such as nuclear and cytological vacuoles and more importantly homogeneous staining of the nuclear chromatin without malignant characteristics. Occasionally it may not be possible to differentiate with any degree of confidence.

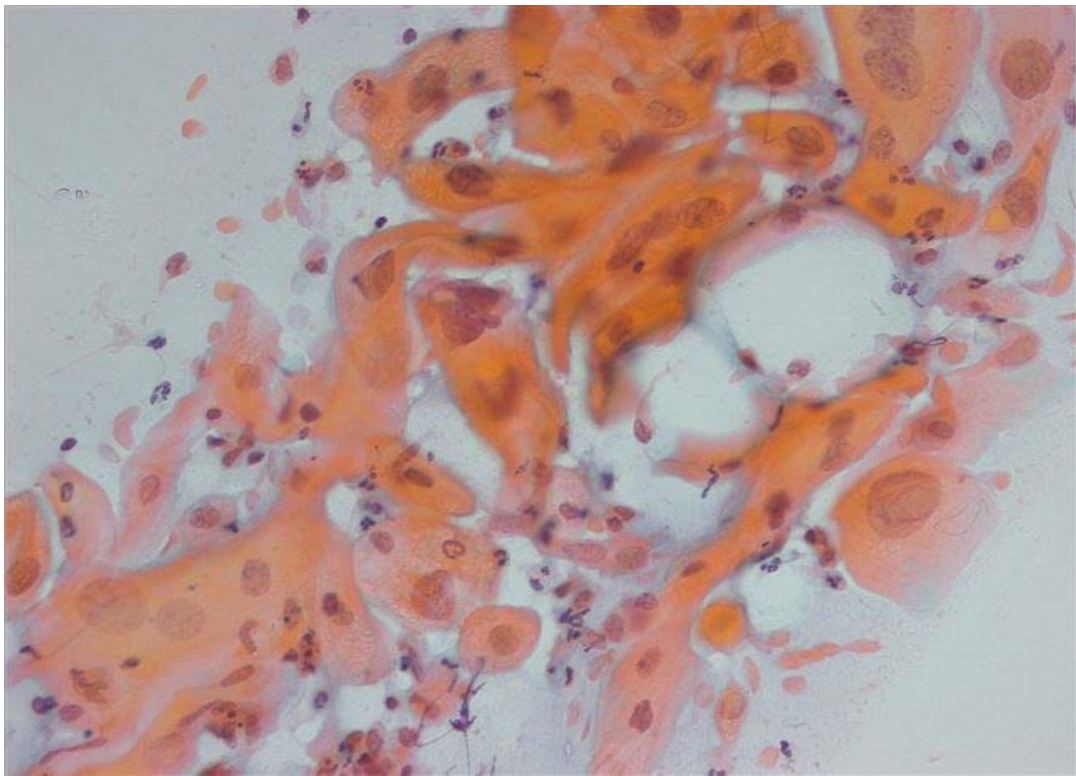


Figure 9. Atypical squamous cells often appear in post-radiation cervical smears and these can cause difficulties in differentiating therapy related changes from residual or recurrent cancer.

It should also be realised that specimens may be received which show the complications of previous irradiation without evidence of residual or relapsed tumour. These can obviously be seen many years after treatment and because of this latency differentiation from relapsed or recurrent tumour may not be possible without biopsy or more definitive surgery. Surgery may well be the appropriate treatment in such situations. In some instances the reason for the symptoms may not be totally clarified by the pathological specimen and functional effects should always be remembered. Lastly the pathologist may be involved in the diagnosis of a radiation-induced tumour that occurs many years after exposure. There is no difference in the morphology of such tumours to help identify aetiology¹⁶⁸.

1.12 Tumour therapy and clinical measurement of toxicity

Radiation is used to treat tumours by killing the component tumour cells. A distinct proportion of cells will be killed with each dose administered. Tumours with high proliferative activity obviously suffer a high rate of cell loss. Tumour cells retain some capacity to repair their radiation damage, but this is limited compared to the surrounding population of normal cells. When designing radiation therapy fields for the treatment of cancer, the radiation oncologist must take into account several important biological and technical factors. These include: likely patterns of regional tumour spread, to ensure coverage of local tumour extensions not detectable with current imaging techniques; uncertainties in positioning the patient for each treatment; and tumour and organ movement during and between treatments. To achieve these aims, normal tissues surrounding the tumour are irradiated, which may result in symptomatic injury. The tolerance of these normal tissues to radiation dictates the dose that is prescribed in the treatment of most malignant diseases.

Fractionation amplifies the differential between normal and tumorous tissue because the former is better able to repair its damage before the next insult. Fractionation also exploits the differential in tumour repopulation (although some tumours accelerate their repopulation after irradiation), allows for redistribution in the tumour cell cycle to a more sensitive phase and allows a degree of reoxygenation. With more recent protocols such as hyper-fractionation a higher overall dose can be given with no increase in the long term side effects. Chemotherapy may alter the cell dynamics and increase radiosensitivity if given appropriately.

When a new cancer therapy is evaluated, the toxic effects on normal tissues must be assessed and compared with standard therapy. A new scoring system has recently

become available: common terminology criteria for adverse events v3.0 (CTCAE, <http://ctep.cancer.gov/reporting/ctc.html>). It was developed from two earlier scoring systems, the common toxicity criteria (CTC), developed by the National Cancer Institute (NCI) for evaluating acute toxicity of new chemotherapeutic agents and acute effects of radiation, and the late effects normal tissue/subjective objective management analytical (LENT/SOMA) for assessing late normal tissue effects and their management¹⁶⁹⁻¹⁷¹. The merged scoring system includes early and late responses, and is applicable to chemotherapy, radiotherapy, surgery, other treatment modalities, and combinations of therapies. It also includes quality-of-life measures. The new scoring system will be useful for assessing the effectiveness of new approaches for preventing or reducing injury to normal tissue.

1.13 Therapeutic possibilities and prospects for the future

Radiation therapy remains a cornerstone of modern cancer management, with an estimated half of all newly diagnosed cancer patients receiving radiotherapy at some point in the course of their disease¹. Compared with surgery, radiation therapy has the advantage of being non-invasive and potentially organ preserving, although the functional outcome might be negatively affected by late side effects. Even in the era of molecular oncology, radiation therapy remains an attractive component of multi-modality therapy because it can be precisely modulated in time and space and provides effective tumour de-bulking in many cases.

All effective cancer therapies that have been developed so far are associated with a risk of various side effects and, as an increasing number of people are cancer survivors, preventing or reducing late side effects has increasingly become a priority. In the United States the National Cancer Institute and the Center for Disease Control estimate that 9.8

million people (3.5% of the population) were alive in 2001 after a diagnosis of non-skin cancer¹⁷². The number of cancer survivors in the United States more than tripled between 1971 and 2001, and this has further stimulated interest in the quality of life of this population. Relatively little is known about this issue, but it has been documented that the burden of late side effects on the physical and social functioning of the individual can be considerable¹⁷³⁻¹⁷⁵.

There is an intimate relationship between radiation-dose fractionation, spatial dose distribution and the clinical outcome of radiation therapy. Effective normal-tissue response modifiers or reliable predictive assays of normal-tissue effects would enable the intensification of tumour dose and/or the application of dose distributions with a higher probability of achieving loco-regional tumour control. This second perspective is becoming particularly exciting owing to the improved technologies for radiation therapy planning and delivery^{176,177}.

It is important to focus research efforts on studying molecular and cellular changes in pathways leading to overt damage and to develop interventions that lessen the incidence and severity of normal tissue injury without compromising tumour control. It is obvious that more must be discovered about the process of wound healing (especially after radiation) if techniques aimed to prevent or repair damage from ionising radiation and other anticancer therapies are to be developed. Also, research to identify molecular targets for the development of new anticancer agents must verify whether those targets exist in normal tissues as well as tumour tissue.

Under the target-cell hypothesis, the most important means of creating a differential between tumour and late normal-tissue effects is modulation of the dose-fractionation schedule. Pharmacological interventions concentrate on cytoprotective compounds such as the free-radical scavenger amifostine¹⁷⁸. Clearly, for this kind of drug to convey a

therapeutic advantage it would require some selectivity for the protection of normal-tissue cells relative to tumour cells. In fact, in the US, the Food and Drug Administration (FDA) has approved amifostine, "to reduce the incidence of moderate to severe xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer, where the radiation port includes a substantial portion of the parotid glands." However, the use of amifostine remains controversial. It is not without side effects of its own, namely hypotension, vomiting and allergic reactions. Furthermore, a substantial body of preclinical data suggest that amifostine induces some degree of tumour protection in addition to normal-tissue protection¹⁷⁹.

As our understanding of the pathogenesis of late radiation effects improves, specific interventional strategies are being developed that target all the contributing pathogenic pathways for late effects¹⁸⁰⁻¹⁸². Early intervention during the initial phase of the cytokine cascade might be highly effective in modulating the subsequent cell and tissue response. One obvious target for the prevention of late radiation complications is the TGF pathway because of its key role in radiation fibrogenesis and its proposed dual role as a suppressor and promoter of malignant progression^{54,56,183}. This makes TGF a high-risk target for intervention on the one hand, but a target with anticancer potential on the other. Several TGF-targeting strategies are in clinical trials as cancer therapies^{52,183,184}, and these include TGF antisense oligonucleotides, TGF antibodies and small-molecule inhibitors of the signalling pathway. Other strategies currently in pre-clinical development involve the silencing of the TGFR2 receptor using a dominant-negative receptor, or inhibiting TGF signalling using the extracellular soluble domain of the TGFR2 receptor.

There is some evidence that this strategy for the prevention or amelioration of radiation fibrogenesis through TGF targeting has some promise. In the pre-clinical study of Giri

and colleagues it was shown that the administration of an antibody against TGF could reduce the severity of bleomycin-induced lung fibrosis¹⁸⁵. Other studies have established that the delivery of soluble TGFR2 receptor by gene therapy with an adenoviral vector¹⁸⁶ or by intra-tracheal instillation¹⁸⁷ reduces the risk of bleomycin- or radiation-induced lung injury in rodent models. Furthermore, the TGF signalling pathway constitutes an alternative or additional potential target: SMAD3 knockout mice show resistance to radiation-induced fibrosis¹⁸⁸, and the Smads constitute potential targets for drug therapies¹⁸⁴. Finally, there are several new small-molecule inhibitors in various stages of development such as SM305¹⁸⁹ and halofuginone¹⁹⁰ that have shown promise in pre-clinical models. Several of these strategies are in clinical trials as potential interventions in fibrotic disease or as prophylaxis against excessive scarring after surgery¹⁹¹. Other cytokines and growth factors will inevitably be investigated as potential targets for radiation induced injury (and other fibrosing conditions for that matter). This includes VEGF and PDGF.

Another logical target to prevent radiation injury include the ROS and RNS pathways. Both Mn SOD delivered by an adenovirus vector¹⁹² and inducible NO synthase inhibition^{193,194} reduce fibrogenesis. Furthermore, there are a number of clinical studies that suggest that radiation-induced fibrosis can be reversed using antioxidants¹⁸⁰. Initially, liposomal Cu and Zn SOD were used but more recently significant regression of clinically marked fibrosis has been seen with pentoxifylline and vitamin E^{195,196}. Treatment could be continued for several months or even years, as necessary for the individual patient, and it was well tolerated. On the other hand, although most patients obtained at least some regression of their fibrosis, few obtained a complete remission, and there was evidence of a rebound effect after the end of treatment¹⁹⁵.

The degree of collagen deposition is a result of the balance between extracellular matrix synthesis and degradation, and the degradation step might be as interesting as the synthesis step as a potential interventional target in fibrogenesis¹⁹⁷. Studies on metalloproteinases and similar proteins should be considered in the future.

In summary, although much of the emphasis over the past 5–10 years has been placed on tissue remodelling as a crucial element in radiation pathogenesis, the damage-induction step (the initial cellular processing of damage) remains relevant as a target for intervention. Interventions that aim to reduce the initial cell killing would also reduce the cellular and tissue-damage response. Several compounds are in clinical trials, including free-radical scavengers, as a means of preventing the side effects of radiotherapy. In fact, the NCI Radiation Research Program sponsored a workshop on this topic and recommended further study in: renin–angiotensin system inhibition (ie, use of ACE inhibitors and angiotensin II receptor antagonists), growth factors and cytokines (particularly TGF β , basic fibroblast growth factor, and keratinocyte growth factor), proteases and their inhibitors, vitamin E and pentoxifylline, penicillamine, eicosanoids, COX2 inhibitors, Cu/ZnSOD and MnSOD, hyperbaric oxygen, and stem-cell transplants. Treatments given before irradiation such as amifostine and expanders of stem-cell populations can also protect normal tissues from acute and late effects¹⁹⁸.

There is also a need to identify surrogate molecular markers such as patterns of gene expression, genetic polymorphisms and imaging patterns that will accurately predict patients at risk for normal tissue injury (such as TGF β and pneumonitis) and to investigate appropriate timing of interventions to arrest or prevent complications. Mechanisms of damage are likely to be tissue specific and may be under genetic control. Efforts to develop and evaluate new therapies to prevent or reduce injury to normal tissue will be facilitated by increased understanding of the mechanisms by

which treatments for radiotoxicity work, and from greater knowledge of why radiation damage does or does not heal. It is obviously crucial on the other hand to ensure that these treatments are effective in the clinical setting and do not protect or give a survival advantage to tumour cells.

1.14 Conclusion

The medical use of radiotherapy to treat cancer inevitably involves exposure of normal tissues. As a result, patients may experience symptoms associated with damage to normal tissue during the course of therapy for a few weeks after therapy or months or years later. Symptoms may be due to cell death or wound healing initiated within irradiated tissue, and may be precipitated by exposure to further injury or trauma. Many factors contribute to risk and severity of normal tissue reactions; these factors are site specific and vary with time after treatment. Treatments that reduce the risk or severity of damage to normal tissue or that facilitate the healing of radiation injury are being developed. These could greatly improve the quality of life of patients treated for cancer.

Radiation therapy has curative or palliative potential in roughly half of all incident solid tumours, and offers organ and function preservation in most cases. Unfortunately, early and late toxicity limits the deliverable intensity of radiotherapy, and might affect the long-term health-related quality of life of the patient. Recent progress in molecular pathology and normal-tissue radiobiology has improved the mechanistic understanding of late normal-tissue effects and shifted the focus from initial-damage induction to damage recognition and tissue remodelling. This has stimulated research into new pharmacological strategies for preventing or reducing the side effects of radiation therapy with emerging strategies now approaching clinical practice.

Chapter 2. Effects of radiation on the prostate gland

2.1 Introduction

Prostate cancer is the commonest cancer of men in the US and is the second most common male cancer worldwide¹⁹⁹. In 2007 there were 218890 new cases of prostate cancer in the United States of America causing 27050 deaths²⁰⁰. This made it the most common malignancy in men and second leading cause of cancer death in the US²⁰⁰. In England and Wales the number of cases registered and death rates have increased and prostate cancer is the second leading cancer related death in men after lung cancer²⁰¹. Treatment of prostate cancer depends on the grade and stage of the disease and in appropriate men hormone manipulation, chemotherapy, surgery, high energy ultrasound or radiation therapy will be used solely, sequentially or in combination. All of these treatments can lead to alterations in the neoplastic and non-neoplastic gland which have been investigated by many authors over the years but few studies have looked at the effects of radiation on the prostate gland that does not harbour a cancer²⁰²⁻²⁰⁷. This first major description of radiation changes in the neoplastic and benign prostate gland was published in 1982 but this included few non-neoplastic cases and no-one has studied the radiation induced changes in the normal prostate gland in any systematic way²⁰⁸.

There are many reasons why it is important to document and understand the changes in the prostate gland associated with radiation. Firstly, prostate biopsies are the mainstay of diagnosis and it is crucial that a full knowledge of the radiation induced changes is appreciated in order to avoid overcalling cancer. Secondly, symptoms after radiation to the lower urinary tract are common and sometimes the reasons for the symptoms cannot be explained. It may be that radiation changes in the prostate or neighbouring structures may provide possible explanations for these symptoms.

The prostate gland is intimately related to the base of the bladder. We therefore looked at prostates in cystoprostatectomy specimens removed from patients who had been treated with radiotherapy for transitional cell carcinoma. For a comparison, prostate tissue removed at TURP for benign prostatic hypertrophy/hyperplasia was used as a control, with age matched cases.

2.1.1 Anatomy of the normal prostate gland

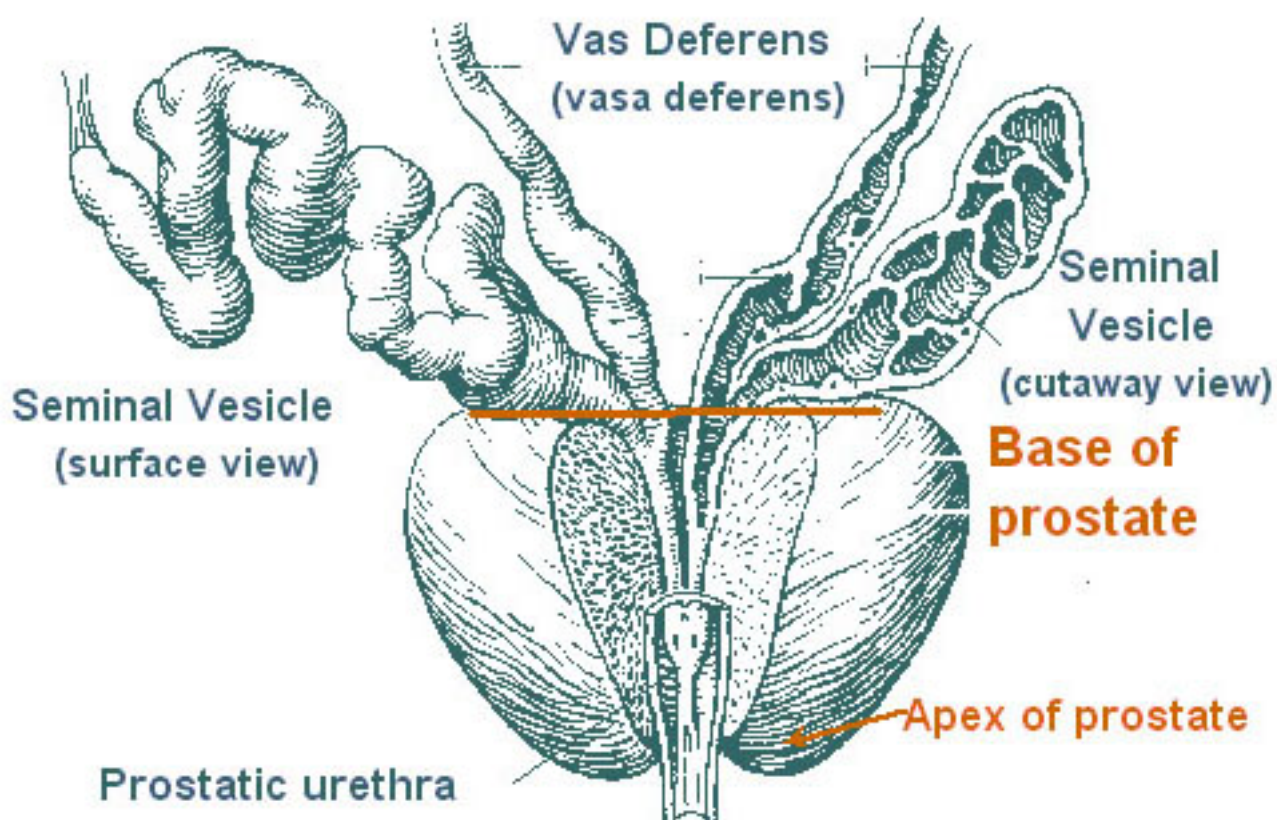


Figure 10. The position and local anatomy of the prostate gland²⁰⁹

The prostate gland develops from epithelial invaginations of the posterior urogenital sinus in utero. It enlarges continuously to reach 20g by around 25-30 years of age. There have been several anatomical descriptions of the internal anatomy of the gland

but currently the most popular depicts several zones encased in a capsule. The zones are useful anatomically and pathologically as the most common disorders of the prostate are frequently found in particular zones. The zones are the peripheral zone, the central zone and the transition zone. Most cancers are found peripherally. The urethra runs through the prostate and the seminal vesicles contribute their secretions to the urethra through gland (Fig. 10). The gland is well innervated.

2.2 Materials and methods

The files of the Pathology Archive of RLH were searched over a 10-year period (1983-1993) looking for all patients who had undergone a radical cystoprostatectomy for the treatment of their bladder cancer. A total of 39 radical samples were identified where there was a specimen available for review. All patients had originally been diagnosed with transitional cell carcinoma of the bladder. The case notes, and in some instances the radiotherapy schedule, were reviewed and all patients had been through the same course of radiotherapy at the London Hospital. The treatment course consisted of 20 daily treatments of 55Gy each day for 5 days over a 4-week period. The protocol was similar for all patients and involved three beam irradiation, one anterior and two posterior oblique beams. The field was determined using CT scan image based planning to build up a three dimensional map of the area to be irradiated. As per convention, the protocol intentionally included a 10-15mm margin of surrounding tissue which would have incorporated the prostate gland. Surgery was necessary because of symptom control or resistant, recurrent or progressive tumour. No patient had had a pre-treatment biopsy of their prostate gland although it was thought that the prostate gland was not involved by the tumour pre-operatively. In each case the time between irradiation and

surgery was known (1 week to 17 years). All cases were male and the age ranged from 41-84 years.

The specimens were all handled in the Department's routine manner. All had a similar assessment at cut-up with a gross description and appropriate block sampling. The pieces of prostate gland tissue retained were random and not directed to any particular area. Between two and five blocks were taken for each case. The tissue was fixed in 10% formalin, processed routinely and embedded in paraffin wax. Sections were subsequently cut at 4-5 microns and stained with haematoxylin and eosin.

The slides from each case were examined by two observers who scored the changes as being either present or absent. If appropriate, the features were graded according to a predetermined scale. The scale was subjective and comprised 0 when the feature was absent, + when there was a mild change, ++ for a moderate change and +++ for a conspicuous, widespread alteration. Changes within glands, stroma, vessels and surrounding structures were all inspected. In particular we looked for glandular atrophy, epithelial cellular changes (especially the nuclei), metaplastic epithelial alterations, stromal inflammation, mural fibrosis, vessel changes and changes within and around nerves. Slides which showed direct involvement by tumour were purposely excluded from the study. In some cases, where present, the seminal vesical was also assessed. In the majority of cases the scores for both observers were identical. When there was a discrepancy however, the cases were re-examined and a final agreed score was given.

In order to relate the findings to a control group, we also looked at 40 TURP specimens removed for benign myoadenomatous hyperplasia. None of these cases included patients with either tumours (known or found at surgery) or re-operations. None had had previous pelvic irradiation. We chose cases from the same time period and same age

range. We did not specifically exclude any cases above or below a certain size/weight of chippings; there was no attempt to correct for this.

For statistics, a Chi-squared test was performed and results showing a p value of less than 0.05% were considered significant.

2.3 Results

The results are outlined in Table 1. The most frequent changes identified within the prostate gland were acinar atrophy (Fig. 11), squamous metaplasia (Fig. 12), epithelial cell cytological and/or nuclear atypia (Fig. 13) and basal cell hyperplasia (Fig. 14). All four of these features were also found in the control group but not as frequently as in the irradiated set. Changes that were much more frequently found in irradiated prostate glands included a foreign body reaction with spillage of corpora amylacea from the ruptured glands into the surrounding stroma (Fig. 15). Inflammation and fibrosis were frequently found in both populations but these were much common in the radiation group (Fig. 13). Intra-prostatic vascular changes were expected but these were surprisingly very common. Some changes such as endothelial cell hypertrophy, foam cells in the intima, intimal hyperplasia/fibrosis, medial hyaline thickening, thrombosis and luminal occlusion were quite common and this combination was rarely seen in the control group (Figs. 14-17). Inflammation, with mononuclear cells, was regularly identified within and around ganglia both within and around the gland itself (Fig. 18). Perineural thickening and inflammation was also conspicuous (Fig. 19). However these types of changes could not be compared with the control group as periprostatic tissue was not regularly represented. The same problem prevented comparison of the seminal vesicle fibrosis which was seen in most of the radiated group patients. It should be pointed out here that the case selection (cystectomy cases) may bias our results to more

exaggerated changes than those that occur in the majority of patients undergoing radiotherapy who do not develop symptoms that require such radical surgery.

Feature	Prostates after irradiation <i>n</i> = 39 (%)	Non-irradiated prostate glands <i>n</i> = 40 (%)	P-values
Glandular atrophy	31 (80)	9 (23)	<0.001
Nuclear atypia in glands	30 (77)	6 (15)	<0.001
Basal cell hyperplasia	30 (77)	17 (43)	0.0018
Squamous metaplasia	23 (59)	3 (8)	<0.001
Foreign body /giant cell reaction to corpora amylacea	17 (44)	2 (5)	0.001
Arterial intimal hyperplasia	36 (92)	9 (23)	<0.001
Marked arterial luminal narrowing	24 (62)	2 (5)	<0.001
Arterial medial thickening and/or hyalinisation	33 (85)	2 (5)	<0.001
Stromal inflammation	35 (90)	23 (58)	0.0012
Marked stromal fibrosis	35 (90)	8 (20)	<0.001

Table 1. Changes in the prostate gland following irradiation

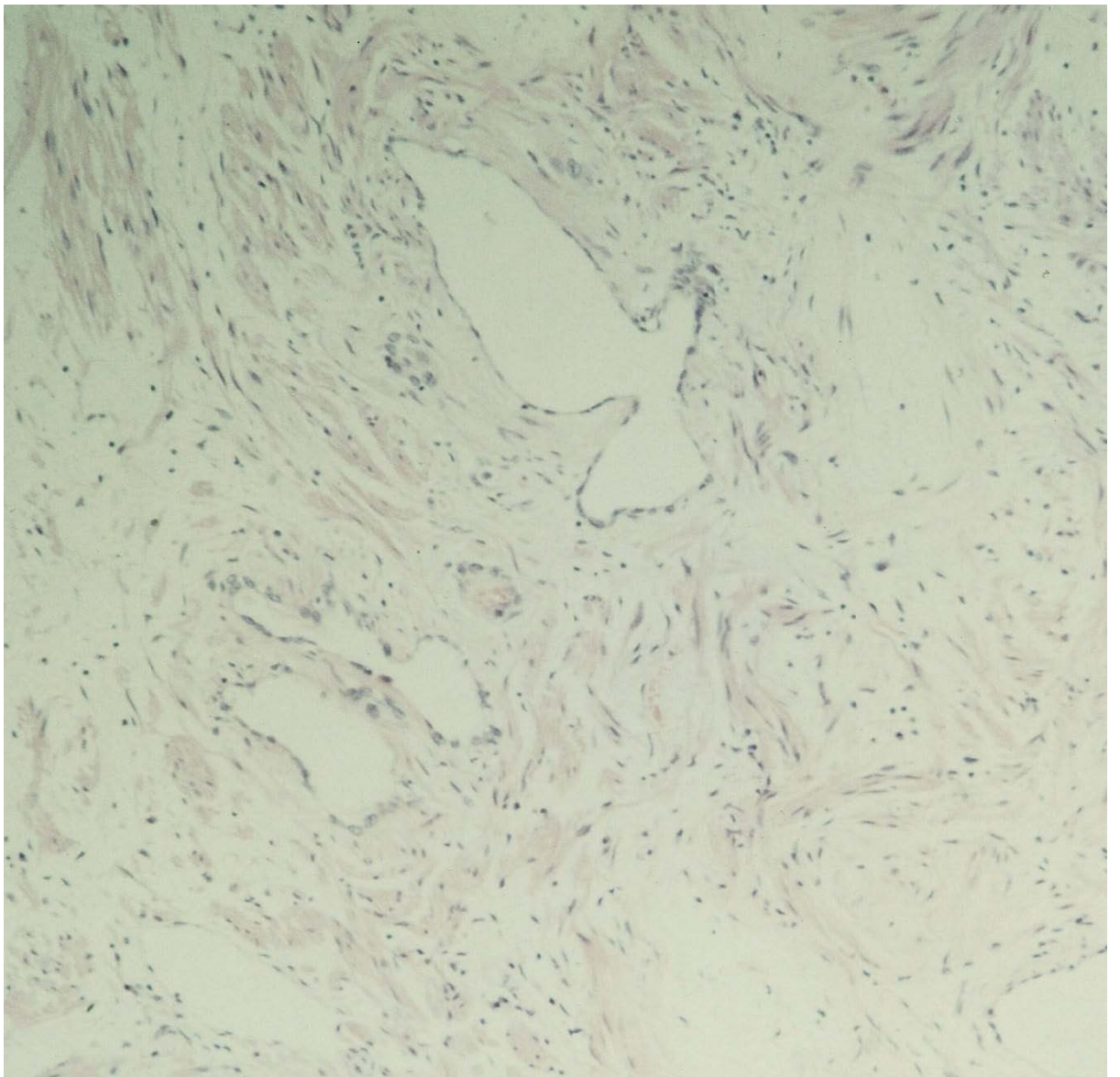


Figure 11. Gland atrophy after prostatic irradiation

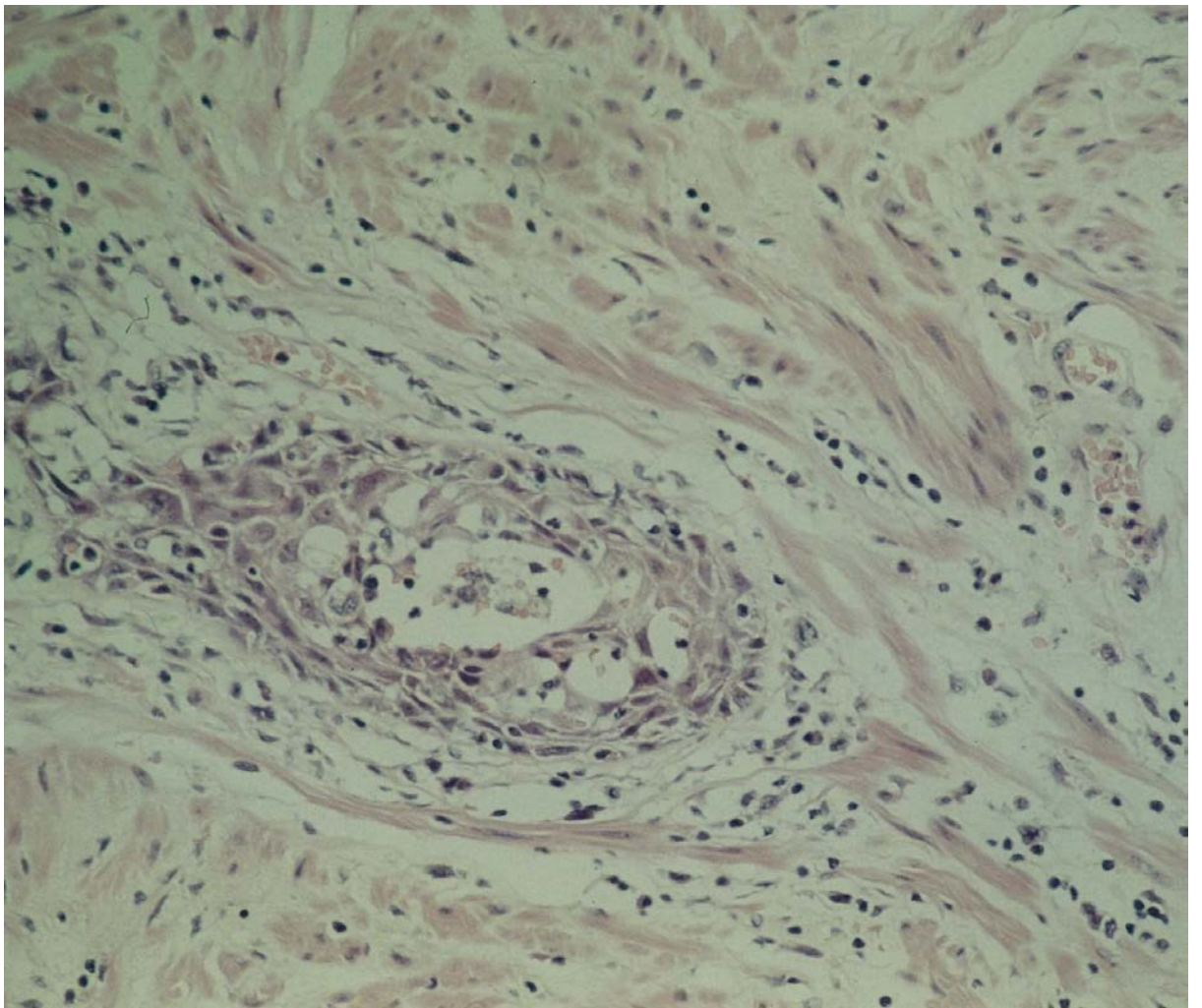


Figure 12. Squamous metaplasia in a prostatic gland after radiotherapy.

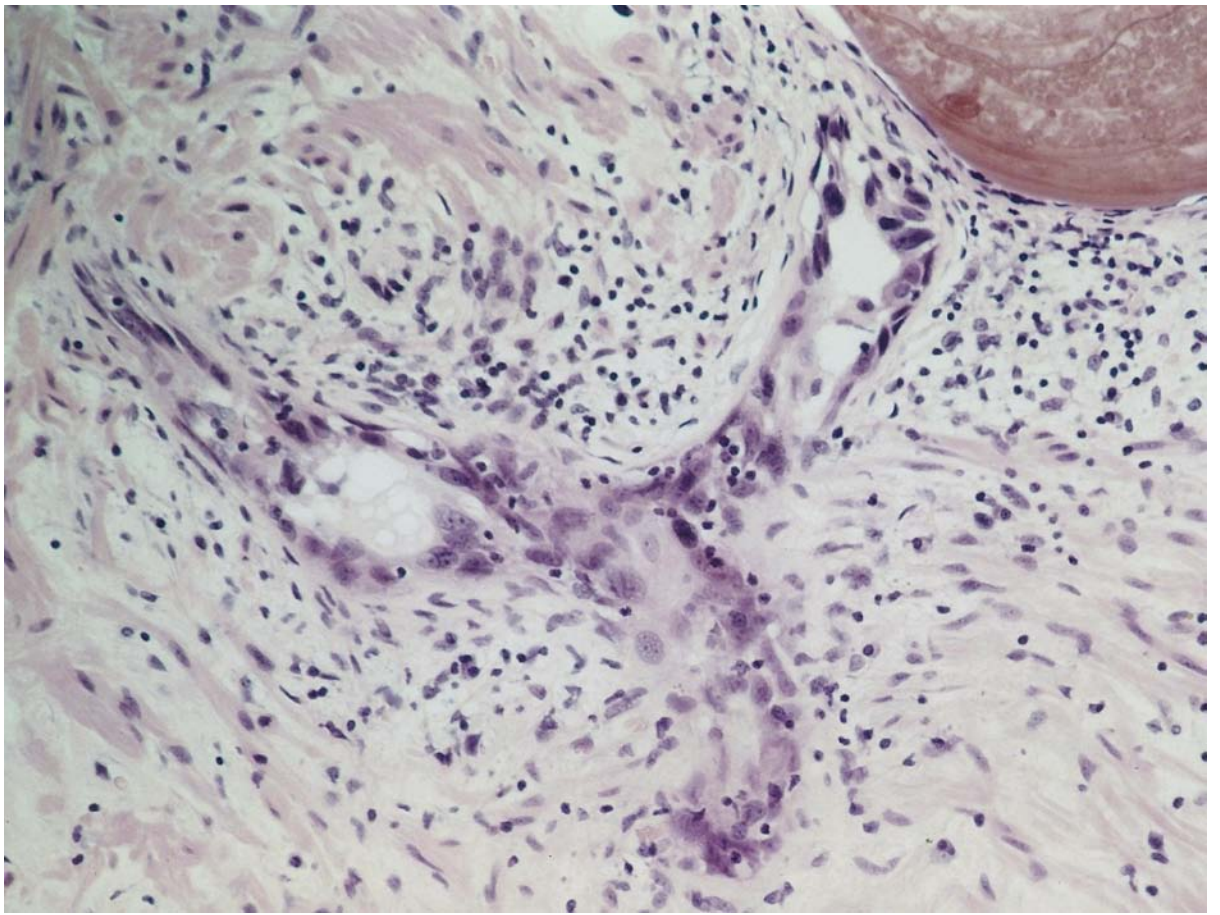


Figure 13. Inflammation and epithelial cell atypia typical of radiation induce changes in the prostate gland.

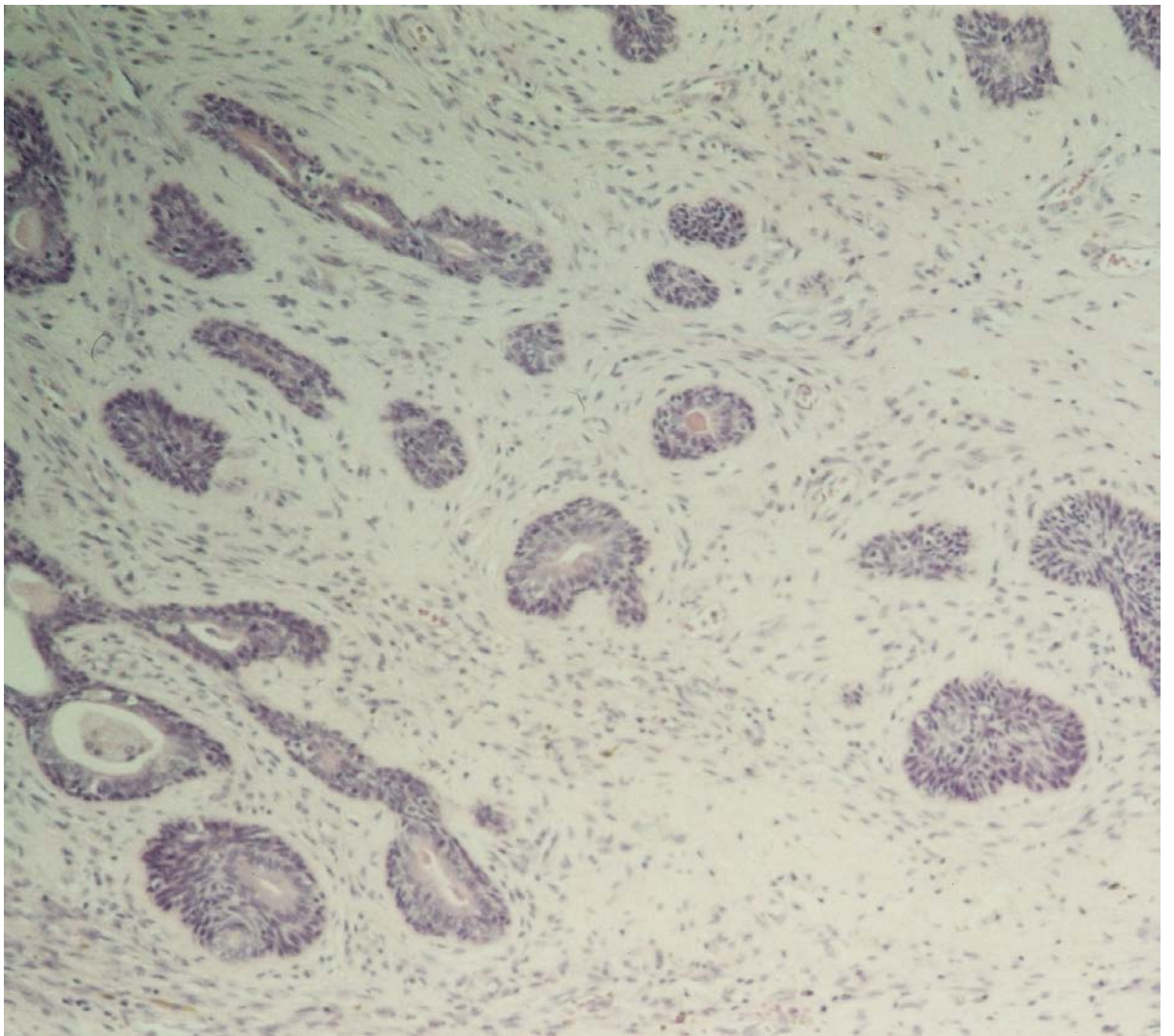


Figure 14. Basal cell hyperplasia is commonly seen in the irradiated prostates.

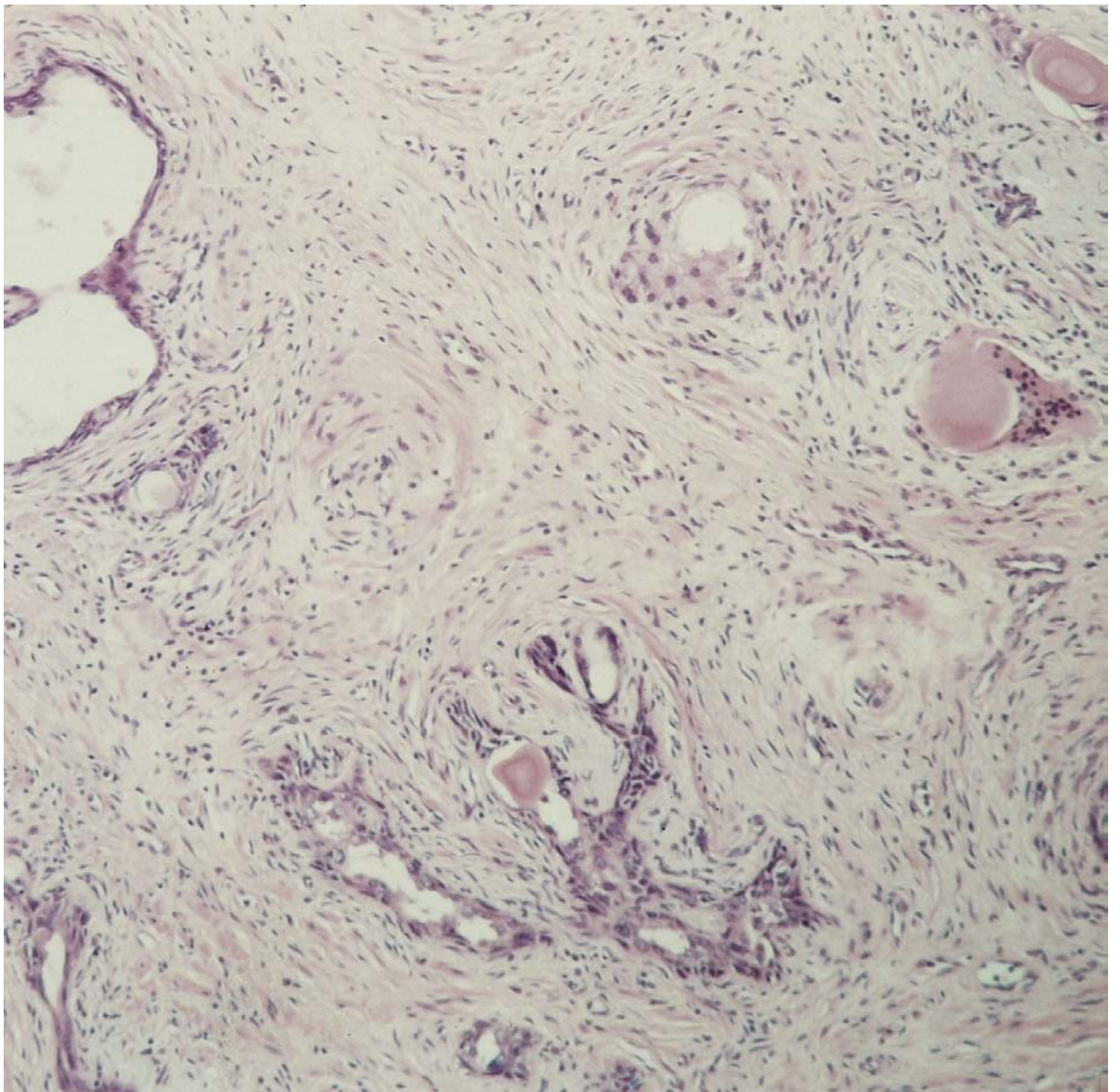


Figure 15. In addition to epithelial hyperplasia and atypia, there was often rupture of corpora amylacea into the periglandular stroma with an associated giant cell response.

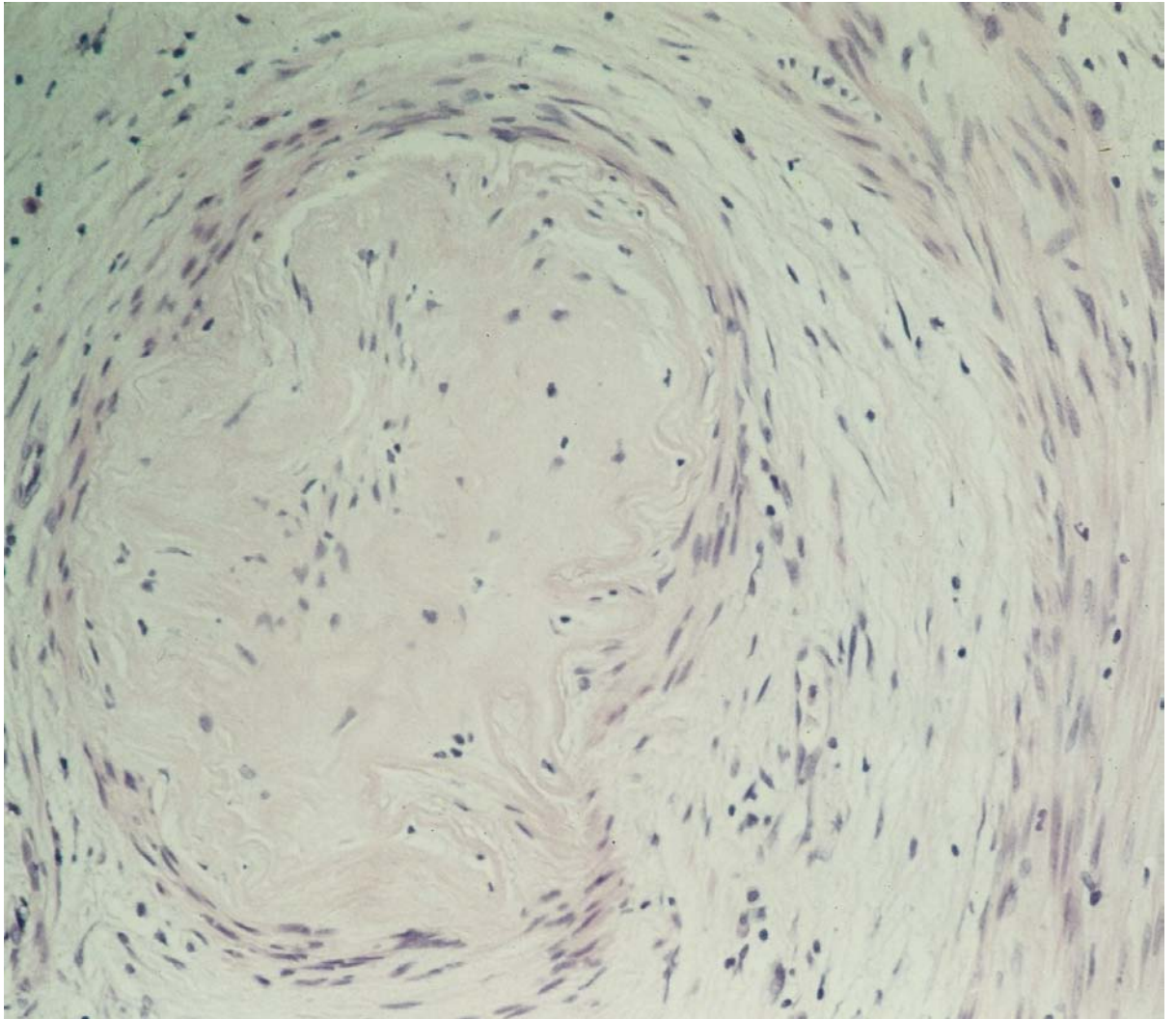


Figure 16. Vessels sometimes show endarteritis obliterans, often quite a long time after irradiation.

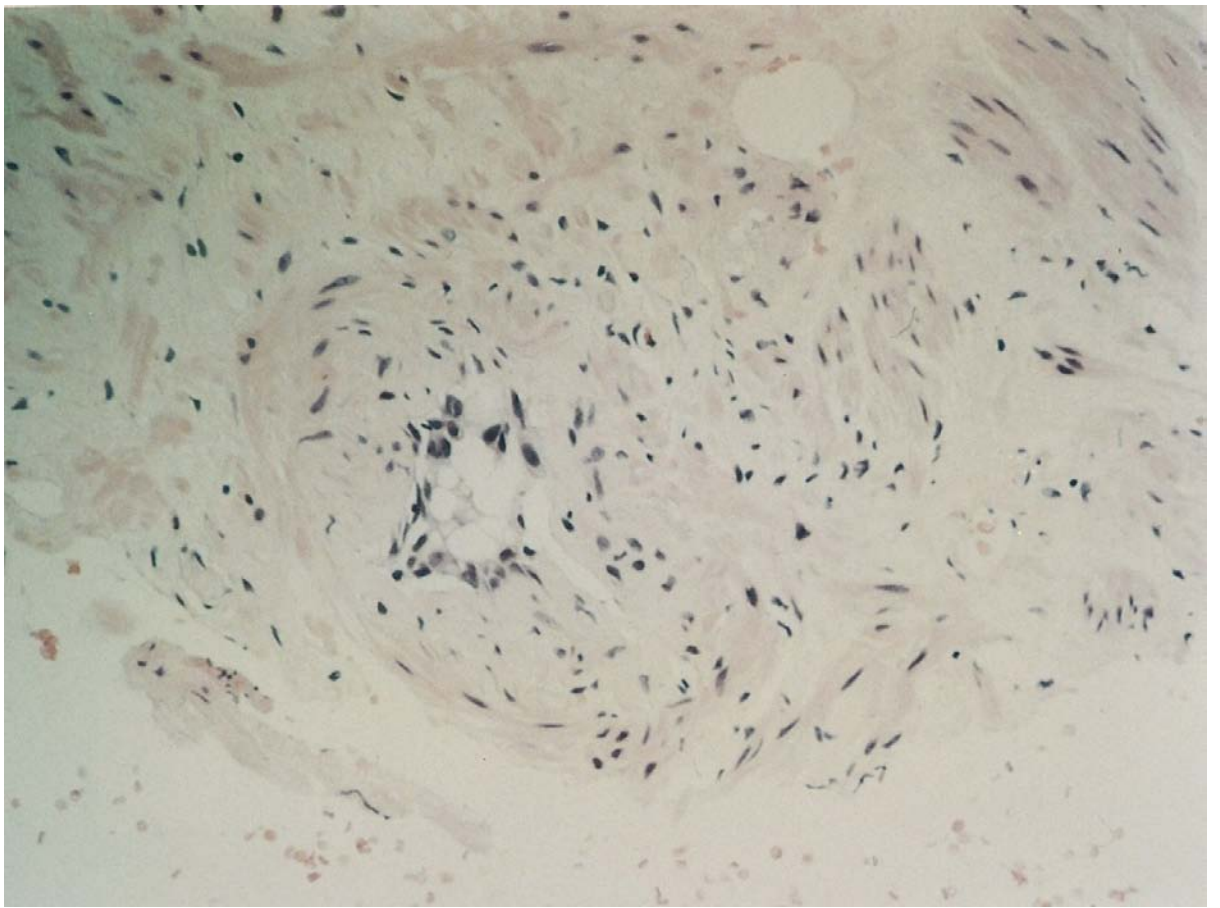


Figure 17. Endothelial cell swelling/hypertrophy was a common finding.

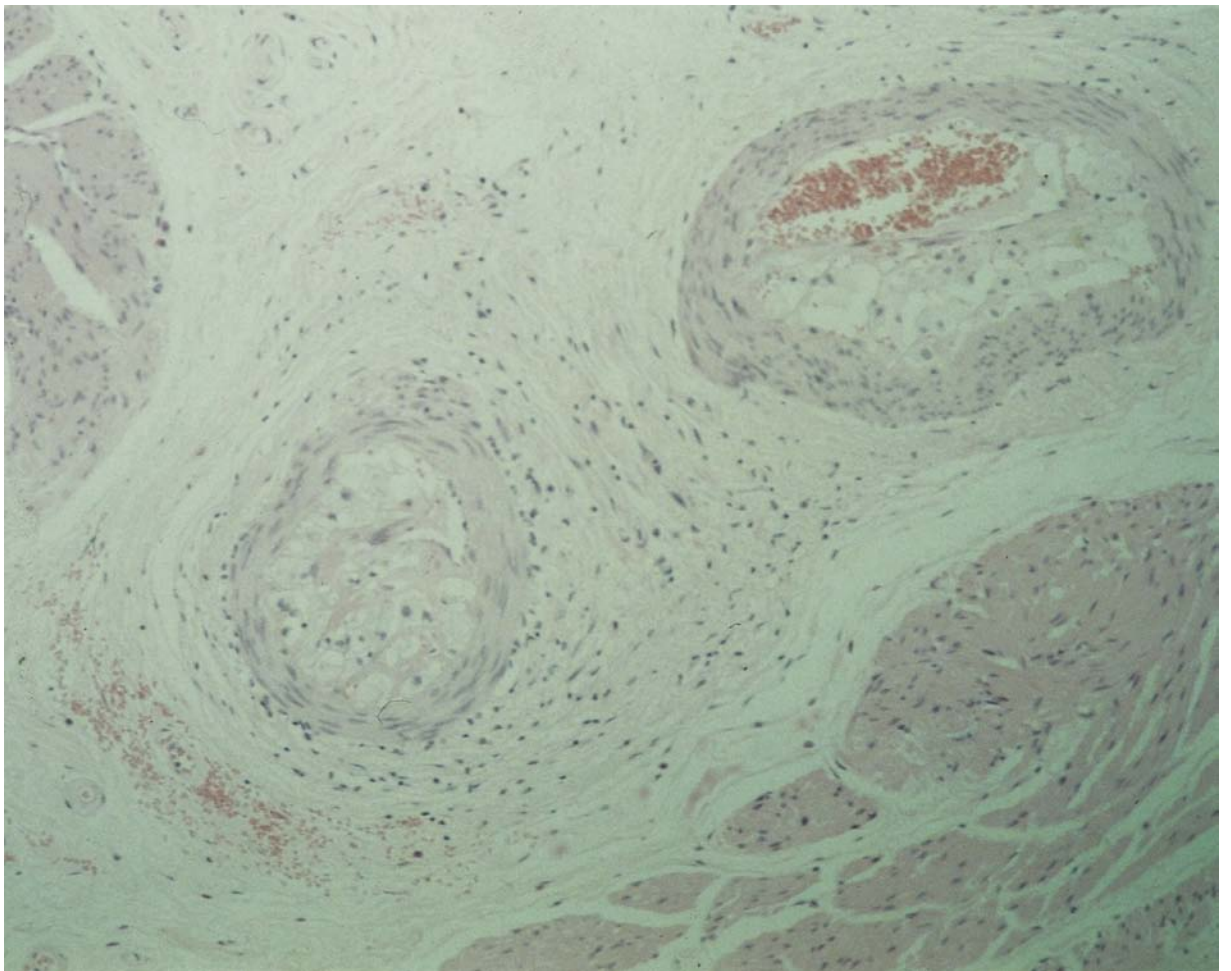


Figure 18. Sometimes atheroma-like accumulation of foamy macrophages was seen beneath the endothelium of small arteries.

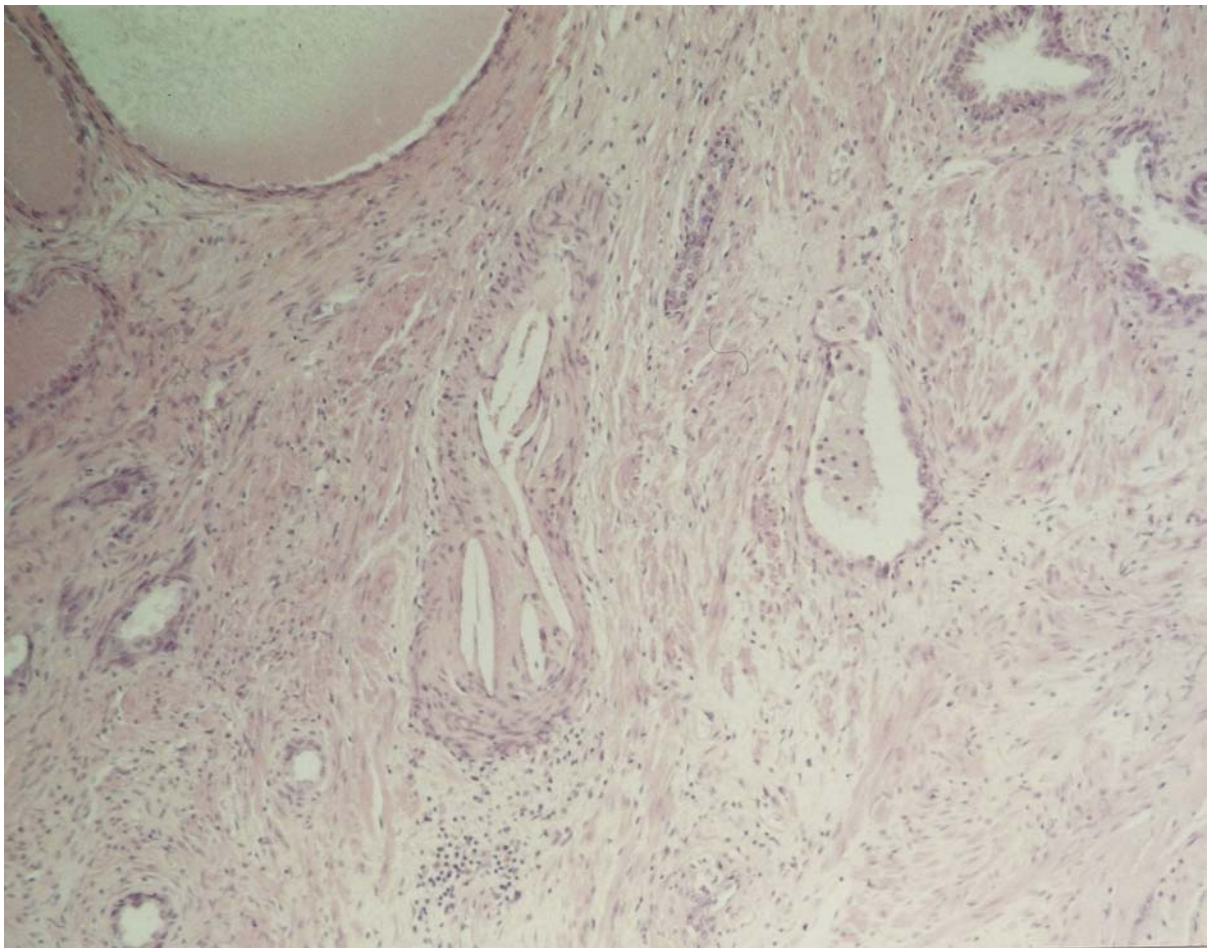


Figure 19. Infrequently cholesterol emboli were noted.

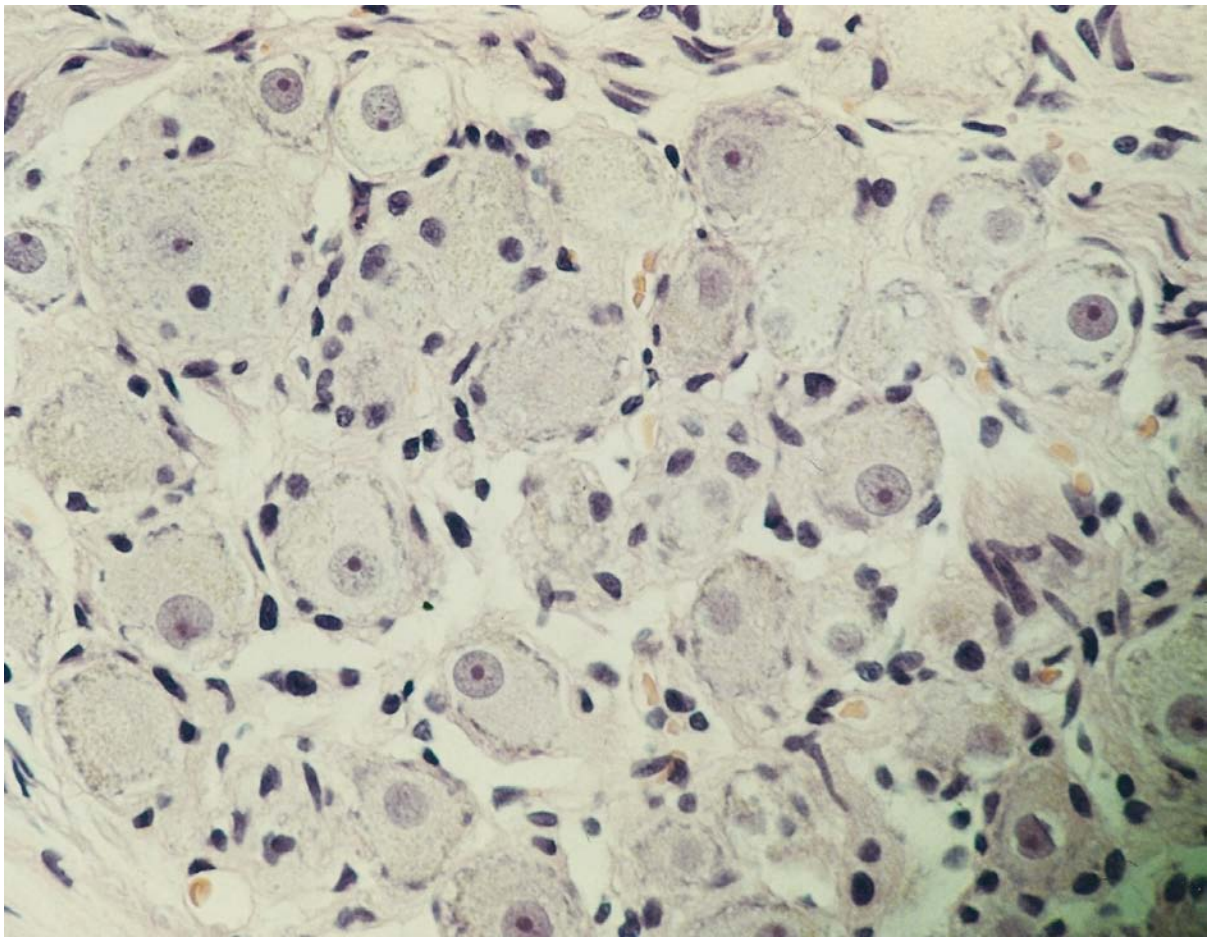


Figure 20. Lymphocytic ganglionitis was frequently seen.

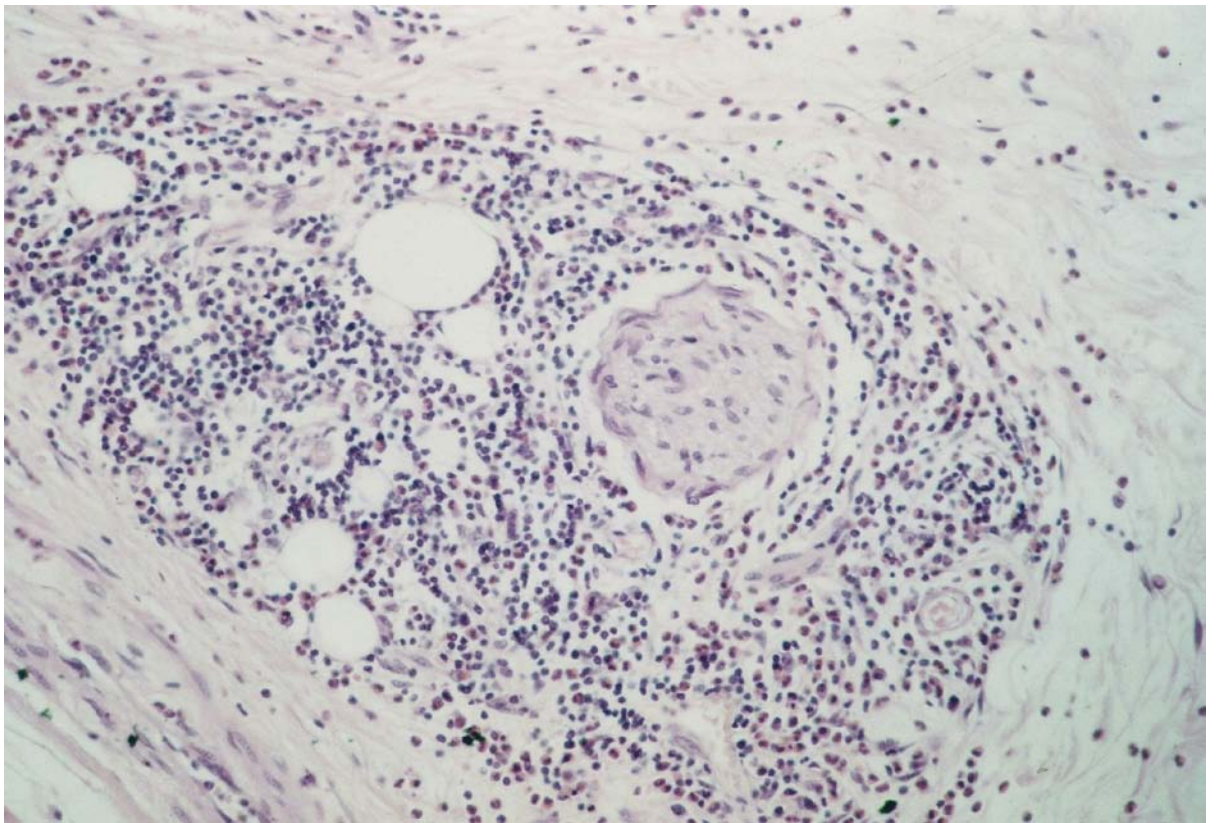


Figure 21. Perineural inflammation (with eosinophils) was often seen.

2.4 Discussion

Radiotherapy is often employed for the treatment of localised prostate cancer and it is also used in advanced, disseminated cases. Radiation therapy is now well established as a key treatment for prostate cancer and there have been many reviews of the types of radiation delivery and suitability of patients for the particular protocols available. Recognised methods include external beam, brachytherapy, IMRT and many other varieties of radiation delivery. The relative benefits of and preference for each of these modalities is beyond the scope of this study but there have been many reviews of this topic²¹⁰⁻²¹⁵. Whilst radiotherapy may be used alone in the treatment of prostate cancer, some advocate a multidisciplinary approach and many patients will benefit from such an approach with radiation as part of the treatment plan in addition to surgery, chemotherapy, hormone therapy or some other method of combination treatment²¹⁶.

Each patient undergoing radiation treatment will require a risk-benefit assessment to establish whether the treatment with radiation is likely to be worthwhile in terms of prognosis and morbidity. The unwanted effects of radiation will factor into this assessment. Toxicity includes of urine retention and proctitis. Most outcomes are measured in terms of symptoms produced and there are scoring systems for this¹⁶⁹.

There is a fairly comprehensive literature on the histological effects of all sorts of treatment (including radiation) on prostate carcinoma but little has been documented on the radiation changes in the normal prostate gland. Knowledge of these changes is of vital importance since one must know the non-neoplastic changes to avoid overcalling cancer. This is especially important when one appreciates that follow up biopsies are frequently performed when assessing response to treatment and predicting tumour progression. It has been suggested that cases with poor outcome prediction from the biopsy morphology may be due to misinterpretation of the biopsy appearance.

There was limited information on the effects of radiation on prostate cancer until fairly recently. In one of the studies performed in 1999²¹⁷, the authors did investigate the changes within benign glands adjacent to prostate cancers but the numbers were very limited. In their cases, they showed that the changes included gland atrophy, cytological atypia and basal cell prominence. Paneth cell like change was seen in 32% of cases, mucinous metaplasia in 21% and blue tinged luminal secretions in 10%. Squamous metaplasia was only found in 6%. Changes in cancerous glands were very variable.

Nilsson and colleagues have looked at the morphological effects of radiation on prostate cancer²¹⁸. This study was primarily focussed on whether their 40 patients with prostate cancer, who were treated by radiation, had evidence of residual tumour and whether this could be differentiated from radiation atypia. They compared their findings to those identified in 10 patients irradiated for non-prostatic pelvic tumour. They found a decreased ratio of tumour glands to stroma, gland atrophy and squamous metaplasia of non-neoplastic glands with or without cytological atypia of the lining epithelial cells. They also identified stromal fibrosis, arterial lumen narrowing due to myointimal proliferation, foam cells in vessel walls, fibrosis and atrophy of the seminal vesicle. Prominent inflammation was noted but this was not helpful in predicting whether the patients had undergone radiation therapy.

Other studies on prostate cancer cases have also specifically looked at the changes within the prostate tissue itself after a variety of local and systemic treatments. For example Bostwick and Meiers looked at a range of at therapy related changes to prostate cancer histology, including a few cases of radiation²¹⁹. Roznovanu and colleagues looked at the hormonal and radiation induced changes in prostate cancers²²⁰. They found similar changes to Nilsson with increased number of atrophic acini, squamous metaplasia and atypical glands. They stated that radiation induced atypia in non-

neoplastic glands after radiation for prostate cancer may persist for many years and therefore be a pitfall for overcalling recurrent cancer.

Magi-Galluzzi's group also described atypia in non-neoplastic prostate glands and have devised a scoring system based on a numerical score 0-3 for nuclear atypia, stromal fibrosis and vascular hyalinization²²¹. When they separated their cases into three groups, depending on the time since irradiation (less than 24 months, 24-48 months and more than 48 months), they found more atypia in interstitial (brachytherapy) and combined cases rather than sole external beam therapy. They also identified more stromal fibrosis in these groups. Vessel changes were the similar in both groups of patients. No change in atypia over time was identified in interstitial and combined although they seemed to show less atypia in the group examined more than 48 months after external beam radiation.

In the study presented here, my aim was to identify and record changes in the non-neoplastic gland where we know a local primary prostatic tumour would not be influencing the appearances. The changes we observed are very similar to those described in the early paper from the Bostwick group²⁰⁸. For example, nuclear atypia in glands was present in 77% of cases studied here and 78% of Bostwick's cases. Squamous metaplasia was present in 59% of our cases and 63% of Bostwick's. A granulomatous reaction to gland rupture and displaced corpora amylacea was identified in 50% of the American cases and 44% of ours. Bostwick did not appear to compare his results to any form of control group.

Many of the changes found in the non-neoplastic glands were seen in both the irradiated and non-irradiated groups including atrophy, chronic inflammation, corpora amylacea and basal cell hyperplasia. This is not particularly surprising as we purposely chose age-matched controls who inevitably included an older population. However, the changes

were more frequently observed in the radiation group than control and they were often more exaggerated. It is possible therefore that the changes observed after radiation reflects acceleration of regular degenerative changes seen in the normal physiological ageing processes. These latter clearly involve apoptosis¹, atrophy²²² and atherosclerosis^{223,224}, all of which are very commonly found in tissues generally after radiotherapy. It could be therefore that radiation induces changes which are similar to, or even identical to, generalised degenerative phenomenon in epithelial and stromal cells, involving nuclear and cytoplasmic structures.

Radiation is also known to be associated with inhibition of DNA synthesis in prostatic epithelial cells²²⁵. In fact, as discussed in Chapter 1, the effects of radiation are generally brought about by direct nuclear injury, free radical induced damage or cytokine induced changes. These are all processes that are implicated in ageing and senescence, which is after all basically an accumulation of structural cellular/system damage over time. Therefore radiation may be inducing premature ageing. It is difficult however to tease out the direct effects from indirect changes associated with ischaemia from vascular injury and it may be the latter which has a more significant effect on the tissues.

It is well recognised that radiation can induce vascular changes in many organs, including changes in large/medium sized arteries such as atherosclerosis. The full spectrum of vascular changes described in many tissues after radiation was observed in the prostate gland. These changes are also found in the prostatic vessels, with conspicuous atheroma-like foam cell accumulation within the intima, endarteritis, medial thickening and intimal hyperplasia all being observed (these are also features of ageing²²⁶). Some of these changes were seen in small vessels as well as medium sized arteries. In the thorax, it is well recognised that atheroma of the coronary arteries is

accelerated and/or increased after radiation to the chest²²⁷. Other vascular changes included thrombosis (presumably due to provocation of the coagulation system by endothelial cell injury).

Stromal fibrosis was especially conspicuous after irradiation (as indeed it was in the prostates examined by Bostwick). This is not surprising since radiation is followed by fibrosis in most organs studied. In the lung this has been shown to be associated with growth factor expression, including release by alveolar macrophages⁸⁹. Similar changes are described in the intestine after irradiation. In culture, endothelial cells have been shown to produce growth factors after irradiation⁷⁴. Such growth factors are potent mitogens and chemotactins for fibroblasts and they increase their production of collagen. It is logical, therefore, that growth factors are involved in the production of radiation-induced fibrosis. We have gone on to show that PDGF expression is increased in the bladder after irradiation and suggest that it may be involved in the contracted bladder that is sometimes seen after radiotherapy (see Chapters 6-8). It is also possible that PDGF may be relevant for some of the vascular injury observed after radiation – there are theories suggesting that local PDGF production may be important in atherogenesis and endothelial cells express PDGF.

Inflammation is a conspicuous feature of early radiation injury. The inflammatory cell profile is unusual in that eosinophils are especially common in a lymphohistiocytic background. Eosinophils were present in the prostate after irradiation but their population was not as florid as in the bladder (see Chapter 5). Mast cells also accumulate after radiation²²⁸ and increased numbers of mast cells were identified in the prostate gland suggesting that radiation provoked this pattern of inflammation rather than it reflecting a local infection/dysfunction.

The presence of mast cells is interesting. These cells contain vasoactive amines and growth factors. The latter includes TGF β . We also describe immunoreactivity for PDGF in mast cells in the bladder and prostate gland after irradiation. Others have also suggested that mast cells contain PDGF^{229,230}. It is tantalising to speculate that the presence of mast cells, with their cytoplasmic fibrogenic factors, may be an important part of the fibrotic process seen after irradiation. After all, mast cells are also increased in other fibro-inflammatory conditions such as scleroderma, interstitial cystitis and atherosclerosis and this surely supports a role for mast cells and their cytokines/growth factors in the pathological manifestations and symptoms encountered in these lesions.

Another particularly interesting finding of this study is that radiation appears to be associated with a range of neural changes. In particular, inflammation was seen around nerves and within ganglia. This time the inflammatory cell population included mainly lymphocytes mainly but again occasional mast cells and eosinophils were admixed. Such changes have been described before in a few superficial bladder mucosal biopsies²³¹ but our study shows that it is a frequent finding not only in mucosal nerves but also in deeper nerves and nerves outside of the pelvic organs themselves. Furthermore, we also found prominent perineural thickening which was more often seen in the irradiated group rather than the control group. The statistical significance of this finding could not be established due to the limited number of cases with nerves outside of the bladder/prostate gland in the control arm. Some growth factors are also present in nerves and the surrounding cells and the relevance of this remains undetermined. The neural changes may be particularly important in the symptoms patients who have undergone radiotherapy complain of, since often no anatomical change is found macroscopically²³².

One of the more common complications of radiotherapy to the pelvis is cystitis, either associated with infection or unassociated. Patients also complain of haematuria often associated with ischaemic injury²³³. However, when specifically asked about these issues, patients report that their quality of life is not greatly affected²³⁴. There may also be other local and related anatomical problems like rectal injury, strictures, lower limb oedema, nerve injury, incontinence and maintenance of erectile potency. Post radiotherapy pain is also seen and this could be related to the neural changes described above. It would be worth pursuing the possibility that neural injury is an important factor in the symptoms patients suffer.

2.5 Summary and conclusion

The prostate gland will inevitably be exposed to radiation when tumours of the pelvic region are irradiated. The subsequent changes include a combination of inflammatory, fibroatrophic and reactive features which may be significant clinically either by producing symptoms or inducing abnormalities that provoke an alteration in subsequent management. Most of the changes are fairly predictable but this study has identified changes that have not previously been recognised and which are crucial to appreciate for two main reasons. Firstly, the findings help to explain some of the morphological changes that have been described by previous studies and secondly, some of the changes may account for some of the post-radiation symptoms suffered by many patients who have undergone pelvic irradiation. This could lead to novel methods of symptom relief and control and deserves more attention in the future.

Chapter 3. Effects of radiation on the human bladder

3.1 Introduction

Radiation is widely used to treat tumours of the pelvis, most frequently cancers of the bladder, prostate and cervix. Radical radiotherapy is associated with a good response rate when used for transitional cell carcinoma of the bladder, but occasionally salvage cystectomy is required²³⁵. Approximately 80% of patients with bladder cancer treated by external beam radiotherapy have long-term survival with intact, well functioning bladders²³⁶. In many cases, however, there is unavoidable injury to the surrounding non-neoplastic tissue. In some cases this includes the structures that make up the lower urinary tract including the bladder itself, the urethra and ureter. Patients develop symptoms soon after, or some considerable time following, radiation treatment and although over 70% of people may develop clinically relevant complications²³⁷ there is a limited amount of pathological data on human tissue with occasional studies on few cases. Despite the extensive literature on the clinical aspects of radiation injury in this area, even with the development of scoring systems, pathological information is limited. In most instances the material relies on superficial biopsies of tissue obtained transurethrally. It is essential therefore that a thorough examination of irradiated bladder tissue be made in order to extend our knowledge of this area and also to search for potential features that might explain the variety of symptoms patients have after pelvic irradiation. There is a large archive of tissue in the files of the Department of Pathology, Royal London Hospital which includes a number of surgical specimens removed and examined as part of the management for bladder cancer. The bladders were removed (cystectomy) for either locally advanced disease, recurrent disease or symptomatic control of complications of radiation.

3.1.1 Structure of the normal bladder

The internal epithelial lining of the bladder (urothelium) consists of 3-7 layers of urothelial or transitional cells resting on a thin connective tissue basement membrane. The cells replicate and proliferate from the base towards the luminal surface. As they migrate superficially, they become larger and take on a more voluminous appearance. At the surface they form 'umbrella cells' with tight junctions between them. There is a polysaccharide layer on the luminal aspect to protect the epithelium and underlying tissue and form a waterproof barrier²³⁸.

Beneath the basement membrane there lies a lamina propria composed of loose connective tissue with occasional smooth muscle cells. Deep to this there is a more prominent muscle layer; the detrusor muscle. This layer of muscle converges near the neck of the bladder to form a more structured arrangement of three circular layers, an inner, an outer and a middle which extend along the urethra in females and end at the limit of the prostate in men. This acts as an involuntary sphincter. The inner layer extends further, along the urethra in men and women.

The bladder is under autonomic control by motor innervation which provides efferent impulses. This occurs through parasympathetic fibres from spinal cord to the detrusor muscle via sacral and pelvic nerves. The trigone, seminal vesicle and ampulla of vas deferens are all under sympathetic control. The pelvic floor and external sphincter are innervated from S2-3 via the pudendal nerve. Afferent or sensory nerves travel via the pelvic nerve and sympathetic pathways to T11-L1.

The bladder's main function is to store urine for voluntary release when required. Storage is accomplished by compliance and requires competent sphincters. There is also an ureteral anti-reflux mechanism to prevent urine passing back into the ureters. The normal bladder capacity is around 400-500ml. On reaching appropriate compliance

afferent fibres send messages to the spinal cord which produces a suitable voiding mechanism. Damage to the bladder may result in reduced capacity to store or void urine and may lead to urinary incontinence. Proprioceptive nerves are also present in the bladder and therefore insult to the bladder may be associated with pain and/or contractions.

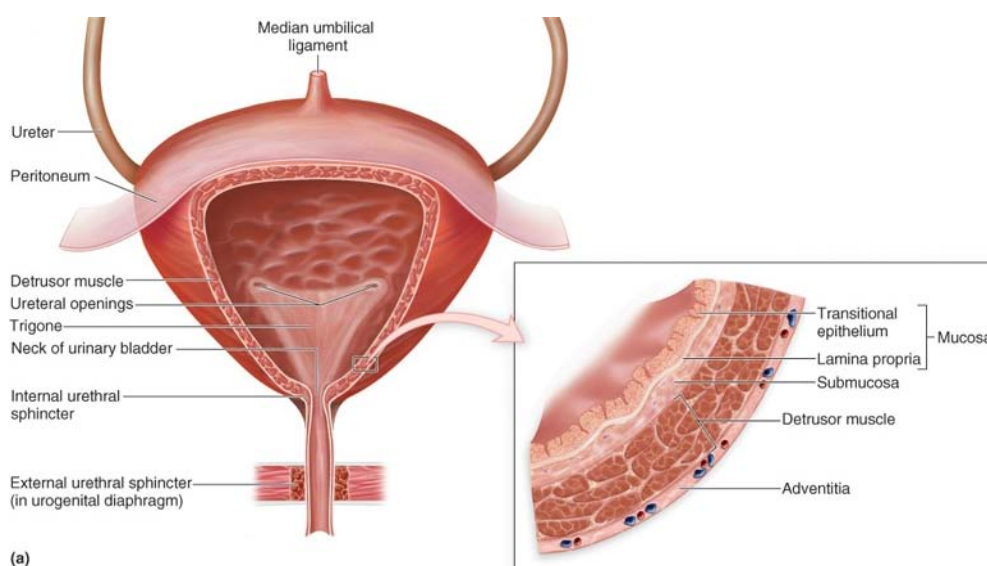


Figure 22. The anatomy and structure of the normal bladder²⁸² (permission for use granted)

3.2 Materials and methods

The files of the Pathology Archive of the Royal London Hospital were interrogated looking for patients with cystoprostatectomy for treatment of their urothelial carcinomas of the bladder for a 10-year period (1984-1993). The database search was then refined to identify those patients who had undergone pre-operative radiotherapy. This originally produced a total of 39 cases. All of the patients had been through the same course of radiotherapy consisting of 20 daily treatments of 55Gy each, over a 4-week period. The age range was 41-84 years. The protocol was similar for all and involved three beam

irradiation, one anterior and two posterior obliques. The field was determined using CT scan image based planning to build up a three dimensional map of the area to be irradiated. The map intentionally included a 10-15mm margin of surrounding tissue which would have incorporated the prostate gland. Subsequent surgery was necessary because of symptom control or resistant, recurrent or progressive tumour. In each case the time between irradiation and surgery was known (1 week to 17 years).

The surgical specimens were all handled in the same routine manner. Briefly, the specimens were fixed in 10% formalin, processed routinely and blocked into paraffin wax. The blocks of tissue were sampled as per our local Department protocol which follows the recommendations outlined in Ackerman¹⁶⁰. Between eight and twenty blocks were taken per case, of which 2-5 contained bladder wall (distant from tumour where possible). Sections were cut at 4-5 microns and stained with haematoxylin and eosin (H&E).

Each case was examined by two observers who scored the changes either present or absent. Changes within the epithelium, lamina propria, stroma, vessels and surrounding structures were looked at and recorded. In particular we looked at epithelial cellular changes, epithelial metaplasia, stromal inflammation, mural fibrosis and vessel changes together with changes within and around nerves. Blocks and slides with direct tumour involvement were excluded. The changes were scored present or absent. Where possible the changes were related to the time since irradiation to see which alterations were seen early, which were late and which were present continuously. In every case there was agreement between observers either individually or after consultation. The alterations were compared with autopsy bladder tissue and cystectomies from patients with interstitial cystitis. However, there were too few of these 'control' cases to perform meaningful statistics. Many of the changes searched for were the same as those

examined in the prostate glands. The bladder wall is a more complicated structure with more anatomical relationships to surrounding tissues than the prostate and therefore there were additional features to study (see Table 2).

3.3 Results

The overall results are presented in Table 2.

The dominant early changes (less than 1 year after irradiation) were eosinophil rich inflammation (Figs. 23 and 24), ulceration, urothelial hyperplasia, transitional cell nuclear atypia, endothelial cell enlargement and smooth muscle cell necrosis. Later changes (over 1 year after radiotherapy) included prominent numbers of mast cells (Fig. 25), fibrosis of the mural smooth muscle, perineural thickening, serosal fibrosis and vessel changes including myointimal inflammation and thickening, and endarteritis obliterans with subintimal foam cells (Figs. 26 and 27). Some of the more common findings throughout included the presence of eosinophils, vascular thrombosis (Fig. 28), perineural inflammation and serosal fat necrosis. The tumour was viable in the majority of cases where residual or recurrent tumour was present.

Feature	Early	Late
Ulceration	+	+
Urothelial hyperplasia	+	-
Urothelial cell atypia	+	+
Squamous metaplasia	+	+
Endothelial cell enlargement	+	-
Arterial intimal hyperplasia	+	+
Fibrointimal thickening	-	+
Endarteritis obliterans	-	+
Subintimal foam cells	-	+
Thrombosis	+	+
Marked arterial luminal narrowing	-	+
Smooth muscle cell necrosis	+	-
Arterial medial thickening and/or hyalinisation	-	+
Stromal inflammation	+	+
Marked stromal fibrosis	-	+
Smooth muscle fibrosis	-	+
Eosinophils	+	+
Mast cells	+	+
Perineural inflammation	+	+
Perineural thickening	+	+
Serosal fat necrosis	+	+
Serosal fibrosis	-	+

Table 2. Features seen in the bladder after local irradiation (listed in no particular order but from inner to outer aspect of the bladder wall).

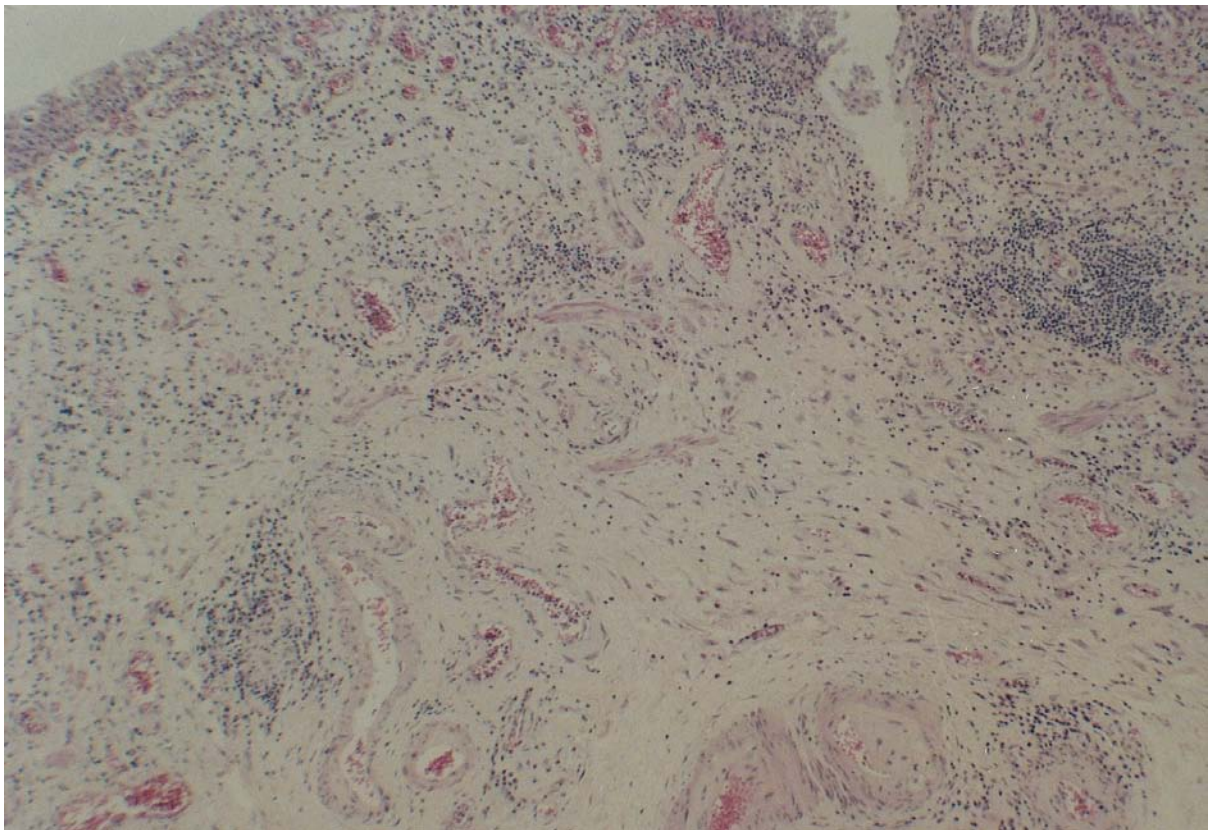


Figure 23. Inflammation was commonly seen in irradiated bladders.

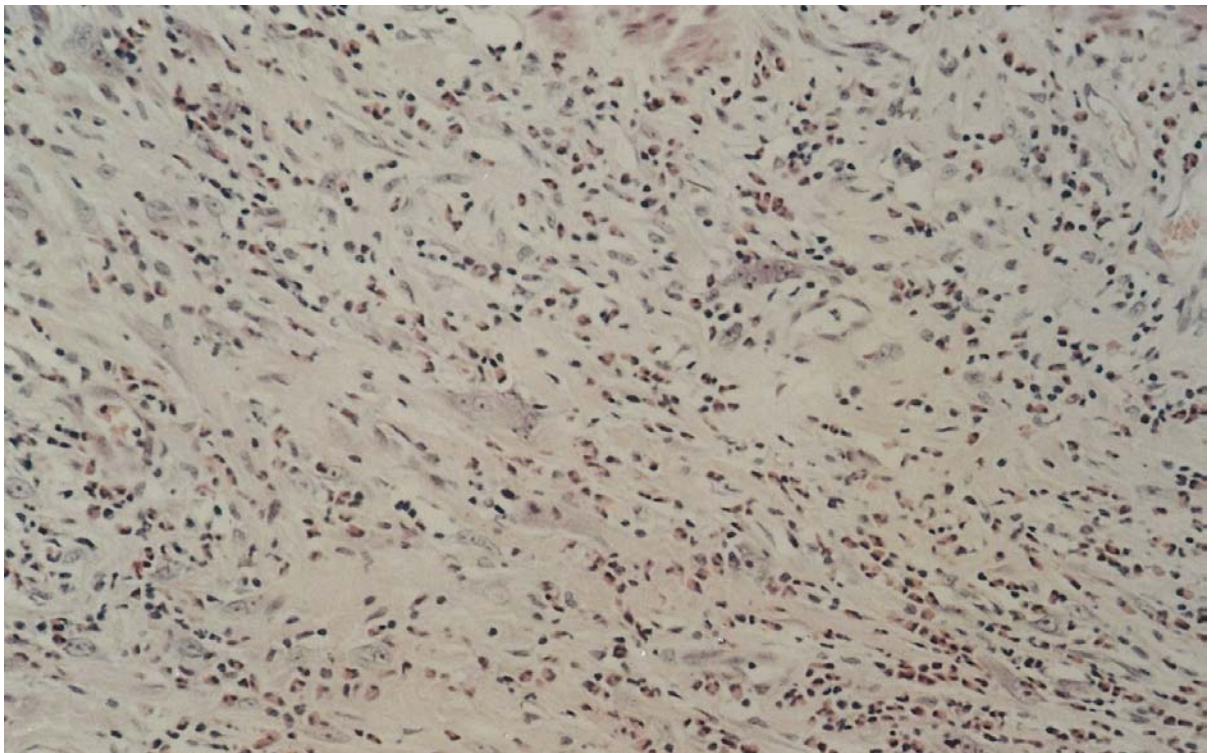


Figure 24. The inflammation was often eosinophil rich. Note the stellate fibroblasts.

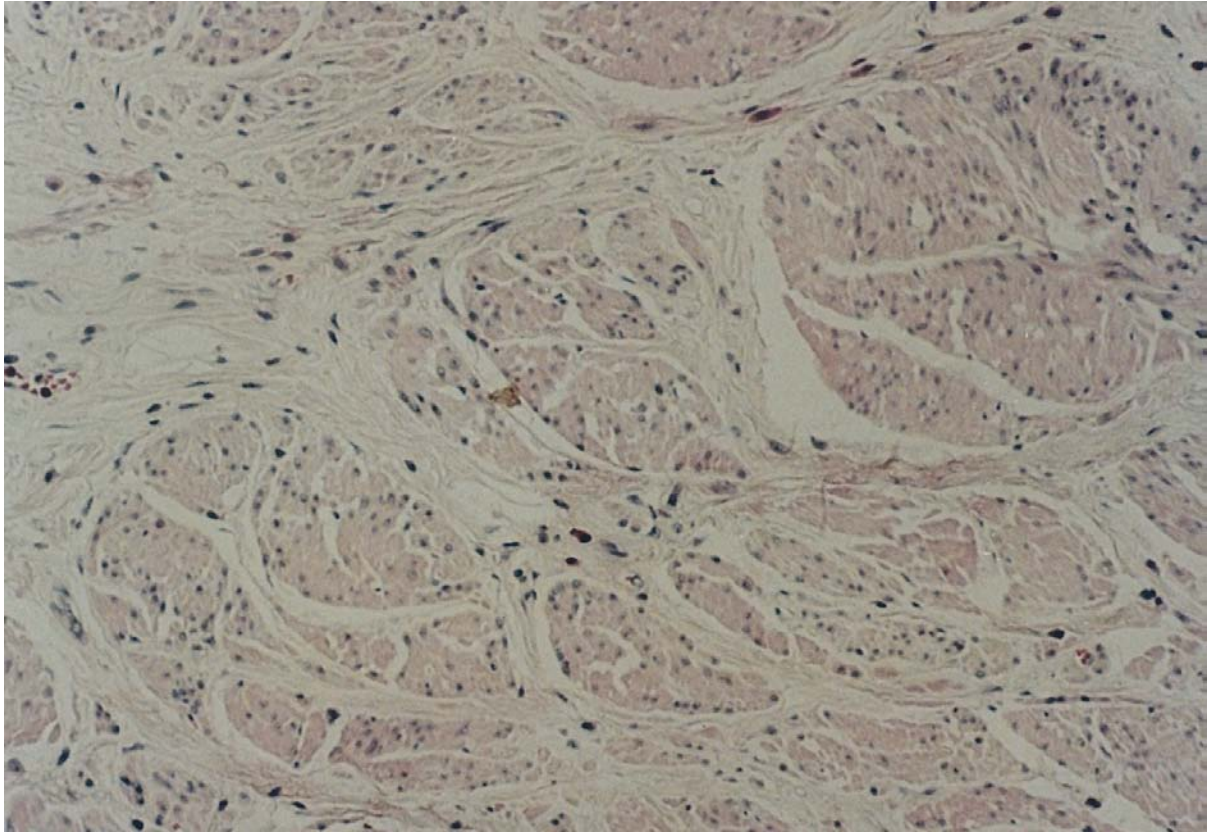


Figure 25. Mast cells were frequently observed in the bladders after radiotherapy.

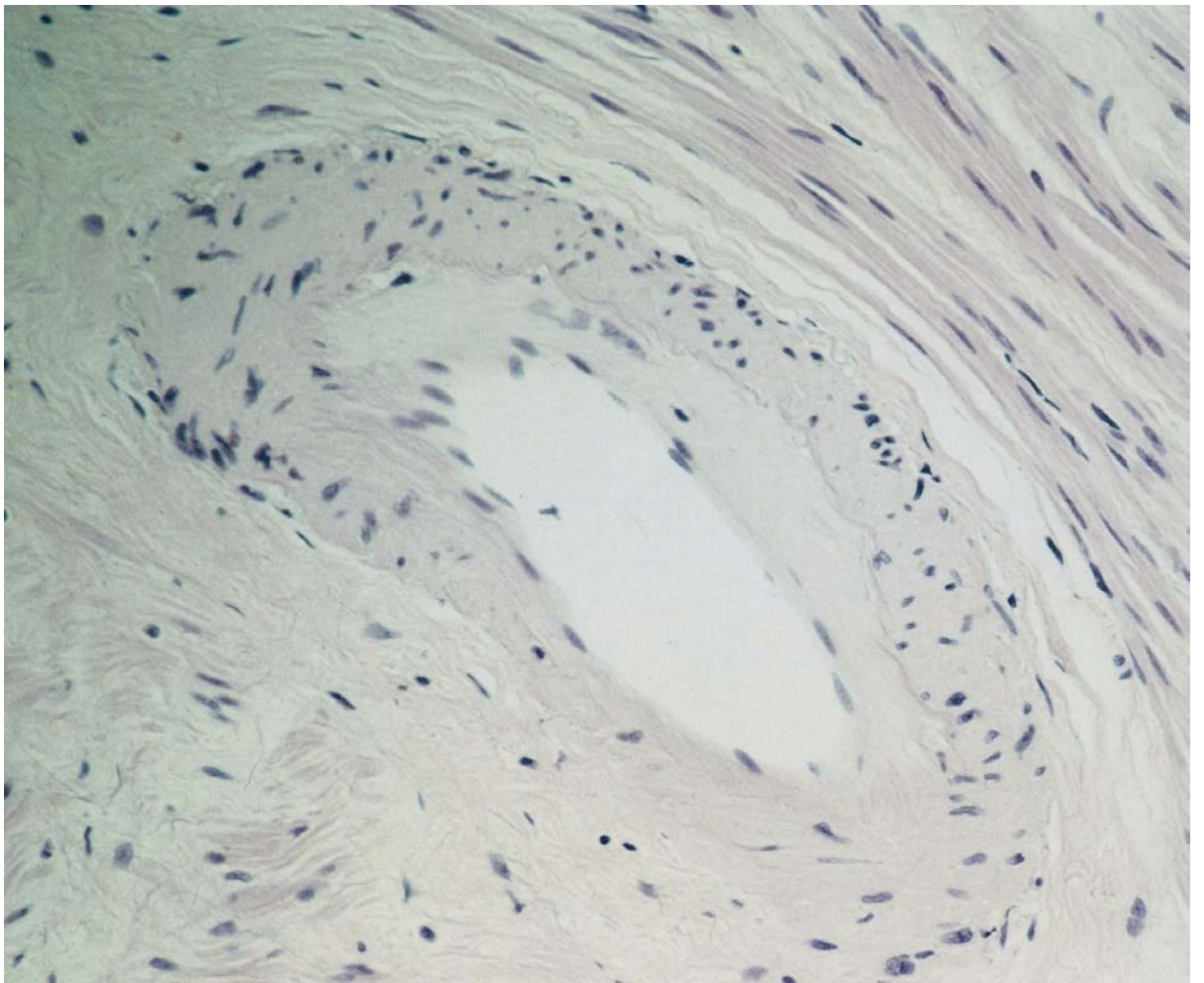


Figure 26. Intramural vessels showed intimal fibrosis.

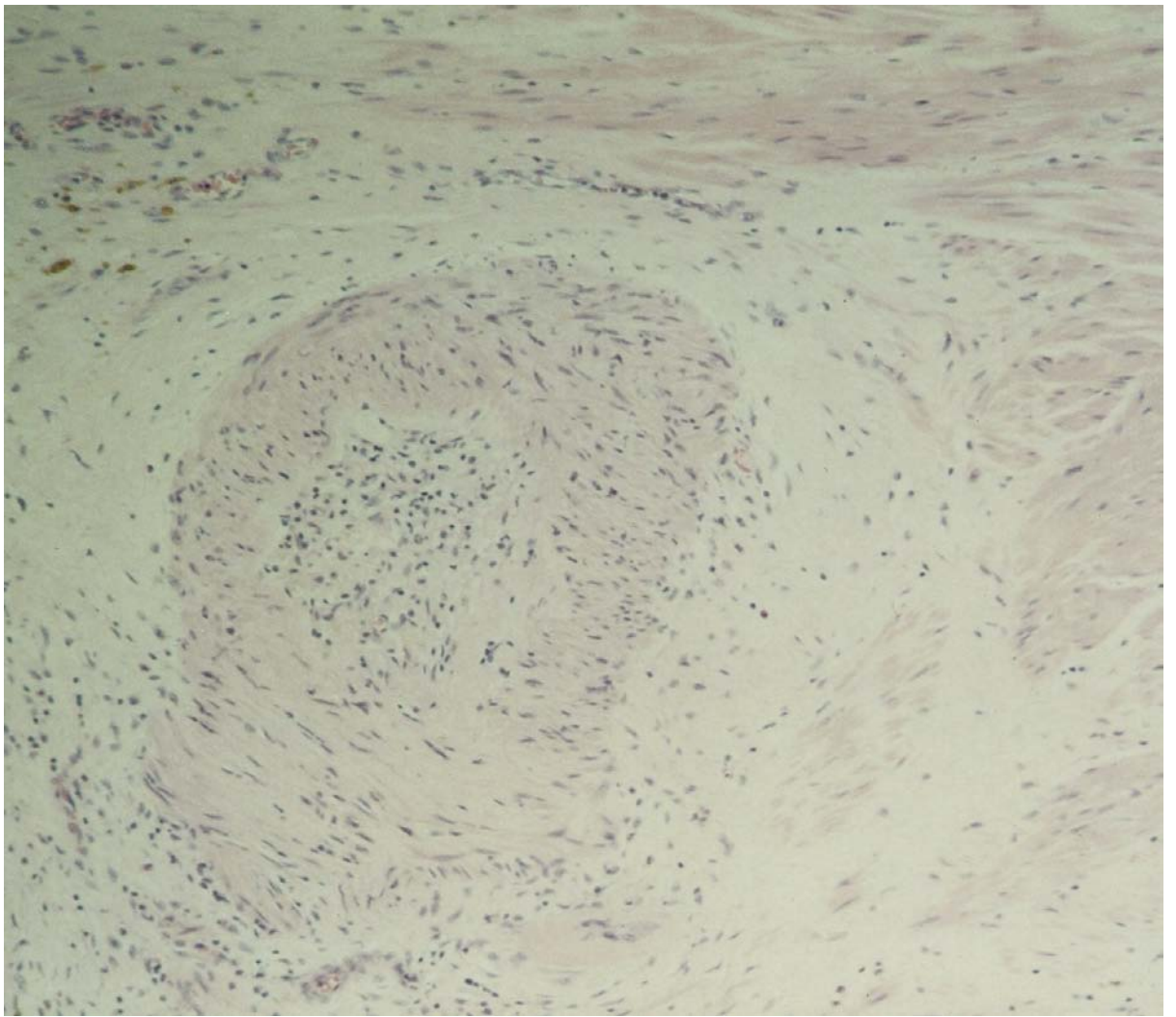


Figure 27. There was sometimes an intimal arteritis as well as fibrosis.

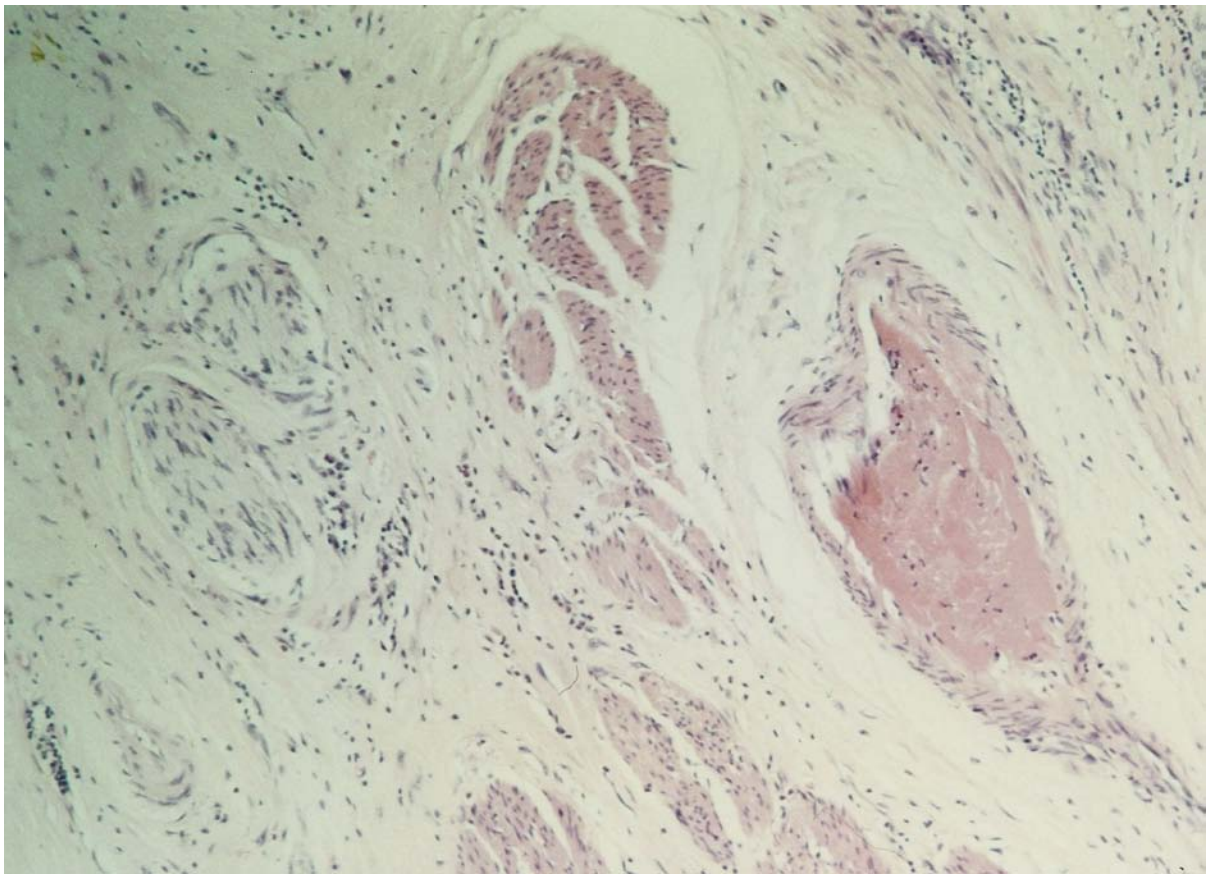


Figure 28. Occasionally the vessels within the bladder wall were thrombosed.

3.4 Discussion

Radiation is frequently used in the treatment of a range of pelvic tumours including transitional cell carcinoma of the bladder. The optimal and most appropriate use of the various techniques in the treatment of bladder cancer has been outlined fairly recently by an International Committee after critical review of the literature available²³⁶. The panel looked at studies describing the use and effectiveness of external beam therapy, interstitial radiotherapy and therapy combined with surgery and/or chemotherapy. They concluded that radiotherapy was an effective treatment for bladder cancer, producing long term disease control and preservation of normal bladder function. Choice of appropriate treatment for the correct individual case was considered paramount with all newly diagnosed patients being assessed in a multidisciplinary setting where the relative merits of surgery, radiotherapy and chemotherapy can be considered. Others have reviewed the most appropriate role and type of radiotherapy for urinary bladder cancer²³⁹. When compared with surgery some suggest that there is no benefit of pre-operative radiotherapy although radiation has the advantage that it can palliate tumour related symptoms in bladder cancer²³⁹.

Others have outlined the usefulness of radiation therapy for the treatment of bladder cancer but all remind us that radiotherapy is often associated with unwanted side effects^{235,240}. The latter looked at 163 patients who were treated with radiotherapy and found that 61% achieved a complete response, depending on T stage, varying from 90% for T1 tumours to 53% for tumours stage T3. A few relapsed in the bladder. There were 18 (11%) partial responders of whom 7 required subsequent salvage cystectomy. All of the patients who did not respond died relatively quickly with a median survival of only

10 months. Their results suggested that radiation before surgery was beneficial in many cases.

Marks and colleagues reviewed the usefulness of radiotherapy and chemotherapy in lower urinary tract lesions and discussed the complications and morphological changes produced by radiotherapy²⁴¹. They reviewed the clinical syndromes associated with radiation treatment of the lower urinary tract and tabulated the complication rates for radiotherapy directed at the pelvis, including urothelial tumours. They also outlined the tolerance doses, options for treatment of side effects and scoring systems for the unwanted effects encountered. Between 3 and 47% of patients treated in a range of studies by radiotherapy for bladder cancer, were reported to have developed significant side effects (greater than or equal to grade 3 in their subjective, objective, management and analytic system). Complication rates after radiotherapy for prostate cancer and cervical cancer were also provided.

There are many other publications on the clinical side effects of radiotherapy used for pelvic tumours. Tomic described 121 patients with muscle invasive or recurrent transitional cell carcinoma who were treated by radiotherapy and cystectomy and found clinically relevant complications in 71-78% of patients (many intestinal)²³⁷. Furthermore, they describe 3% mortality from these complications. On the other hand, late toxicity appears to be less of a problem after three-dimensional conformal radiotherapy and intensity modulated radiotherapy (IMRT) for prostate cancer²⁴².

In an Italian series of complications following radiation treatment of bladder cancer, Tonoli et al found 459 cases with grade 1-2 toxicity of which the majority were urinary 23%, and 6% were late and enteric²⁴³. It appears that some of the complications, including radiation cystitis, can be alleviated by hyperbaric oxygen. Three women with radiation cystitis given hyperbaric oxygen were found to cease their macrohaematuria

and cystoscopy showed that the macroscopic and microscopic bladder inflammation improved but the telangiectasia did not resolve completely²⁴⁴.

There is a fairly well established history of animal studies of radiation injury in the bladder including investigations on dogs, rabbits, mice and rats. In the dog, complications of irradiation for pelvic tumours were seen in 20 of 51 dogs irradiated (39%)²⁴⁵. The complications included skin necrosis (7), chronic colitis (4), strictures (4), osteopaenia (2), rectal perforation (1), bladder thickening (1), iliosacral osteosarcoma (1), and one each of pelvic limb oedema and perianal pain.

In rabbits, epithelial and muscular changes were found to be dose dependent, but the submucosal and extramuscular changes were not²⁴⁶. The transitional epithelium was either atrophic or hyperplastic, umbrella cells were always present and there was fibrosis in the submucosa and muscle layers. Blood vessel and lymphatic changes were seen in some of the animals. Rabbits studied by angiographic, microangiographic and tissue microscopy showed similar changes with atrophy of mucosa, epithelial desquamation, cellular atypia and ulceration and that these irradiation changes appear earlier when increased doses of radiation are used²⁴⁷.

Antonakopoulos et al. looked at changes in the rat bladder after X-irradiation²⁴⁸. By light and electron microscopy they detected basal cell damage in the urothelium early on which extended to the intermediate cells a little later and by 6 months the epithelium was generally hyperplastic. The blood vessels showed changes with endothelial cell and smooth muscle cell damage. Fibroblasts appeared to show increased secretion with abundant collagen deposition leading to severe fibrosis of bladder wall. After a few months muscle cells degenerated, became disorganised and some were even necrotic. By 20 months nearly 60% of surviving animals developed transitional cell carcinomas. In another rat model of post-radiation bladder dysfunction, two phases of reduced

bladder compliance were noted, the first at 4 weeks and the second at 6 months. Histologically, at 6 months after irradiation, increased mast cell density was identified with fibrosis in around half of the cases. Electron microscopy showed smooth muscle cell injury and selective degeneration of unmyelinated axon profiles²⁴⁹. Similar changes were observed in mouse bladders, which could be reversed by acetylsalicylic acid which suggested that prostaglandins may be involved in the radiation response²⁵⁰.

In humans, the histological changes associated with radiation injury in the bladder are generally poorly documented and most of the studies previously performed have only looked at superficial bladder mucosal biopsies. Consequently, the pathogenesis of many radiation related symptoms remain unexplained²⁵¹. Superficial biopsies have been investigated previously because the easiest way to take samples for histology is obviously via cystoscopy. One of the largest series of cases is described in the article by Suresh et al.²³¹. They investigated the changes in superficial biopsies of 15 patients together with 3 cystectomies. All patients had received radiotherapy as part of their treatment for cervical and uterine cancer. The time interval varied from 6 months to 4 years. Symptoms included urinary frequency, dysuria, incontinence and haematuria. Histological changes on the surface included ulceration, cellular atypia, proliferation, squamous metaplasia, cytoplasmic vacuolation. In the stroma and/or submucosa there was oedema, fibrosis, atypical stromal cells, vascular changes, deep epithelial inclusions (cystitis cystica) and a conspicuous inflammatory cell infiltrate. When the authors compared the differences in specimens taken before and after 12 months from the date of radiotherapy, they found that the epithelial changes were prominent in the early cases and the stromal changes dominated the later cases. However, there was a continuing spectrum of epithelial changes which persisted many years after initial radiation. Inflammation was present throughout, but there was 'no predilection for eosinophils in

infiltrate'. The inflammation was thought to be the cause of the fibrosis which developed quite early, but which persisted into later stages.

Some post-radiation changes can cause confusion with recurrent or residual tumour. A review of such radiation and chemotherapy related changes described the issues with clues to avoid confusion with urothelial carcinomas²⁵². One particular clue for differentiation between the two is the finding of characteristic chemotherapy and/or radiotherapy changes nearby which can help to avoid misinterpretation. Furthermore, 'polypoid' or 'papillary cystitis' is another mimic of transitional cell carcinoma that has been described in 41 of 155 cases originally diagnosed as neoplasms by Lane and Epstein²⁵³. Two of these cases followed previous radiotherapy. These changes were not seen in our cases.

In order to enhance the current literature on radiation changes to the bladder, we reviewed a large series of cystectomy specimens removed after a standard course of radiotherapy. Many of the findings described in previous animal and human studies were confirmed, but alterations in deeper structures were also identified. Some of these might explain some of the symptoms experienced by patients after radiotherapy, others are novel. The significance of some of the changes is uncertain. Furthermore we were able to categorise our cases into arbitrary groups on the basis of time since irradiation. We found differences between changes seen early and late phases. Additionally, the progression of many of the changes from the early period to later could be demonstrated.

The changes found in the bladder wall are outlined in the results section and will not be repeated here. Briefly however, there were consistent epithelial changes, such as hyperplasia, cellular atypia and metaplasias; almost universal necro-inflammatory changes throughout the bladder wall; frequent vascular changes and also changes in the

nerves of many of the cases. The early post-radiation period was dominated by the inflammation, ulceration and necrosis (although these were frequently seen to a lesser extent throughout the study period) and later the picture was dominated by the vascular compromise associated with arterial narrowing and obstruction. Some features were seen in virtually every case, such as conspicuous eosinophils, vascular changes and perineural changes.

The presence of cellular atypia is well described after irradiation, especially within epithelial cells. Radiation is also known to cause tumours, usually after a significant latent period of several years. It is likely that these two are related although it is interesting to note that cellular atypia is a frequent finding after bladder irradiation but development of urothelial carcinomas is not²⁵⁴. Researchers following a group of people exposed to radiation after the Chernobyl incident have demonstrated DNA damage in urothelial cells of those exposed²⁵⁵. They found significant activation of DNA damage repair mechanisms (base and nucleotide excision repair) which were thought to be induced by oxidative stress generated by ionising radiation. Some patients developed chronic proliferative atypical cystitis, others had multifocal dysplasia and carcinoma in situ. Ten of their cohort developed transitional cell carcinoma, although all were small and superficial. All of the findings were significantly higher than in their control group.

The morphological changes described integrate well with the current theories of the pathogenesis of radiation injury, including that seen in the bladder. This has been illustrated fully in the previous chapters. The role of the inflammation, with a range of inflammatory cytokine including eicosanoic acid derivatives and growth factors is a fundamental part of the process. Whether eosinophils have a special relevance in radiation injury remains unknown but they appear to be a consistent feature of the reaction of tissues to irradiation. Furthermore, direct and indirect nucleic acid damage is

also important and it introduces the concept of radiation inducing accelerated ageing as discussed in the chapter on the prostate gland. The key features of radiation changes and the pathogenesis of these changes is leading to realistic treatment options for patients who develop complications after radiotherapy such as cytokine inhibitors and growth factor influencers. In some, but not all, bladders, the tumour was persistent and viable after radiotherapy. Clearly it is impossible to determine whether some of the changes observed were related to the presence of the tumour. Another potential contributor to post-radiation effects on the bladder is drug-related injury although this is reported not to increase the risk of clinical complications, above radiotherapy alone²⁵⁶. The clinical and pathological effects of radiotherapy are said to be dose related although this could not be determined in this study since all patients have received the same standard course of therapy.

Two potentially important new findings from this study included the changes to pelvic nerves and the presence of mast cells. The latter may go some way to explaining the unresolved symptoms that some individuals suffer, in particular the local pain. The pelvic nerve changes included perineural fibrosis and perineural inflammation with ganglionitis. Mast cells have been described in a range of fibrosing conditions around the body including scleroderma and scar tissue. Mast cell numbers were increased in the bladder after irradiation. They also appear to be sources of growth factors, in particular PDGF²⁵⁷. The latter is a potent chemotactic agent and activator of fibroblasts, leading to stromal fibrosis. Increased mast cell density has previously been described in association with bladder irradiation in animals²⁴⁹. This rat model of radiation injury to the bladder found that half of the rats irradiated had a reduced index of bladder compliance beginning at 4 weeks after treatment which progressed at 3-4 months and persisted at 6 months. Biopsy at 6 months showed increased numbers of mast cells in the irradiated group with degenerative changes in smooth muscle cells and selective

degeneration of unmyelinated axons. The results suggest a link between mast cells and post-radiation fibrosis and also subsequent neuronal injury.

Increased mast cells have also been described in the detrusor muscle of patients with spina bifida where recurrent infections lead to mural fibrosis²⁵⁸. These patients also had evidence of mast cell degranulation and increased collagen deposition, which were thought to be directly related.

3.5 Summary and conclusion

Radiation is commonly used to treat cancers of the pelvic organs. Complications are relatively common and sometimes these involve the lower urinary tract. Many of the complications can be explained by the necro-inflammatory and fibro-atrophic processes seen within tissues exposed to irradiation anywhere in the body. Others may be related to the neurological changes and other novel findings discovered during this investigation. The pathogenesis of radiation related injury to the bladder is very likely to be similar to radiation injury in general and reflect cellular toxicity followed by a series of inflammatory and growth promoting events which lead to the processes described above. In some unfortunate individuals radiation may also be carcinogenic. A better understanding of the progressive changes that are involved in radiation injury will pave the way for targeted therapy which could reduce or even reverse the unwanted effects of radiation on the bladder.

Chapter 4. Fibrosis and fibronectin in the bladder after irradiation

4.1 Introduction

Most superficial bladder tumours can be treated by chemotherapy or removed by minor surgical procedures, muscle invasive tumours often require radiotherapy. This not only has an effect on the tumour but also damages the surrounding normal tissue. Early (reversible) or late (irreversible) side effects may develop²⁵⁹. The primary role of the vasculature in the development of these effects has been known for some time^{260,261}, and the degeneration of the smooth muscle cells in the tunica media and its replacement by hyaline material is a common finding in human radiation pathology, as is the development of fibrosis to the irradiated urinary tract²⁶². The latter is due to the excessive synthesis of ECM components, including collagen types I and III, elastin, glycosaminoglycans, and the basement membrane constituents (fibronectin, collagen IV and laminin). The latter three components provide structural support for bladder mucosal cells and influence cell migration, attachment, differentiation and proliferation.

More than 30 years ago the World Health Organization proposed to define fibrosis “as the presence of excess collagen due to new fiber formation”²⁶³. The same is true for radiation fibrosis that was classically considered as a chronic and progressive process in which normal tissue is replaced by fixed and irreversible fibrotic tissue. This view has however been challenged and fibrosis has been recently redefined as a dynamic process resembling chronic wound healing²⁶⁴⁻²⁶⁶.

Fibronectin is a large multifunctional high molecular weight glycoprotein which is bound to cell surfaces by integrins. It is an important participant in tissue morphogenesis and repair and exists in several forms created by both alternative splicing and post-translational modifications. The ED-B+ (extra domain B) form is restricted to embryonic tissues, but it has been shown to subsequently reappear in

granulation tissue, liver cirrhosis, fibromatosis and in tumour stroma²⁶⁷⁻²⁷⁰. Its expression is stimulated by TGF- β and has been linked with tumour angiogenesis and behaviour with regards to invasiveness and metastatic potential²⁷¹. More importantly, several recent studies have underlined its importance in relation to cell survival after radiation damage²⁷²⁻²⁷⁶.

Historically there have been very few studies concerning the histological changes induced by irradiation of the urinary bladder^{248,277}. Other researchers have concentrated on the histological changes in the bladder biopsy after irradiation and have been descriptive²³¹. Radiation induced late changes have been studied in various animal models, but the time after radiation was short (6-12 months)²⁷⁸.

This study therefore aims to quantify the degree of fibrosis in the bladder wall at various times after irradiation, and to compare it with a range of control groups. Furthermore, given its close involvement with repair and fibrosis in other situations, and more recently determined roles in modulating cell cycle arrest and survival after radiation, the expression of fibronectin in the various components of the irradiated bladder wall will be examined.

4.2 Materials and methods

Ninety-four cystectomy specimens were included in this study group and comprised: 56 pre-operatively irradiated bladder tumours, 18 non-irradiated bladder tumours, 6 interstitial cystitis and 14 post-mortem bladder specimens. The irradiated bladder group was further subdivided into 19 cases, 26 cases and 11 cases, each group of which had undergone cystectomy 0.25-12 months, 12-36 months and 36-98 months after irradiation respectively. The specimens were obtained from patients attending The Royal London Hospital between 1981 and 1995, and ranged in age from 41-84 years

(mean 58.7years). All 56 irradiated patients had received a standard course of radical radiotherapy (20 treatments of a 55 Gray dose over 4 weeks), and required cystectomy because of either recurrent tumour or urinary symptoms. The time between radiotherapy and subsequent surgery ranged from 0.25 to 98 months (mean 23.8).

4.2.1 Immunohistochemical and tinctorial analysis

Cystectomy tissue blocks (distant from the tumour) were retrieved from the archives, and 3 micrometer sections were cut and placed on Superfrost slides for immunohistochemical localisation of fibronectin and on ordinary microscope slides for both haematoxylin and eosin (H&E) and tinctorial staining of collagen.

Avidin-biotin-peroxidase complex (ABC) method was used in conjunction with monoclonal mouse anti-human fibronectin antibody (NCL-FIB, Novocastra, UK) at a dilution of 1:200. Normal kidney was used as a positive control, and the negative control had the primary antibody omitted. Picosirus red was used for collagen localisation as it gave greater contrast with the background tissue components than haematoxylin van Gieson. Furthermore it has been shown to stain collagen stoichiometrically under similar conditions and reflects the amount of fibrosis present^{279,280}. Positivity for fibronectin was defined as a convincing brown colouration of the component tissue which was clearly different from the background counterstain (usually comparing to the smooth muscle present which was negative). All cases were assessed by two observers and any cases which were considered particularly difficult and agreement was not reached independently were read together and the consensus answer was decided to be the final result.

In order to validate the results, suitable positive control tissues were performed alongside each antibody run. Normal kidney tissue was used as a control as per the

manufacturers recommendations. Negative controls included omission of the primary antibody.

4.2.2 Morphometric image analysis

A JVC 3-CCD Model KY-F55BE video camera mounted on a Zeiss microscope was used. The video output was connected to a microcomputer which contained the Zeiss KS400 image analysis software package (version 3). The light source was controlled by a photo diode voltage regulator (7.90V), which maintained a light source of constant intensity. Histological images of human urinary bladder were visualised using a x 10 eyepiece and x 6.3/0.16 objective lens at an Optivar setting of 1.25. Two dimensional Red-Green-Blue (RGB) colour images were acquired using the live video camera displayed on screen and optimally adjusted using the focus on the camera and microscope.

The picrosirius stained slides were subjected to morphometric image analysis in order to measure the amount of collagen present. This was used as a marker of fibrosis in all the study groups; the distribution of collagen throughout the lamina propria (LP) was compared to that within the MP. For each case, 4 tumour free random sample areas within both the LP and MP were analysed. In order to determine intra and interoperator reproducibility the area of interest in 10 random cases was randomly remeasured and the analysis revealed consistent results. The image was thresholded (definition of minimal pixel intensity to be included in the calculation) and pointset described (definition of the parts of an image to be included in the calculation; Figs 30 and 31). The total stained area and field area were measured, the information transferred to an Excel spreadsheet and the interactive statistical visualisation package JMP (SAS Institute, USA) was used to provide ANOVA (One Way Analysis of Variance) and

Dunnett's test analysis to compare irradiated and nonirradiated groups against the control post mortem groups. P values less than 0.05 were considered as significant.

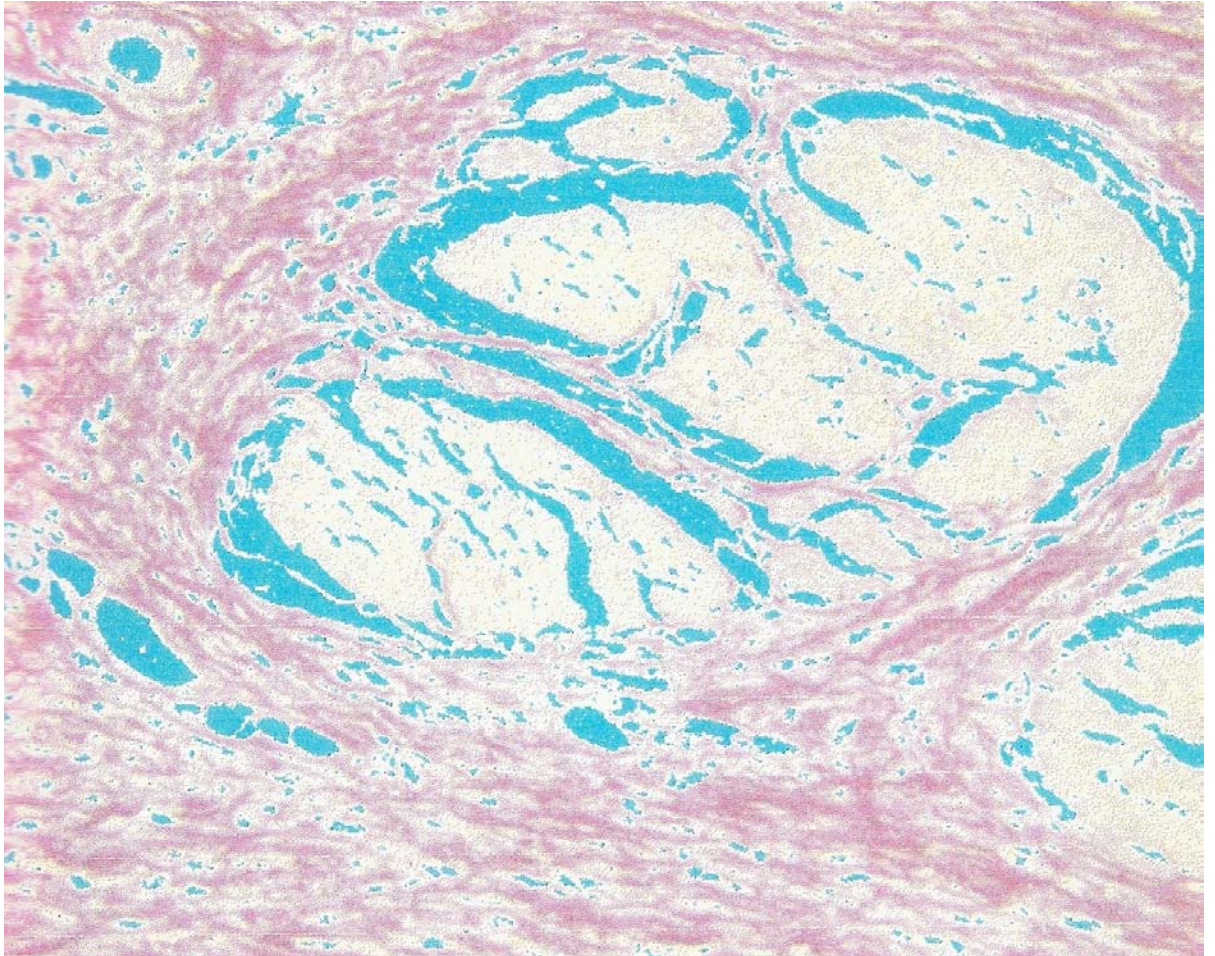


Figure 29. The captured image highlighted with blue to exclude the gaps in the tissue associated with processing

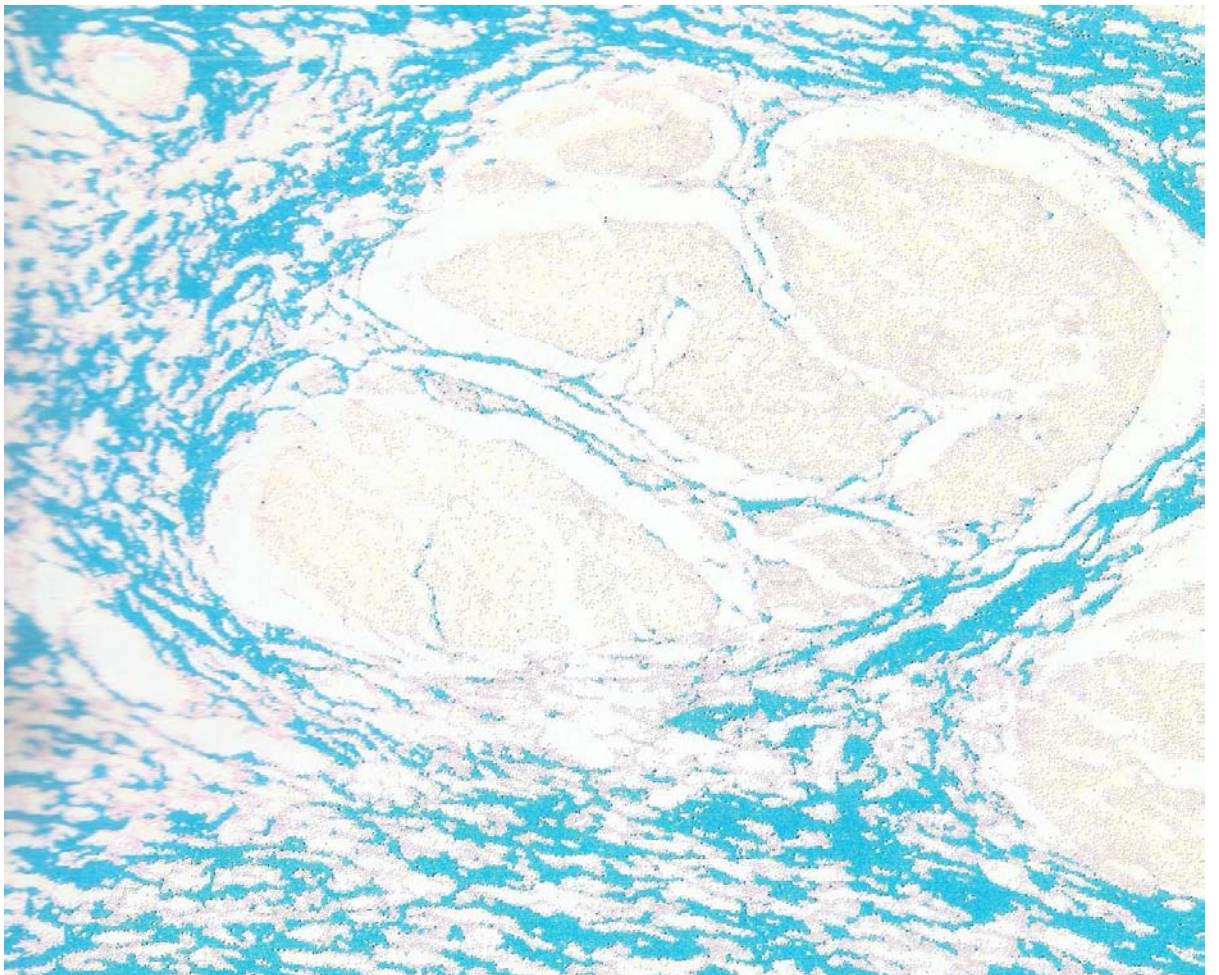


Figure 30. The blue pixels now show the areas of collagen to be calculated

4.3 Results

4.3.1 Fibronectin immunohistochemistry

The general stroma, basement membrane (BM), fibroblasts and endothelial cells of both the mucosal and MP layers were analysed. Immunoreactivity was microscopically evaluated in two different areas of the sections and scored as either positive or negative. The results are summarised in Table 3 and provided in Appendix 2.

Of 14 control non-irradiated post-mortem cases 3 (22.4%) showed mucosal stromal and 4 (35%) showed mucosal fibroblast positivity for fibronectin. In contrast MP general stroma, BM and fibroblasts were negative, as was the BM of the mucosa. A similar finding was seen in the cystitis cases with the MP general stroma, BM and fibroblasts again being negative, where as the mucosal general stroma 5(83.3%), BM 1(16.6%) and fibroblasts 1(16.6%) were positive.

One of the key findings was that in the irradiated and non irradiated tumour groups there was a diffuse increase in fibronectin positivity (Fig. 31) particularly in the MP general stroma, BM and fibroblasts, all three of which were negative in the post mortem and control groups. The increase in expression was more prominent in the irradiated group. This group showed the highest levels of fibronectin expression in all the four components analysed both in the mucosa and the MP (Table 4).

The other notable feature was the consistently high level of fibronectin expression by endothelial cells in all the groups studied, both in the mucosa and MP. Again the highest levels of fibronectin expression (92.8% and 94.6% for mucosal and MP endothelial cells respectively), are in the irradiated group.

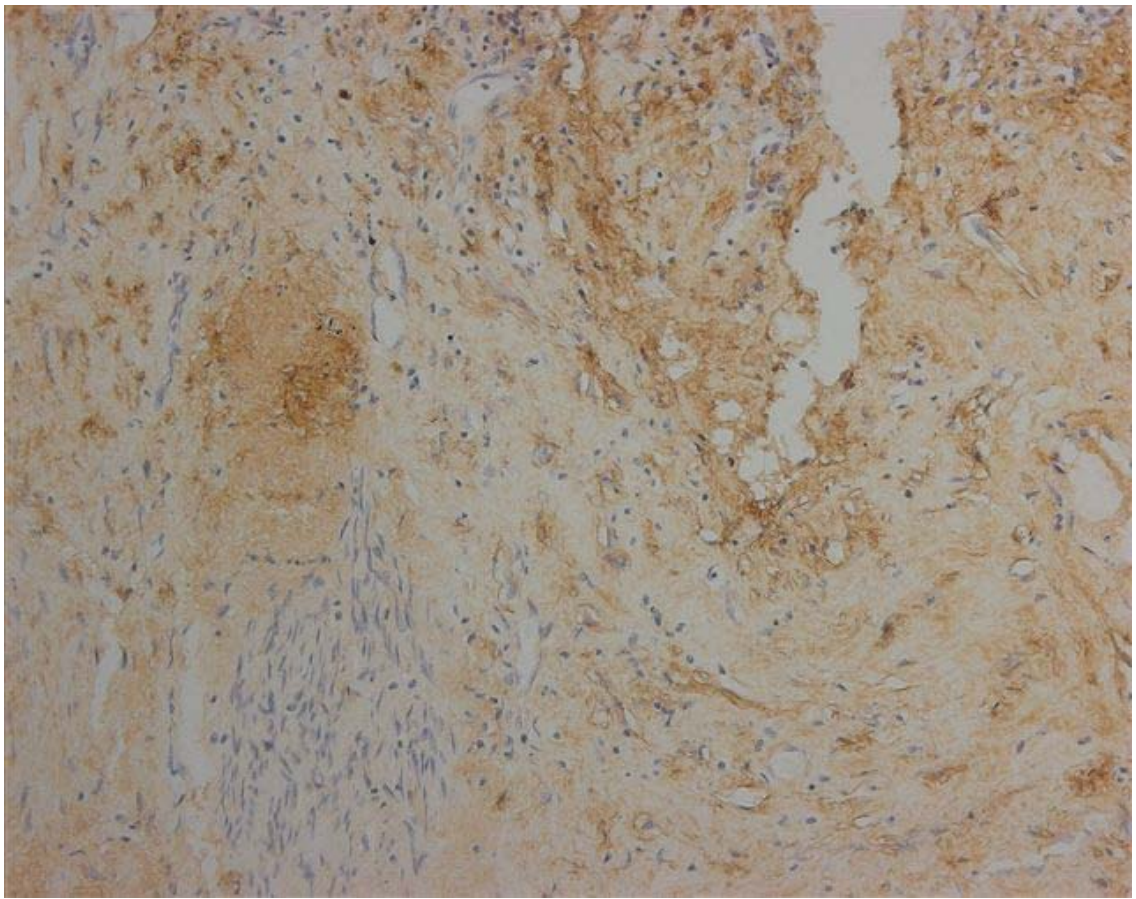


Figure 31. Positive staining for fibronectin in the superficial part of the bladder wall after radiotherapy. Note the smooth muscle bottom left which is negative.

The irradiated bladder tumour group was subdivided according to the timing of the cystectomy post irradiation. Group A included 0.25 to 12months, B 12-36 months and C 36-98 months. The fibronectin expression in the mucosal and MP layers of the bladder for each group are detailed in Tables 4 and 5. Of note again is the consistent high expression of fibronectin by the endothelial cells in both sites which does not drop below 88.4% at any stage and is 100% in both sites in group A (0.25-12months). In the mucosal and MP basement membrane the percentage of fibronectin expression appears to be negatively correlated with the time post irradiation.

4.3.2 Collagen quantification

Although there was a steady increase in the amount of mucosal and MP collagen between the three post irradiation time groups (Graph 1), this was not statistically significant. However, the degree of MP fibrosis in both the irradiated and non irradiated tumour groups was significantly higher ($p < 0.05$) when compared with the post-mortem group, Graph 2.

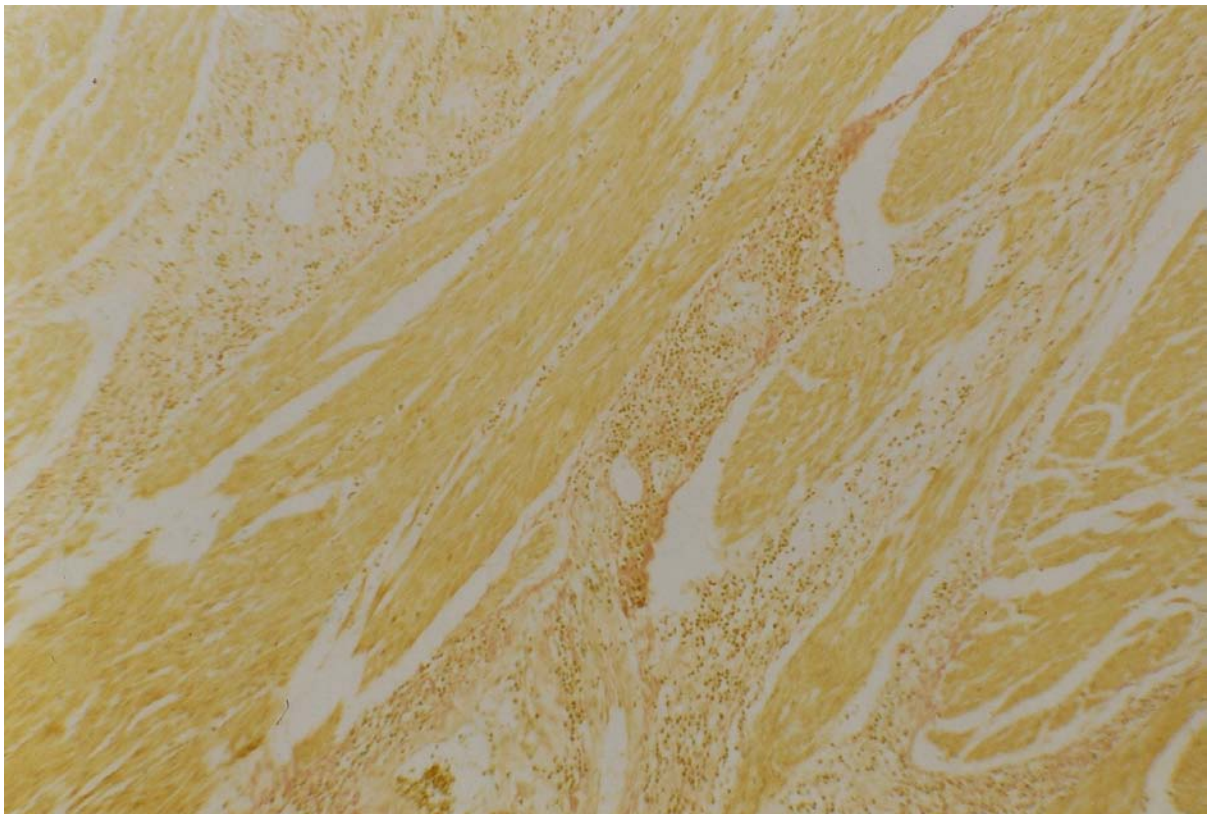
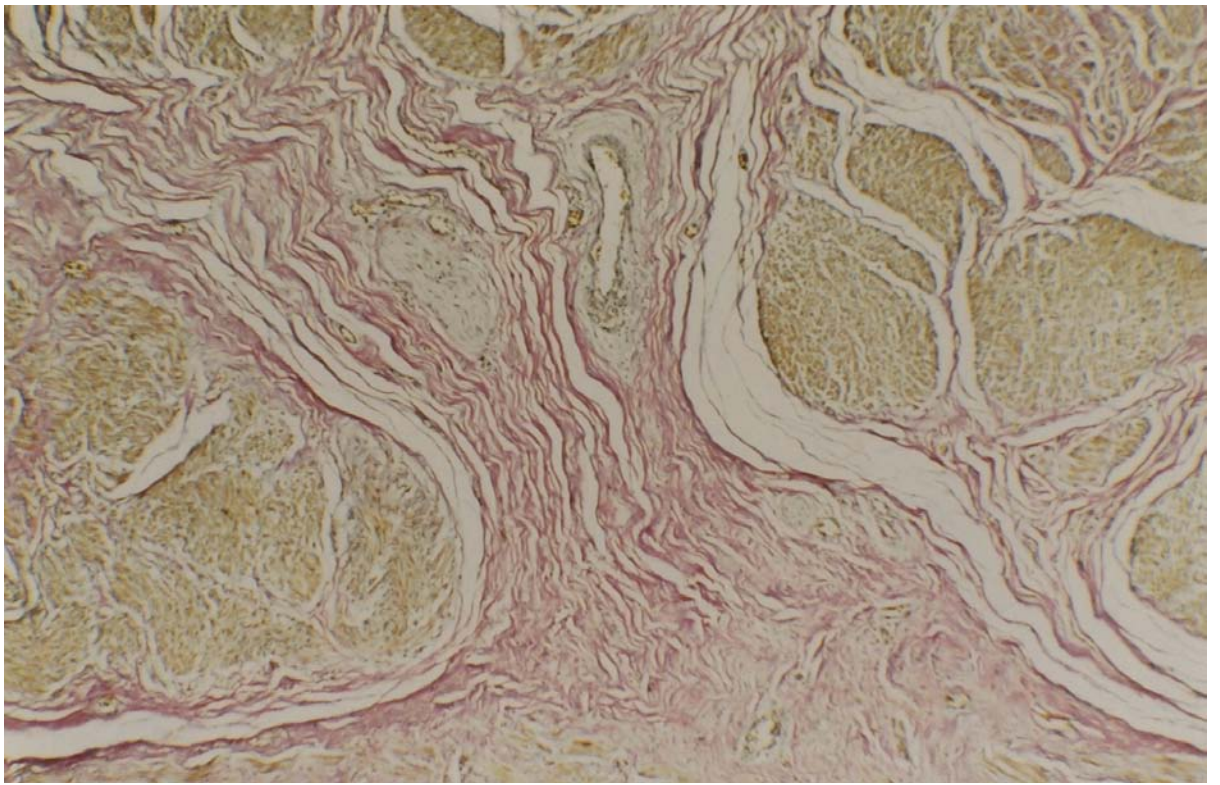


Figure 32. a. Picrosirius red to highlight collagen. b. EVG is not as good in this situation

Group	No. of cases	General stroma	Basement membrane	Fibroblasts	Endothelial cells	General stroma	Basement membrane	Fibroblasts	Endothelial cells
Post-mortem	14	3(22.4%)	0	4(35%)	13(92.8%)	0	0	0	11(78.5%)
Cystitis	6	5(83.3%)	1(16.6%)	1(16.6%)	6(100%)	0	0	0	4(66.6%)
Nonirradiated	18	13(66.6%)	3(16.6%)	3(16.6%)	13(72%)	5(22.2%)	1(5.5%)	2(11%)	14(77.7%)
Irradiated	56	40(71.4%)	15(26.7%)	20(35.7%)	52(92.8%)	33(58.9%)	10(17.8%)	15(26.7%)	53(94.6%)
Total	92								

Table 3. Number and percentage of cases expressing fibronectin.

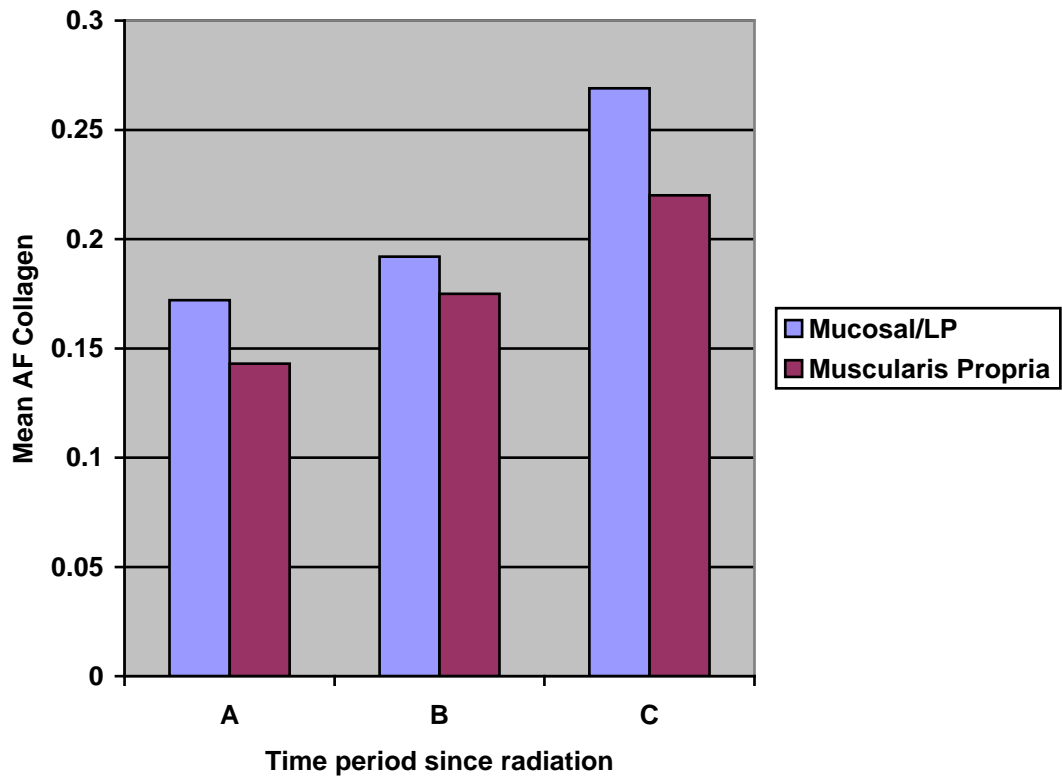
Mucosal/LP layer component	Time Period		
	A	B	C
General stroma	15(78.9%)	15(57.6%)	10(90.9%)
Basement membrane	6(31.5%)	7(26.9%)	2(18.2%)
Fibroblasts	7(36.8%)	10(38.4%)	3(27.7%)
Endothelial cells	19(100%)	23(88.4%)	10(90.9%)
TOTAL (56)	19	26	11

Table 4. Fibronectin expression in mucosal layer of irradiated bladder and its positivity at different time periods since radiotherapy.

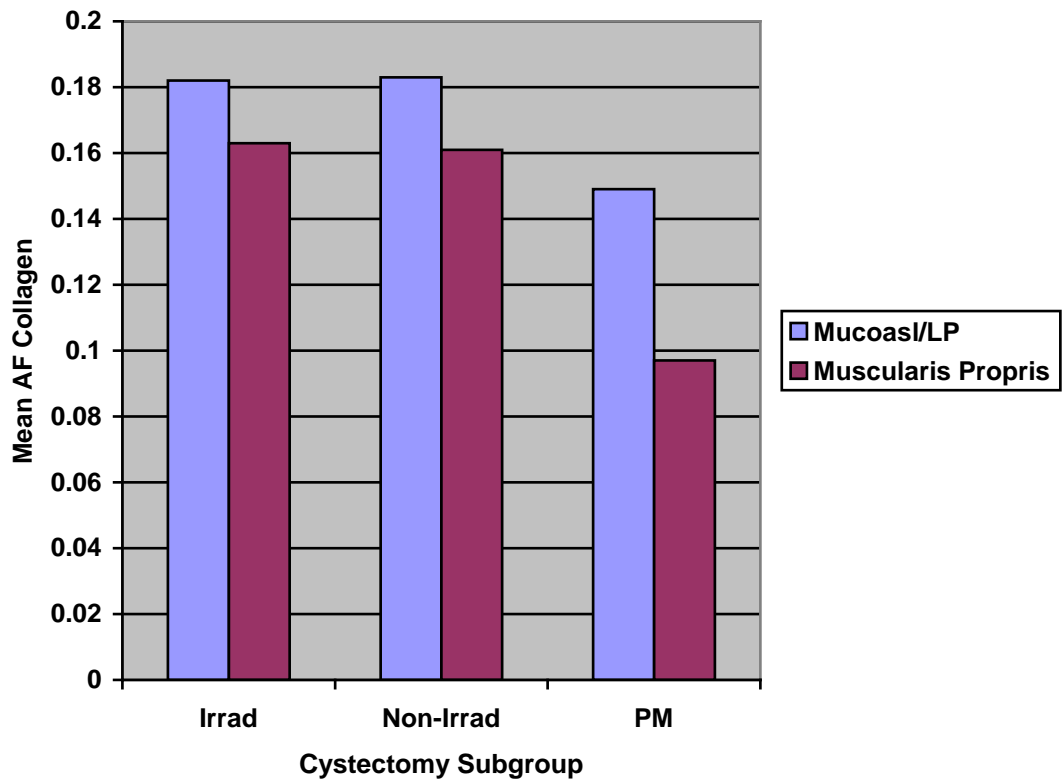
Muscularis Propria Component	Time Period		
	A	B	C
General stroma	10(52.6%)	15(57.6%)	8(72.7%)
Fibroblasts	5(26.3%)	7(26.9%)	3(27.7%)
Endothelial cells	19(100%)	23(88.4%)	11(100%)
TOTAL (56)	19	26	11

Table 5. Fibronectin expression in the intermuscular layer of irradiated bladder and its positivity at different time periods since radiotherapy.

Graph 1: Area fraction of collagen in mucosa/LP and muscularis propria of irradiated bladder group and its relation to post-irradiation time period



Graph 2: Comparison of the mean area fraction of collagen in the mucosal/LP and muscularis propria of irradiated, non irradiated and post-mortem cystectomy specimens.



This was not the case for mucosal fibrosis, which showed no significant difference between the normal post-mortem group and the irradiated/nonirradiated tumour groups. When the mean area fraction of mucosal and MP fibrosis was grouped together and analysed the difference in fibrosis was again significant ($p < 0.05$), when comparing the post mortem group with both the irradiated and non irradiated tumour groups. Due to sample size (six), the intersitital cystitis group was excluded from this analysis.

4.4 Discussion

In this study we have analysed the expression of fibronectin by various components of the bladder wall at various times after irradiation. This ECM molecule, a component of the basement membrane, has in recent years been recognised to play an important role in cellular responses injury. We have also quantified the amount and location of fibrosis in the post irradiated bladder. Fibrosis is characterised by the deposition of collagen, fibronectin and glycosaminoglycans by fibroblasts in response to various cytokines including platelet derived growth factor (PDGF), transforming growth factor Beta (TGF-B), fibroblast growth factors(FGF), interleukin 1(IL-1) and tumour necrosis factor (TNF). Many of the above are produced both by tumours and chronic inflammatory states, whether infective, or induced by other insults such as radiation²⁸¹⁻²⁸³.

Cell responses to radiation are modulated in part by cell-ECM interactions with the type of ECM component able to influence the percentage of cells that arrest in G1 phase²⁷³. In the skin, fibronectin has been shown to be upregulated in response to UV light²⁸⁴. Others have shown that lung cancer cell lines which are irradiated on fibronectin show significantly greater survival when compared with cells irradiated on polystyrene or

other substrates. The authors suggest this may be due to the increased time spent in G2 arrest²⁷⁵. The same lung cancer cell lines grown on fibronectin have been shown to have a reduced response to single dose chemotherapy regimens²⁷⁴. Bcr-abl transfected cells pre-adhered to immobilized fibronectin have a significant survival advantage after gamma irradiation when compared to the same cells grown on laminin, polylysine or suspension²⁷⁶. Thus, in each case, fibronectin appears to confer specific survival advantages in multiple irradiated cell lines.

It is not just a radiation insult that has been shown to cause fibronectin upregulation. The fibronectin gene may be a natural target for hepatitis surface antigen, via the NF kappa B pathway, leading to the accumulation of fibronectin in the liver in Hepatitis B infection²⁸⁵. Hepatic stellate cells are known to express many ECM proteins including collagen IV, entactin and fibronectin and have been implicated as one of the prime culprits in hepatic fibrosis and cirrhosis. The finding that complement (C5a) specifically elevated fibronectin mRNA in these cells²⁸⁶, suggests that fibronectin may also be involved in mediating the damage and subsequent fibrosis due to inflammation. Based on the above findings one could propose that most tissues may upregulate fibronectin expression after irradiation or an infective insult. To date there is very limited literature on this but fibronectin expression has been shown to be upregulated in the connective tissue stroma of the irradiated rat submandibular gland^{287,288}. We have also demonstrated increased fibronectin expression in the stromal components of the bladder wall after radiation particularly in the MP layer. This is not surprising given the above previous findings. However the non-irradiated tumour group also showed a diffuse increase in fibronectin expression by all the elements examined. This may be due to factors released by the tumour, such as TGF- β . Paracrine modulation of ECM components by tumour cells has been demonstrated in the past²⁸⁹.

The relative restriction of fibronectin staining to the mucosa in the normal post-mortem and cystitis cases seen in this study, is interesting, and may reflect its continuous role in the deposition and remodelling of other matrix proteins²⁹⁰.

Endothelial cells of all groups exhibited high levels of fibronectin immunoreactivity in both the mucosal and MP layers. This corroborates a previous study which demonstrated the presence of FN immunoreactivity in the subendothelial area in capillaries, venules and arterioles of the normal human myocardium²⁹¹. This high level of endothelial fibronectin immunoreactivity may reflect their key role in inflammation, and the sensitivity of the vascular system to radiation²⁶⁰. Vascular damage may be the most consistent pathogenic mechanism in delayed radiation injury and fibrosis. The appearance of fibronectin immunoreactivity in the stroma, basement membrane and fibroblasts of the MP may be a step in the process of MP fibrosis. Such a step wise cascade post irradiation has been suggested in pulmonary fibrosis²⁹². The mechanisms by which FN is up-regulated may, however, be quite different in the irradiated versus the non irradiated cystectomy groups as already alluded to.

The finding of a significant quantified increase in MP fibrosis in the irradiated cystectomy specimens when compared with post mortem controls, although not previously published, is to be expected given that fibrosis is a known sequelae of radiation therapy that has been documented in many different tissues²⁹³. Few papers have addressed the issue of whether there is a temporal increase in the degree of fibrosis. Rubio et al. have looked at this issue in the irradiated distal ileum of the rat²⁹⁴. They showed a dose-time dependant increase in the amount of collagen in the all layers of the bowel wall, however we have not seen any such papers concerning the human bladder, where the clinical problem that fibrosis causes may result in a cystectomy. Although we also found a steady increase in the amount of LP and MP collagen between the three post irradiation time groups (Graph 1), this was not statistically

significant. This interpretation may be limited by the sample size. Furthermore, we cannot be sure that this steady increase in the degree of fibrosis after irradiation is due to the latter alone, as the non-irradiated group also showed a similar steady increase in MP fibrosis.

We found preferential fibrosis in the MP and this significant increase in MP fibrosis was also found in the non-irradiated tumour group. Of further note, was that with regards to mucosal fibrosis, there was no statistically significant difference between any of the study groups. The increase in MP fibrosis may be linked to the up-regulation of fibronectin by MP constituents, especially fibroblasts that do not normally express the molecule. The lack of a significant increase in mucosal fibrosis is interesting, and may potentially be due to local mucosal factors that prevent or inhibit the development of fibrosis to a greater extent than in the MP. Interesting in this regard is the suggestion of an inverse correlation between BM fibronectin expression and time since irradiation. Furthermore the mucosa and lamina propria are less robust and more vulnerable, when compared with the MP and do not cope as well in tissue handling and processing. This can be a source of error in morphometry studies²⁹⁵.

In summary there was an increase in MP fibronectin expression in irradiated and non-irradiated cystectomy specimens when compared with post mortem and cystitis control bladders. Similarly there was a site specific significant increase in MP fibrosis in both the irradiated and non-irradiated cystectomy groups. Vascular endothelial cells consistently expressed the highest levels of fibronectin in both the irradiated groups and also in all of the control groups. The observed upregulation of fibronectin by fibroblasts and other constituents of the MP may be a precursor to subsequent fibrosis in this area. This may be due to the direct effects of radiotherapy, tumour related factors or most likely a combination of both.

Chapter 5. Platelet-derived growth factor

5.1 Introduction

Platelet-derived growth factor (PDGF) is one of many growth factors found in the human body. It is a highly conserved protein, being found throughout the animal kingdom. It was named in 1982 although it was originally identified in 1974 as a constituent of whole blood serum that was absent in cell-free plasma^{296,297}. It was subsequently purified from human platelets^{298,299} and was found to act as a serum growth factor for fibroblasts, smooth muscle cells and glial cells³⁰⁰. Soon after its discovery it was realised that PDGF protein was composed of two polypeptide chains A and B. Mitogenic and active in physiological states mainly during development, with activities in wound healing, angiogenesis, fibrosis, atheroma and carcinogenesis. In the first decade of the 21st century it became clear that PDGF was more complex than originally appreciated. Researchers became aware of two further chains, C and D. The function of these additional PDGF proteins largely remains a mystery in human physiology and disease. However it has become clear that PDGFs are closely related structurally and functionally to VEGFs. These potent neovascularising agents are conserved throughout the animal kingdom and have many similarities to PDGFs. All of these growth factors are dimers, formed of polypeptide chains with disulphide links/bonds. They are classified according to their receptor binding. Platelet-derived growth factor is a family of heterodimeric or homodimeric isoforms of A- and B-polypeptide chains that are synthesized as precursor molecules undergoing proteolytic maturation. The synthesis of PDGF isoforms is carefully regulated, and their action on receptors is modulated by interaction with components in the matrix as well as with soluble binding proteins.

5.2 Structure and expression of PDGFs

PDGF-A was first characterised by Betscholtz by cDNA cloning in 1986³⁰¹. At first it was thought that most cells secreting PDGF, expressed PDGF-A and secreted PDGF-AA homodimers²⁹⁶. Later, PDGF-BB homodimers were produced by SSV-transformed or PDGF-B-expressing cells and this led to the realisation that the PDGF family consisted of at least three proteins—PDGF-AA, PDGF-AB, and PDGF-BB—encoded by two genes, *PDGF-A* and *PDGF-B*²⁹⁸. This view lasted for more than 15 years until combinations of genomic and biochemical efforts identified two additional PDGF genes and proteins; PDGF-C^{303,304} and PDGF-D³⁰⁵, although relatively little is known currently about these more recent additions to the family. All isoforms of PDGF are disulfide-linked dimers of two related polypeptide chains, designated A, B, C and D, which are assembled as heterodimers (PDGF-AB) or homodimers (PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD)³⁰⁶.

The genes for the A- and B-chains of PDGF are found on chromosomes 7 and 22, respectively^{301,307}. Both genes are organized in a similar manner with seven exons. In each case exon 1 encodes the signal sequence, exons 2 and 3 encode precursor sequences that are removed during processing, exons 4 and 5 encode most of the mature protein and exon 7 is mainly non-coding³⁰⁸. Exon 6 encodes a COOH-terminal sequence that may be removed during the maturation of the B-chain; the A-chain occurs as two different splice forms, with and without the exon 6-encoded sequence. The C and D chains are similar in that they share related CUB domains (Fig.33).

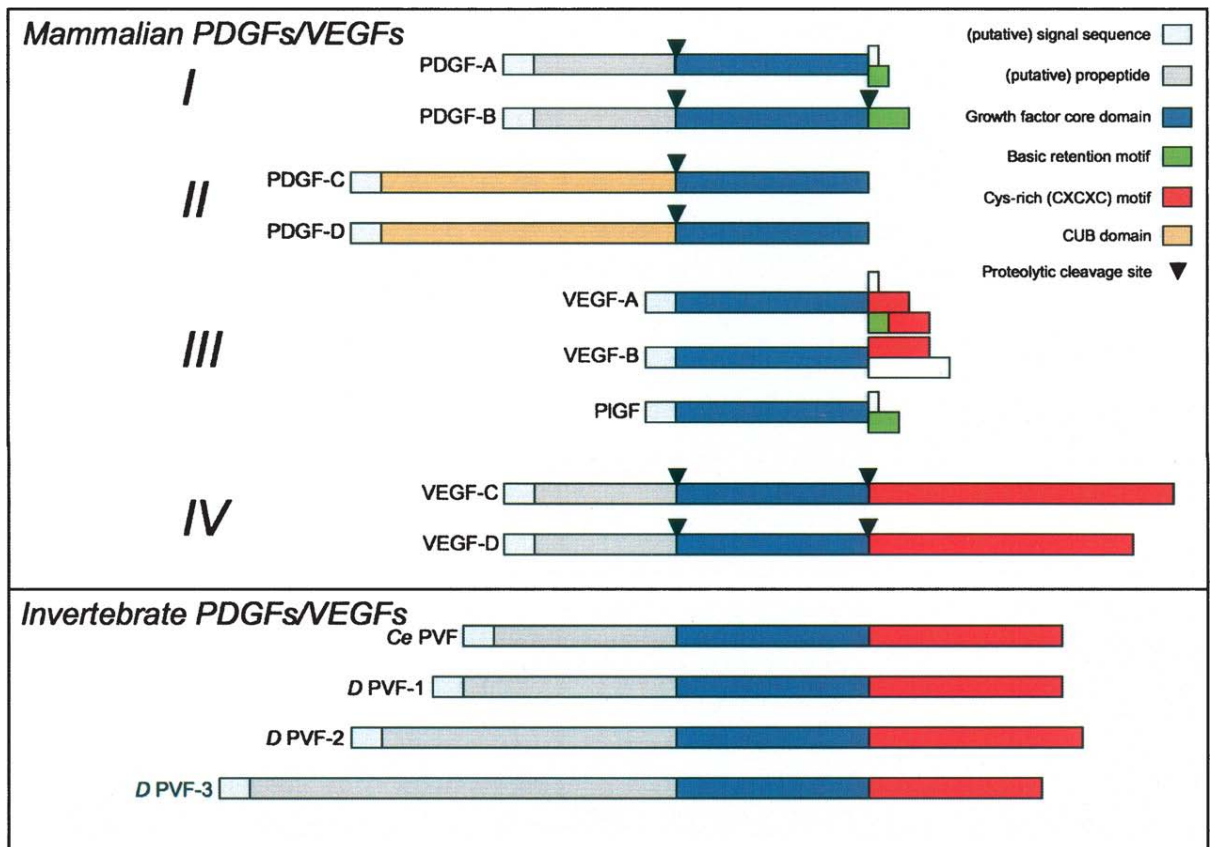


Figure 33. The PDGF and VEGF family of growth factors³⁰² (with permission)

5.3 Location of PDGFs

Platelet-derived growth factors can be produced by many different cell types. There appears to be no regular expression of PDGFs but synthesis is often increased in response to external stimuli, such as exposure to low oxygen tension³⁹³, thrombin^{310,311}, or stimulation by other growth factors and cytokines³¹². Expression of PDGF-A also increases in human uterine smooth muscle cells during the physiological hypertrophy of pregnancy³¹³. Although the α -granules of platelets are a major storage site for PDGF, recent studies have shown that PDGF can be synthesized by a number of different cell types such as macrophages, epithelial and endothelial cells³¹⁴⁻³¹⁷. Most cell types expressing PDGF are capable of making both A- and B-chains, but the expression of the two chains is independently regulated at the transcriptional as well as post-

transcriptional levels³⁰⁸. Generally, PDGF B is mainly found in endothelial cells, megakaryocytes and neurons. PDGF A and PDGF C are located in epithelial cells, muscle and muscle progenitor cells. At present it is unclear where PDGF D is predominantly found but it has been seen in fibroblasts and SMCs at certain locations (possibly suggesting autocrine functions via PDGFR- β). Therefore PDGFs are generally produced by predictable groups of cells and they act locally³⁰⁹.

5.4 Production, secretion and binding of PDGF

PDGF biosynthesis and processing is controlled at multiple levels and is different for the various PDGFs. PDGF-A and PDGF-B become disulfide-linked into dimers and are released as propeptides. PDGF-A and PDGF-B are similar in that they contain N-terminal pro-domains that are removed within the cell by a number of convertases including furin³¹⁸. This N-terminal processing is essential for PDGF-A to acquire receptor-binding ability and it is presumed that PDGF-B also requires N-terminal cleavage to become active. PDGF-C and PDGF-D have been less studied in this regard because of their novelty. However, it is known that PDGF-C and PDGF-D are processed differently. They are not altered intracellularly but are instead secreted as whole (but inactive) proteins³¹⁹. Activation occurs outside of the cell and requires proteolytic dissociation of the CUB domain. Experimentally, plasmin and tissue plasminogen activator (tPA) have been shown to remove the CUB domain in PDGF-C, rendering it biologically active³²⁰. The activation factors in vivo remain to be identified, but tPA is a likely contender for PDGF-C and plasmin may cleave and activate PDGF-D. It remains to be established if some of the overlapping expression patterns for PDGF-A and PDGF-C result from common transcription regulatory mechanisms. Little

is currently known about the transcriptional regulation of *PDGF-C* and *PDGF-D* and the *PDGFRs*.

5.5 Extracellular distribution of PDGFs

PDGF, in common with many other growth factors and cytokines, shows an uneven distribution within connective tissue. The precise localisation and retention in the interstitium depends on coordinated binding to extracellular matrix components. The main trapping mechanism for PDGF-A and PDGF-B appears to be accomplished by the positively charged C-terminal motifs (so-called retention motifs) which are rich in basic amino acid residues. The presence of the retention motif is determined by alternative splicing in PDGF-A and by alternative C-terminal proteolytic processing in PDGF-B. Alternative splicing of the PDGF-A transcript is cell type-specific. PDGF-C and PDGF-D lack basic retention motifs, but the CUB domains described earlier are thought to be important in protein–protein and protein–carbohydrate interactions in general. Therefore CUB may regulate the extracellular distribution of PDGF-C and PDGF-D.

Much of the work on PDGF retention has come from studies of PDGF interaction with heparan sulfate proteoglycans (HSPGs) and phenotypic analysis of PDGF-B retention motif knockout mice. PDGFs bind to heparin and HSPGs in common with other growth factors and morphogens with critical functions during development such as hedgehog and Wnt signaling pathways³²¹⁻³²³. Targeted deletion of the PDGF-B retention motif in mice or reduced heparan sulfate (HS) N-sulfation leads to pericyte detachment from the microvessel wall. This is probably caused by attenuated PDGF-BB binding to HS. This suggests that PDGF-BB secreted from endothelial cells interacts with HS at the endothelial surface or in the periendothelial matrix which would lead to local deposits of PDGF-BB. PDGF-BB, is thought to be critical for the correct investment of pericytes

in the vessel wall. HS on endothelial cells may function to enhance PDGF-BB-mediated PDGFR- β signalling in neighbouring pericytes.

PDGF also binds to several non-HSPG extracellular proteins, but the physiological relevance of these interactions is unclear. These include α -2-macroglobulin³²⁴, which might act as a scavenger for PDGF-B through low-density lipoprotein (LDL) receptor-related protein (LRP) receptors on macrophages and other cells³²⁵. PDGF-B also binds to SPARC and adiponectin, which may trap the growth factor to heparin sulphate proteoglycans and heparin. Thrombin might also draw PDGF-B to its locality. C-terminal proteolytic processing of PDGF-B may take place intracellularly or extracellularly. The endogenous protease(s) responsible for C-terminal cleavage of PDGF-B has not been identified, but it may be thrombin³²⁶. Cells transfected with PDGF-B expression vectors secrete soluble PDGF-BB into the conditioned medium. However, a major part of endogenously expressed PDGF-B becomes trapped on the cell surface or in the extracellular matrix, where it subsequently can be released by thrombin. C and D probably bind via the CUB domain and when this is missing the factors become soluble.

5.6 PDGF receptors

PDGFs exert their effects by interacting with two structurally related receptors on the target cell surface. These receptors are both proteins with tyrosine kinase activity, designated alpha and beta. The α - and β -receptors have similar overall structure with molecular sizes of ~170 and 180 kDa, respectively. The extracellular component of each receptor contains five immunoglobulin-like domains. The intracellular part of the receptor comprises a tyrosine kinase domain that contains a characteristic sequence which is different to other kinases³²⁷⁻³²⁹. The basic structure of PDGF receptors is

similar to those of other growth factor receptors such as colony stimulating factor-1 (CSF-1) receptor³³⁰ and the stem cell factor (SCF) receptor³³¹. The human alpha-receptor gene is localized on chromosome 4q12, close to the genes for the SCF receptor and VEGF receptor-2³³², and the beta-receptor gene is on chromosome 5³²⁹ close to the CSF-1 receptor gene³³³.

Because PDGF isoforms are dimeric molecules, they can bind two receptors simultaneously and thus dimerize receptors upon binding³³⁴⁻³³⁶. The alpha-receptor binds both the A-, B- and C-chains of PDGF with high affinity, whereas the beta-receptor binds only the B- and D-chains with high affinity. Therefore, PDGF-AA induces alpha-receptor homodimers, PDGF-AB alpha-receptor homodimers or alpha/beta-receptor heterodimers, and PDGF-BB all three dimeric combinations of alpha- and beta-receptors³³⁷⁻³³⁹. Theoretically the possible PDGF–PDGFR interactions are multiple and complex and include the formation of receptor heterodimers. However, in vivo there is functional evidence for only a few interactions; i.e., those of PDGF-AA and PDGF-CC via PDGFR- α , and PDGF-BB via PDGFR- β . It is likely that PDGF-DD acts through PDGFR- β in vivo, but evidence for this is currently lacking. PDGF ligand dimerization of the receptors leads to subsequent autophosphorylation of the PDGF receptor tyrosine kinase (RTK). Activated RTK phosphorylates numerous molecules that initiate intracellular signalling cascades (discussed later).

5.7 Location of PDGF receptors

There are differences between α - and β -receptors in their binding specificity of PDGF isoforms and in the signals they transduce and so the response of a cell to PDGF stimulation will be determined by which of the two receptor types the cell expresses.

The classical target cells for PDGF, fibroblasts and smooth muscle cells, express both α - and β -receptors, but generally higher levels of β -receptors. Other cell types such as the O-2A glial precursor cells and human platelets express only α -receptors whereas other cell types express only β -receptors. PDGFR- α is expressed in mesenchymal cells with particularly strong expression of PDGFR- α in subtypes of mesenchymal progenitors in lung, skin, and intestine and in oligodendrocyte progenitors. PDGFR- β is expressed in mesenchyme, particularly in vascular SMCs (vSMCs) and pericytes.

The distribution of platelet-derived growth factor receptors is not uniform over the surface of the cell membrane. They are found mostly in caveolae (distinct membrane invaginations) which help endocytosis³⁴⁰. Binding internalizes the ligand-receptor complex to produce endosomes³⁴¹. A small proportion of the PDGF-receptor complex subsequently dissociates, and the receptor recycles to the cell membrane. PDGF receptors also undergo cytoplasmic degradation in proteasomes after ubiquitination³⁴². Most of the ligand-receptor complex however is degraded upon fusion of the endosomes with lysosomes. The rate of deactivation of PDGF receptors is an important parameter in the regulation of the mitogenic response. The internalization of PDGF receptors is a controlled process that is dependent on the kinase activity of the receptor³⁴³ as well as on the interaction between the receptor and PI 3-kinase³⁴⁴ and other molecules³⁴⁵.

5.8 Cellular Effects Mediated by PDGF Receptors

Isoforms of PDGF exert their cellular effects by inducing tyrosine kinase receptors, resulting in cell growth, chemotaxis, actin reorganization and prevention of apoptosis. The level of receptors at the cell surface can be modulated by external stimuli. Most of our current knowledge concerns the functional roles of PDGF receptor homodimers.

Both α - and β -receptor homodimers transduce potent mitogenic signals and both receptors mediate an increase in intracellular Ca^{2+} concentration. PDGFR activation can inhibit gap junctional communication between cells³⁴⁶ and exert an antiapoptotic effect³⁴⁷. There is, however, a difference between the receptors regarding their effects on the actin filament system and activation of chemotaxis. The α -receptor activates chemotaxis whilst activation of the β -receptors inhibits chemotaxis of certain cell types including fibroblasts and smooth muscle cells, but stimulates chemotaxis of certain other cell types³⁴⁸.

5.9 PDGFR-induced signalling pathways

Of particular interest to cancer biologist was the discovery in 1983 that B chain of PDGF had close homology with v-sis SSV oncogene³⁰². Subsequent investigations led to the discovery that c-sis was in fact PDGFB. Scientists found that PDGF could act in an autocrine way to transform SSV in virus and this introduced the important concept of a fundamental relationship between growth factors and neoplastic cell transformation. This exciting discovery provided the impetus for much of the subsequent research on which our current theories of carcinogenesis are based. Both PDGFR- α and PDGFR- β both tyrosine kinase type receptors which engage several well-characterized signalling pathways such as Ras-MAPK, PI3K, and PLC- γ ; all of which are involved in multiple cellular and developmental responses. PDGFRs attach to Ras-MAPK mainly through the adaptor proteins Grb2 and Shc. Grb2 binds the activated PDGFR through its SH2 domain and complexes Sos1 through its SH3 domains. Sos1 in turn activates Ras, leading to downstream activation of Raf-1 and the MAPK cascade. MAPK signaling activates gene transcription, leading to stimulation of cell growth, differentiation, and migration^{349,350}.

PI3K is a family of enzymes phosphorylating phosphoinositides. Effectors of PI3K signalling include serine/threonine kinases such as Akt/PKB, some members of the PKC family, p70 S6 kinase and small GTPases of the Rho family. Activation of the PI3K pathway by PDGFRs promotes actin reorganization, directed cell movements, stimulation of cell growth and inhibition of apoptosis³⁵¹. When PLC- γ binds PDGFRs it results activation through phosphorylation, leading to mobilization of intracellular calcium ions and the activation of PKC³⁵². The effects of PDGFR-mediated PLC- γ activation include stimulation of cell growth and motility³⁵³.

PDGFRs also engage several other signalling molecules, including enzymes, adaptors and transcription factors. For instance, activation of the Src TK promotes Myc transcription and mitogenic responses³⁵⁴. In addition, members of the Fer and Fes TK family bind to PDGFRs and PKC- δ is phosphorylated by PDGFR- β , leading to its activation and translocation to the cell membrane. This signal may be involved in cell differentiation. The adaptors Nck and Crk bind to PDGFRs through their SH2 domain and are involved in activation of JNK³⁵⁵. The adaptor Grb7 contains a SH2 domain and binds PDGFR- β ³⁵⁶. STAT transcription factors may bind to PDGF receptors, leading to their phosphorylation and activation³⁵⁷.

PDGF receptors interact also with integrins, which promote cell proliferation, migration and survival³⁵⁸. This interaction helps localizing PDGFRs and interacting molecules at focal adhesions, which are sites where several signalling pathways initiate and cross-talk³⁵⁹. Recently, Na⁺/H⁺ exchanger regulatory factors (NHERFs) were shown to bind PDGFR- β and link it with focal adherence kinase and the cortical actin cytoskeleton³⁶⁰, as well as to N-cadherin³⁶¹ and the phosphatase PTEN³⁶².

Overall, the best characterised mechanisms by which PDGF down-streaming signalling mediates cellular responses involve the activation of the ras/MAPK pathway, which can

functionally increase cellular proliferation, migration and differentiation³⁶³, and the PI3K/Akt pathway, which promotes cell survival³⁶⁴. Both pathways are of crucial importance for tumour resistance to radiotherapy and chemotherapy. Furthermore, platelet-derived growth factor (PDGF) exerts its potent mitogen and chemotactic effects in a variety of mesenchymal cells such as fibroblasts, vascular smooth muscle cells, glomerular mesangial cells and brain glial cells³⁶⁵⁻³⁶⁸ making PDGF a potential key molecule for tissue rebuilding in response to physiological and non-physiological conditions.

5.10 Control of PDGFR signaling

There is an effective negative feedback mechanism by which ligand occupancy promotes endocytotic receptor internalisation and lysosomal degradation, thereby limiting the duration of PDGFR signalling. Furthermore, stimulatory and inhibitory signals arise in parallel and the ultimate response depends on the balance between these signals. The SHP-2 tyrosine phosphatase binds PDGFR through its SH2 domain and dephosphorylates the receptor and its substrates³⁶⁹. Ras-GAP, which negatively regulates Ras, also binds PDGFR- β through its SH2 domain³⁷⁰. Recycling of PDGFR- β , but not PDGFR- α , was recently observed in cells deficient for the phosphatase TC-PTP, which is a negative regulator of PDGFR- β phosphorylation. However, lysosomal degradation of PDGFR- β depends on interactions with c-Cbl and receptor ubiquitination. The adaptor protein Alix, which interacts with the C-terminal domain of PDGFR- β , facilitates ubiquitination and degradation of c-Cbl, thereby inhibiting PDGFR- β down-regulation³⁷¹.

5.11 Other cellular responses to PDGFR-mediated signaling

Some of the cellular responses to PDGFs take place rapidly (within seconds to minutes) of PDGFR activation and are independent of gene expression and protein synthesis. The cellular response to PDGFR- α and PDGFR- β activation is similar but not identical. Both receptors stimulate rearrangement of actin filaments, but only PDGFR- β promotes formation of circular ruffles. PDGFR- β also mobilizes calcium ions more efficiently than PDGFR- α ³⁷². PDGFR- β inhibits gap junctional communication between cells through phosphorylation of the gap junction protein connexin 43³⁷³. It is unclear whether this ability is shared with PDGFR- α .

In addition to the rapid post-transcriptional responses, PDGFRs (like other RTKs) induce fast transcriptional changes involving immediate early genes (IEGs)³⁷⁴. IEGs are direct targets of the transcription factors that get activated (by post-translational modification) through various signalling pathways. The IEG responses are probably necessary for many of the long-term effects of PDGFs but the extent to which the IEG responses contribute specificity to PDGFR signalling is currently unclear. Different RTKs induce virtually identical sets of IEGs but different signalling pathways activated by PDGFR- β induce overlapping sets of IEGs. It seems that quantitative rather than qualitative differences in the IEG responses mediate the specific responses to different RTKs and signalling pathways. In contrast, PDGFR- α shows strikingly different roles of the different downstream signalling pathways. For PDGFR- β , disruption of signalling through PI3K alone has no, or only minor, developmental consequences³⁷⁵. In contrast, PI3K is absolutely crucial for PDGFR- α function during development³⁷⁶.

Different IEGs appear to cooperate in their regulation of downstream cellular and developmental events. The large overlap between the signalling pathways, IEGs and biological processes suggest that specificity is generated through a combination of

quantitative differences in magnitude and duration of the responses occurring at different levels in the signalling cascade. On the other hand, a major part of the specificity of the PDGFRs in developmental functions depends on cell type- and context-specific PDGFR expression. The point in time during development and location may be more important than the specificity of the receptor. It is interesting to note that knockout mice models show that PDGFR- β signalling can fully substitute for PDGFR- α signalling if it is expressed at the right place and time³⁷⁷. Conversely, PDGFR- α signalling can only partially compensate for the loss of PDGFR- β signalling. Overall it is felt that specificity of PDGFR signalling is achieved through a combination of cell type-specific expression and differential engagement of downstream signalling pathways.

5.12 Physiological and pathological functions of PDGFs and PDGFRs

5.12.1 General

PDGF primarily acts in a paracrine manner on local cells although in tumours it may well also take on an autocrine function. PDGFs are generally produced by discrete populations of cells that act locally to drive different cellular responses. They do not appear to be important for routine homeostatic processes; there is limited evidence that they perform a role in normal adult human physiology. However, expression increases after inflammation and in culture. PDGF expression in cultured cells is found after exposure to a variety of stimuli including hypoxia, thrombin, cytokines and growth factors, including PDGF itself³⁰². Several factors induce PDGFR expression, including TGF- β , oestrogen (probably linked to hypertrophic smooth muscle responses in the pregnant uterus), interleukin-1 α (IL-1 α), basic fibroblast growth factor-2 (FGF-2), tumour necrosis factor- α , and lipopolysaccharide.

On the other hand many investigators have described increased expression and/or activity of PDGF in basic pathological or disease processes, including fibrosis, wound healing, atherosclerosis, glomerulonephritis and fibromatosis. Moreover, aberrant production of PDGF and autocrine stimulation may be an important mechanism in the neoplastic conversion of PDGF receptor-positive cells³⁰². This opens a door for pharmacological exploitation using antagonists PDGFR targeting with inhibitors. Currently, pharmacological studies of PDGF and R inhibition are ongoing looking at gene inactivation, blocking antibodies and receptor blockers such as imatinib (Gleevec) in GISTs, and AML but the action of such compounds is currently non-specific.

5.12.2 Development and embryogenesis

PDGF is particularly important in cell migration, organogenesis and epithelial-mesenchymal interactions. The developmental roles of PDGFs and PDGFRs have been unravelled mainly through experiments with gene-targeted mice. A large number of knockout, knock-in, or transgenic mutants have been used to identify specific cell types that are primary targets of PDGF signalling during development. This has led to many important discoveries. The critical roles of microvascular pericytes³⁷⁸, lung alveolar smooth muscle cells³⁷⁹ and gastrointestinal villus cluster cells³⁸⁰ were all identified by analysis of PDGF-B and PDGF-A knockout mice. PDGF signalling is vital for directing various populations of cells in developmental processes such as the movement of oligodendrocyte precursors in the spinal cord, of neural crest mesenchymal cells toward the branchial pouches and cardiac outflow tract, and of pericytes along newly formed angiogenic sprouts. The exact mechanisms by which the cells manoeuvre, and how PDGF regulates this process, remains obscure, however.

PDGF-B expression by endothelial cells recruits local pericytes³⁸¹. Pericytes expressing PDGFRs migrate along steep gradient of PDGF-B in the peri-endothelial compartment to endothelial cells and thus initiate intimate association with the abluminal surface of the endothelial cells³⁸¹. Pericyte-deficiency promotes a range of microvascular changes, such as endothelial hyperplasia, vessel dilation, leakage and rupture, leading to capillary microaneurysms, and lethal microhemorrhage³⁷⁸. Despite structural and functional abnormalities in the microvasculature, mice embryos deficient of up to 90% pericytes are compatible with embryonic and postnatal survival, while loss of more than 95% of the pericytes is lethal^{378,382}. This suggests that a rather low threshold density of pericytes is required for basal microvascular function.

During post-implantation development, PDGF-A expression occurs in epithelia, nervous tissue, myotome, and vascular and visceral smooth muscle. PDGFR- α , in contrast, is expressed by most mesenchymal cell populations; often in a reciprocal pattern compared with PDGF-A³⁸³. Some epithelial PDGFR- α expression has been identified, such as lens epithelium³⁸⁴, limb apical ectodermal ridge and the early epithelial somite³⁸⁵. PDGF-A appears to be the epithelial-derived factor promoting proliferation and movement of nearby PDGFR- α -positive mesenchymal cells. PDGF-A/PDGFR- α is a common signalling partnership in epithelial–mesenchymal interaction, acting in concert with other signalling molecules such as hedgehogs, FGFs, BMPs and Wnts.

PDGFR- β appears to be the dominant PDGFR involved in vascular pathology, whereas the literature suggests a pivotal role for PDGFR- α signalling in various types of mesenchymal cell/fibroblast-driven pathologies. These differential pathological roles for the two PDGFRs resemble the developmental roles summarized above, in which PDGFR- β signalling has a key role in vascular mural cell formation, whereas PDGFR- α

has both general and specific roles in the development of various mesenchymal and fibroblastic cell compartments.

5.12.3 PDGF and disease

In general, two main types of cell respond in a pathological fashion to PDGFs, smooth muscle cells and fibroblasts, promoting vessel wall pathologies and fibrotic tissue scarring, respectively. In addition, PDGF appears to be involved in the development of several neoplastic lesions.

5.12.3.1 Neoplasia

Hanahan and Weinberg assign six capabilities to cancer cells; self-sufficiency in growth signals, insensitivity to anti-growth signals, escape from apoptosis, sustained angiogenesis, tissue invasion and metastasis,³⁸⁶ and limitless replicative potential³⁸⁶. Numerous studies have demonstrated that such self-sufficiency may be established in certain cell types through autocrine growth stimulatory loops involving PDGF-B/PDGFR- β signalling³⁰². However, autocrine PDGF stimulation in vitro does not seem to provide a cell with a fully malignant phenotype. PDGF-B-producing retroviruses require cooperation with other genetic changes caused by retroviral insertions in order to induce malignant behaviour^{387,388}. Thus, autocrine PDGF signalling can contribute to tumorigenesis by driving proliferative expansion of clones of preneoplastic and/or genetically unstable cells, which become fully malignant through further genetic alteration. Not only can PDGF induce a cell-autonomous proliferative stimulus, but it can also play a role in invasion and metastasis of certain epithelial cancers. Furthermore, paracrine PDGF signalling is likely to influence recruitment of different types of tumour stromal cell, especially fibroblasts and pericytes, which can affect tumour growth, survival and metastasis. PDGF can stimulate EMT and may also

promote metastasis by secreting CCL5, which induces metastatic behaviour. Tumour cell-derived PDGF-B and PDGF-D may promote detachment of pericytes, which may facilitate metastasis. Tumour fibroblasts may further produce factors that directly act on the tumour cells to promote their proliferation and migration such as HGF, CXCL12, FGF2, and FGF7. Tumour fibroblasts may also secrete angiogenic factors that help in sustaining tumour angiogenesis. Tumour angiogenesis is crucial to the supply of oxygen and nutrients to an expanding tumour mass. Moreover, by regulating stroma cell function, PDGF may limit drug delivery to tumours through effects on interstitial tissue pressure. PDGF may therefore be implicated causally (and hence constitute a putative drug target) in at least three of the six Hanahan/Weinberg's cancer cell traits—self-sufficient growth, angiogenesis, and metastasis—and furthermore in resistance to cytotoxic therapy.

Autocrine PDGF loops are thought to be important in different types of malignant brain tumour of glial origin (gliomas). Such loops involving PDGF-A or -B and their respective receptors have been observed in many malignant and low-grade astrocytomas, while the activation of PDGF autocrine loops was suggested to be an early event in the pathogenesis of malignant astrocytomas³⁸⁹. Virtually all tested human glioma cell lines and fresh tumour isolates express multiple PDGFs and PDGFRs^{390,391}, especially PDGF-A, PDGF-C, and PDGFR- α , but also PDGF-B, PDGF-D, and PDGFR- β ³⁹². In various experimental systems, overexpression of PDGFs has been shown to cause the formation of glioma-like tumours^{388,393,394}. Inhibition of PDGF/PDGFRs has been shown to slow down glioma cell growth in experimental models^{394,395} which has led to clinical trials of inhibitors of PDGFR signalling (such as imatinib) for high-grade glioma patients³⁹⁶. PDGFR antagonists also prevent glioma formation in a mouse xenograft model^{390,397,398}. DNA synthesis in meningiomas cell lines can also be inhibited through an antagonist of PDGF³⁹⁷.

The mechanism by which PDGF expression is activated in gliomas is poorly understood. It is possible that TGF β signalling, through Smad2/3/4, activates PDGF-B transcription and secretion, and that the effect of TGF β on glioma cell proliferation depends on PDGF-B/PDGFR signalling^{399,400}. The response to TGF β appears to be correlated with the methylation status of the PDGF-B gene suggesting that the proliferative response to TGF β in human gliomas, and perhaps also the poor prognosis associated with TGF β pathway activation in this disease, is mediated by PDGF-B expression and function. Furthermore, glioma tumour stem cells appear to respond to PDGFs⁴⁰¹. Adult neural stem cells in the peri-ventricular zone, which is capable of generating both oligodendrocytes and neurons, were shown to express PDGFR- α and respond to PDGF by proliferation, leading to the formation of glioma-like hyperplasias.

There are several ways by which genetic alterations may lead to overexpression or altered function of the gene product in tumours. For example gene amplification, translocation and activating mutations have all been described in human tumours with high PDGF expression. There are several reports on *PDGFR-A* amplifications, especially in glioblastomas^{392,401,402}. Overexpression of PDGFR- α appears to precede amplification of the gene. Up-regulation of PDGFR- α protein is detected in most gliomas with the more malignant tumours showing the higher expression. *PDGFR-A* amplifications have also been described in anaplastic oligodendrogliomas⁴⁰³, oesophageal squamous cell carcinoma⁴⁰⁴ and pulmonary artery intimal sarcomas⁴⁰⁵. In each of these tumours, it is speculated that paracrine stimulation can drive proliferative expansion of genetically unstable cells leading to tumour formation rather than PDGF directly causing the tumour itself.

Other non-neurological tumours have been also associated with PDGF and/or receptor abnormalities. An activating mutation of PDGF-alpha R has been described in some

GISTs, whilst alterations of the PDGFB chain are seen in dermatofibrosarcoma protuberans (DFSP) and PDGF-beta R can be detected in AML, some hypereosinophilic syndromes (HES) and mastocytosis.

Most GISTs carry activating mutations in the c-kit receptor tyrosine kinase and hence imatinib is currently used to treat these patients. In 35% of GISTs with wild-type c-kit, gain-of-function mutations in *PDGFR-A* have been detected^{406,407}. It seems that c-kit and PDGFR- α mutations are mutually exclusive and 85%–90% of all GISTs carry one of the two mutations. However, the alternative defects lead to similar alterations of the downstream signaling cascades and cytogenetic changes. PDGFR- α mutations are found either in the juxtamembrane region or in any of the two TK domains and lead to constitutive activation of the receptor. Signalling through PI3K and STAT3 is thought to be of most importance for this activation⁴⁰⁸.

More than 95% of all DFSPs have a unique translocation of the *PDGF-B* gene, which results in a fusion of collagen type 1 α 1 chain (*COL1A1*) and *PDGF-B* genes. This leads to the *PDGF-B* exon 2 being under the control of the *COL1A1* promoter⁴⁰⁹, resulting in over-expression of PDGF-B in skin fibroblasts (which normally strongly express COL1A1) This creates an autocrine stimulatory loop that drives cell proliferation and fibrosis. Furthermore, when transplanted onto mice, DFSP tumour growth is inhibited by imatinib⁴¹⁰.

Most translocations involving *PDGFRs* result in a constitutively active receptor. At least six different translocations of the *PDGFR-B* gene have been reported in myeloid disorders and leukaemias⁴¹¹. One of the most commonly found translocations involves fusion of the *ETV6-PDGFR-B* genes. The *ETV6-PDGFR-B* fusion results in a protein containing the transmembrane and catalytic intracellular part of the receptor, a protein that cannot bind to PDGF but is nevertheless constitutively active.

Fusion of the *FIP1L1* and *PDGFR-A* genes is found in a subset of patients with hypereosinophilic syndrome (HES)^{412,413}. The *FIP1L1-PDGFR-A* fusion gene has also been identified in systemic mastocytosis⁴¹⁴. The *FIP1L1-PDGFR-A* fusion protein is a constitutively active tyrosine kinase that can transform haematopoietic cells and lead to myeloproliferative disease.

PDGF production in carcinomas is generally thought to act in a paracrine fashion to promote tumour stroma, but it is also possible that autocrine signalling plays a role in carcinoma development in conjunction with aberrant PDGFR expression⁴¹⁵⁻⁴¹⁷. Expression of PDGF has been found to correlate with advanced tumour stages and unfavourable prognosis in human breast carcinoma⁴¹⁸. Furthermore, increased expression of genes of the PDGF pathway has been correlated with EMT⁴¹⁹. PDGF-neutralizing antibodies, dominant-negative PDGFR expression, or imatinib, have recently been shown to influence EMT and metastasis in mouse breast cancers⁴²⁰. Thus, PDGF expression in epithelial cancers may have a role in EMT and tumour dissemination.

PDGF expression is commonly found in the neoplastic component of different human tumours. PDGFR expression is also regularly found in the stromal compartment (endothelial cells, pericytes or fibroblasts) of several types of carcinoma such as skin, breast, colorectal, lung, pancreas, prostate, and ovarian cancers^{298,421}. Through its effects on tumour stroma, PDGF signalling may directly or indirectly promote tumor growth, blood perfusion, metastatic dissemination and drug resistance. PDGFR- β expression in solid tumours occurs mainly in vascular smooth muscle cells. PDGF-BB or PDGF-DD expression in experimental fibrosarcoma or melanoma has been shown to enhance pericyte recruitment to tumour vessels, correlating with tumour vessel stabilization, tumour cell survival and tumour growth³⁸¹. Pericyte recruitment in tumour angiogenesis

is as important as during development. Pharmacological inhibition of PDGFR- β signalling in tumour pericytes of mouse models was synergistic with inhibition of VEGFR signalling in endothelial cells leading to suppression of tumour growth⁴²².

It appears that the quantity of recruited pericytes affects tumour vessel function and tumour growth in most cancers, but the quality of the pericyte coating influences haematogeneous tumour dissemination. Low pericyte coverage is commonly observed in tumour vasculature⁴²³⁻⁴²⁵ and low expression of smooth muscle markers correlates with metastatic propensity in human solid tumours⁴²⁶. Based on these findings, it would appear dangerous to inhibit pericyte behaviour as part of an anti-tumour angiogenesis strategy, as it might simultaneously impair tumour vessel function and increase metastasis⁴²⁷ but further experiments are required to establish possible benefits and drawbacks of therapy aimed at inhibiting tumour pericyte recruitment.

Tumour fibroblasts are generally considered a passive component of tumours. However, it is becoming clear that these cells may affect the malignant behaviour of the neoplastic cells and contribute to tumour progression and resistance to therapy. PDGF-B-driven recruitment of fibroblasts increases the progression of immortal human keratinocytes from a non-tumorigenic to a tumorigenic phenotype⁴²⁸. It is likely that hepatocyte growth factor is the likely mediator of this effect⁴²⁹. In addition, tumour cell-derived PDGF-AA promotes recruitment of PDGFR- α -positive fibroblasts, which in turn produce VEGF-A which can rescue tumour angiogenesis⁴³⁰. The production of angiogenic growth factors such as PDGFB by tumour fibroblasts may lead to prominent recruitment of both fibroblastic and vascular stroma components⁴³¹. In models of human lung cancer, PDGF-A and PDGF-C have been shown to recruit PDGFR- α -positive tumour fibroblasts and thereby promote tumour growth⁴³². Furthermore, PDGF-driven fibroblast recruitment drives tumour cell proliferation in a model of mouse cervical

cancer, by the secretion of FGF2 and FGF7 by recruited fibroblasts⁴³³. In this model, PDGFR inhibition by imatinib or PDGFR antibodies, or inhibition of the stroma-derived FGFs through ligand trapping, were both effective in reducing tumour growth. Other studies have demonstrated the importance of fibroblast secretion of SDF-1/CXCL12 in supporting tumour growth through binding of CXCR4 on tumour cells⁴³⁴. Cancer fibroblast-derived CCL5/RANTES may enhance the metastatic potential of tumour cells⁴³⁵. It could be that SDF-1- and CCL5-positive stromal fibroblasts are recruited into the tumours by tumour cell-derived PDGFs.

Solid tumours commonly display high interstitial fluid pressure (IFP), which reduces fluid convection rate across the capillary wall. Increased IFP has been suggested to interfere with drug uptake in malignancies. It has been suggested that PDGFR- β signalling regulates IFP in normal tissues³⁷⁵ and PDGFR inhibition by imatinib has been shown to enhance the uptake and/or anti-tumour effects of cytotoxic drugs⁴³⁶⁻⁴⁴⁰.

Overexpression of PDGF has been observed in fibromatoses (desmoid tumours), while inhibition of PDGF signalling by imatinib is reported to induce an overall 1 year tumour control rate of 36.8% in a phase II clinical study⁴⁴¹. Thus, inhibition of PDGF may be an attractive therapy option, alone or combined with surgery or/and radiotherapy in refractory cases. Another example for an important role of PDGF in oncogenesis is overexpression of PDGF and c-kit observed in Leydig cell tumours. Treatment with imatinib can largely inhibit Leydig cell tumour growth in an allograft mouse model by inhibition of PDGF and c-kit signalling with no drug-resistance development during imatinib treatment⁴⁴². The clinical success of imatinib/gleevec, a triple tyrosine kinase inhibitor of c-kit, PDGF and c-Abl signalling, in chronic myeloid leukaemia⁴⁴³ and gastrointestinal stromal tumours⁴⁴⁴ has accelerated the development of molecular

targeted cancer therapy. It is highly likely that many more antitumoral substances of this class will be developed and discovered in the near future.

Angiogenesis is an important event in tumour growth as tumours require a sufficient supply of nutrients and oxygen for sustenance and growth⁴⁴⁵. Hypoxic tumour cells secrete cytokines, including VEGF, PDGF, basic fibroblast growth factor (bFGF), insulin growth factor (IGF), to stimulate neovascular formation⁴⁴⁵. However, the neovasculature in tumours is very different from normal physiological vessels. Disorganised growth leads to vessel malformations including dilation, tortuosity, leakage, rupture and the formation of microaneurysms³⁷⁸. Since small numbers of pericytes in tumour vessels may be critical for vessel integrity and function³⁷⁸, targeting pericytes in tumours may be an attractive and efficacious way for anti-angiogenic therapy. Inhibition of PDGF signalling (and also VEGF signalling) induces tumour vessel regression by direct anti-angiogenic effect to endothelial cells and pericytes and by inhibiting pericyte mediated endothelial cell survival mechanisms⁴⁴⁶. Therefore, on the one hand PDGF increases survival and proliferation of endothelial cells and on the other, PDGF regulates vessel growth via pericyte recruitment and association to newly formed vessels.

PDGF's crucial role in tumour propagation appears to relate to its inherent angiogenic activity⁴⁴⁷. During angiogenesis, PDGF has been shown to be essential for the stability of normal blood vessel formation by recruiting pericytes and smooth muscle cells to the developing vessels³⁷⁸. PDGF-B expression by endothelial cells recruits pericytes through a short-range paracrine mode³⁸¹. Pericytes expressing PDGFRs migrate along a steep gradient of PDGF-B in the peri-endothelial compartment to endothelial cells and thus initiate intimate association with the abluminal surface of the endothelial cells³⁸¹. Pericyte-deficiency promotes a range of microvascular changes, such as endothelial

hyperplasia, vessel dilation, leakage and rupture, leading to capillary microaneurysms and microhemorrhage³⁷⁸. In animal models, loss of more than 95% of the pericytes is lethal while mice embryos deficient in up to 90% pericytes are compatible with embryonic and postnatal survival^{381,382}. This is seen in knockout mice models such as PDGF-B $-/-$ or PDGFR- β $-/-$.

Angiogenesis is critical for tumour survival and growth, since tumours located more than 100–200 μm from a blood vessel need neovascular formation to ensure a sufficient supply of nutrients and oxygen⁴⁴⁵. Tumour cells in hypoxia secrete cytokines, including VEGF, PDGF, basic fibroblast growth factor (bFGF), insulin growth factor (IGF), to stimulate neovascular formation⁴⁴⁵. However, neovasculature in tumours differs from normal physiologic vessels. The rapid and disorganised growth leads to vessel malformation including vessel dilation, tortuosity, leakage, rupture and formation of microaneurysms identical to those described above in pericyte deficient mice³⁷⁸. Interestingly, these hallmarks of microvascular malformation in tumours were found to be identical with the alterations found in pointing to a pericyte-deficiency in the disordered neovascular formation in tumours³⁷⁸. Since small numbers of pericytes in tumour vessels may be critical for vessel integrity and function³⁷⁸, targeting pericytes in tumours may be an attractive and efficacious way for anti-angiogenic therapy.

Recent data from experiments *in vivo* imply that targeting pericytes actually provides additional benefits⁴⁴⁸. Until now, endothelial cells were considered to be the most important target for anti-angiogenic therapies⁴⁴⁹. Inhibiting VEGF in endothelial cells reduces endothelial cell survival, proliferation, tube formation and invasion *in vitro*⁴⁴⁹. However, it has recently been shown that endothelial cells are resistant to the inhibitory effect of SU5416 by blocking VEGFR *in vivo* through pericyte mediated escape strategies via the Ang-1/Tie2 pathway⁴⁴⁶. Combined inhibition of VEGF and PDGF

signaling enforces tumour vessel regression by a direct anti-angiogenic effect on endothelial cells and pericytes and by inhibiting pericyte mediated endothelial cell survival mechanisms⁴⁴⁶. Others have also shown that tumour vessels lacking pericytes are more dependent on VEGF for their survival than are vessels invested by pericytes⁴⁴⁸. In fact, sorafenib and sunitinib/SU11248 act as anti-angiogenic agents by inhibiting VEGFR-2/-3, PDGFR- β , Flt-3, and c-KIT. Both drugs exert clear clinical effects in patients with renal cell carcinoma which are most likely mediated via anti-angiogenic effects^{450,451}.

In summary, PDGF has at least two distinct functions in pro-angiogenic signalling. Firstly, PDGF increases survival and proliferation of endothelial cells secondly, it regulates vessel growth via pericyte recruitment and organisation in newly formed vessels.

As has been outlined previously ionising radiation not only has a direct effect on tumour cells but radiation induced damage of endothelial cells plays a major role in tissue damage and antitumoral efficacy⁴⁴⁸. Thus, ionising radiation can be considered as a potent anti-angiogenic agent⁴⁴⁸. On the other hand, tumour cells are capable of producing pro-angiogenic cytokines including VEGF, PDGF and FGF in response to ionising radiation⁴⁴⁹⁻⁴⁵². These pro-angiogenic cytokines could protect endothelial cells and vessels from radiation-induced damage and consequently ensure supply of oxygen and nutrients for tumour cells^{447,448,453}. Secretion of PDGF can also be stimulated by irradiation of stromal cells, such as endothelial and fibroblast cells⁴⁵⁴. Elevated expression of these growth factors correlates with higher vessel density and negative clinically prognosis in various tumours⁴⁵⁵ and often this type of tumour possesses relative resistance to radiation therapy⁴⁵⁶. Inhibition of pro-angiogenic signaling by tyrosine kinase inhibitors can therefore augment the radiation induced damage to

endothelial cells and abolishes the tumor cells mediated protection. Moreover, these inhibitors can prevent the re-growth of endothelial cells and neovascular formation⁴⁵⁴. Consequently, anti-angiogenic substances targeting VEGF and PDGF may increase anti-angiogenic activity of ionising radiation and possess a potent antitumoral synergy with radiation.

This dual role for PDGF signaling in oncogenesis and angiogenesis does seem to be genuine and it has been demonstrated in gliomas. PDGFR- α has been detected in gliomas using in situ hybridization and immunohistochemistry to indicate the presence of autocrine and paracrine loops in glioma cells and in endothelial cells within the tumour mass. Therefore, treatment with imatinib/gleevec may disrupt the PDGF/PDGFR autocrine loop by specifically inhibiting phosphorylation of PDGFR and thereby exerting a synergistic antitumoral effect with ionising radiation (i.e it acts as a radiosensitiser⁴⁵⁷) and it may also inhibit hypoxia-induced angiogenesis and reinforce the anti-angiogenic effect of radiation⁴⁵⁸.

5.12.3.2 Vascular disorders

5.12.3.2.1 Atherosclerosis

PDGF expression has been reported in virtually every cell type of the atherosclerotic arterial wall, as well as in the infiltrating inflammatory cells⁴⁵⁹. All PDGFs (A–D) and, in particular A and B have been found in atherosclerotic lesions at increased levels compared with the normal vessel wall⁴⁵⁹. PDGFR- α and PDGFR- β are also expressed at increased levels in smooth muscle cells of the media of arteries diseased by atheroma. The role of PDGF and PDGFR is not completely clear. Several conditions associated with cardiovascular disease affect PDGF or PDGFR gene expression, such as increased blood pressure and α -adrenergic stimulation of the vessel wall (which enhances vascular

PDGF-A expression^{460,461}), reduced endothelial shear stress (which induces both PDGF-A and PDGF-B⁴⁶²) and hypercholesterolaemia (which dramatically increases PDGF-A and PDGF-B expression in circulating mononuclear cells⁴⁶²).

In the response-to-injury hypothesis of atherosclerosis, PDGF released from aggregating platelets at sites of endothelial injury was thought to be important in the migration of smooth muscle cells from the media into the intima, as well as in the subsequent proliferation of the smooth muscle cells at this site^{463,464}. The current view of atherogenesis is more focussed on local chronic inflammation. Lipoprotein retention and the infiltration and activation of different types of immune cells are believed to play key roles⁴⁶⁵. PDGF may still be important as one of many factors secreted in the vessel wall by activated immune cells. In fact, in different animal models of acute arterial injury by balloon catheterization, neointimal smooth muscle cell accumulation was reduced by the administration of various PDGF pathway inhibitors, including neutralizing PDGF (AB) antibodies⁴⁶⁶, PDGFR kinase inhibitors^{467,468} and PDGFR-neutralizing antibodies⁴⁶⁹. Restenosis following angioplasty of atherosclerotic vessels in animal models and chronic cardiac transplant rejection-induced atherosclerosis in rats can also be inhibited by PDGFR- inhibitors. Atherosclerotic lesions can be inhibited in ApoE-deficient mice by neutralizing PDGFR- β antibodies⁴⁷⁰ and a PDGFR inhibitor⁴⁷¹. On the other hand, infusion of PDGF-BB, PDGF-C or PDGF-D, or local transfection of a PDGF-B expression vector can produce increased smooth muscle cell proliferation and intimal thickening in models of arterial injury^{472,473}. There is currently no evidence for a role for PDGFR- α in neointimal proliferative responses to acute or chronic arterial injury.

There is support for a functional role for PDGF-B and PDGFR- β in atherogenesis/restenosis from genetic models of PDGF-B or PDGFR- β deficiency.

Carotid ligation experiments in *pdgfrb*^{+/+} and *pdgfrb*^{-/-} chimeras demonstrated a low proportion of *pdgfrb*^{-/-} cells in the neointima but an increased contribution to the media. This suggests impaired migration of medial smooth muscle cells to the intima in the absence of functional PDGFR- β ⁴⁷⁴. Furthermore, bone marrow transplantation of PDGF-B-deficient marrow into lethally irradiated, ApoE-deficient recipients and the subsequent lack of PDGF-B production by infiltrating inflammatory cells led to a small and transient inhibition of neointimal fibrous cap formation⁴⁷¹. The reason for the relatively modest effect may be that PDGF-B has the opposing effects (on atherosclerosis) by being both anti-inflammatory and stimulatory to smooth muscle cell proliferation in the vessel wall⁴⁷⁵.

Additional support for PDGF-B and PDGFR- β 's central role in atherogenesis comes from studies of LRP1⁴⁷⁶⁻⁴⁷⁹. LRP1 is a multifunctional transmembrane receptor, which is expressed by many cell types and binds a biologically diverse array of ligands. LRP1 is tyrosine phosphorylated in response to PDGF-BB. It seems that LRP1 normally limits PDGFR- β expression and function in medial smooth muscle cells by inhibition of thrombospondin-mediated TGF β activation and signalling through Smad2/3, leading to decreased PDGFR- β expression⁴⁸⁰ and LRP1-dependent endocytosis and c-Cbl-mediated ubiquitinylation of PDGFR- β ⁴⁸¹.

5.12.3.2.2 Pulmonary hypertension

Primary pulmonary hypertension (PH) is a severe condition that can lead to right ventricular failure and death. The etiology and pathogenesis of PH is not well understood although pulmonary artery smooth muscle cell hyperplasia is a hallmark of the disease and increased expression of PDGFs and PDGFRs has been described in various experimental animal models of PH^{482,483} and in humans⁴⁸⁴. The basis for

increased PDGFR signalling in PH is uncertain but it is possible that there are links through ApoE and adiponectin. The combined action of ApoE deficiency and insulin resistance can promote development of PH in mice. In this model, ApoE and adiponectin suppressed proliferation of and inhibited PDGF signalling in pulmonary artery smooth muscle cells⁴⁸⁵. ApoE promotes down-regulation of PDGFR- β ⁴⁷⁹, possibly by binding to LRP1 (see above). Adiponectin appears to bind PDGF-B and scavenge it^{486,487}. The cellular origin of the proliferative lesion in PH remains unclear. Increased expression of PDGFR- β in PH has been observed in perivascular fibroblast-like cells, suggesting that these cells may differentiate into smooth muscle cells in conjunction with pulmonary hypertension⁴⁸⁸.

Imatinib has recently been shown to reverse experimental hypertension in two animal models of PH⁴⁸⁴ and early results of imatinib in a human case of PH are promising⁴⁸⁹. Imatinib reversed pulmonary arterial smooth muscle cell proliferation and neointimal formation and these effects may reflect inhibition of PDGFR signalling. On the other hand, imatinib is not a completely specific inhibitor of PDGFRs. However, other targets of the drug such as Abl, Kit, and Flt3 are not currently implicated in medial smooth muscle cell growth.

5.12.3.2.3 Retinal vascular disease

PDGF-B has been implicated in ischaemic retinopathies such as proliferative diabetic retinopathy, proliferative vitreoretinopathy and choroidal neovascularization. Intraocular injection of PDGF-B-inhibiting aptamers can protect against retinal detachment in a rabbit model of proliferative retinopathy⁴⁹⁰. Inhibition of PDGFR signalling either using antibodies or imatinib has been shown to enhance the therapeutic effect of anti-VEGF-A treatment in multiple mouse models of ocular

neovascularization⁴⁹¹. Focal neovascular retinal disease resembling proliferative diabetic retinopathy has been reported in two different mouse mutants with PDGF-B deficiency with an accompanying loss of retinal pericytes^{492,493}. In diabetic mice, loss of one copy of the *pdgfb* gene also enhanced retinal pericyte loss and endothelial death⁴⁹⁴. Overall, it appears that PDGF-B may play a role in the pathogenesis of vitreoretinopathy, while pericyte loss (caused by PDGF-B deficiency) may lead to the development of a diabetic retinopathy-like condition. Pericyte loss is a hallmark of human diabetic retinopathy⁴⁹⁵ and may be causally involved in its pathogenesis, but it is not known if diabetes-induced pericyte loss reflects PDGF signalling in the retina.

5.12.3.3 Diseases associated with fibrosis

PDGF signalling has been implicated in several fibrotic conditions and is assumed to play a role in driving proliferation of cells with a myofibroblast phenotype. Tissue fibrosis involves excessive deposition of extracellular matrix material, which might be partly due to increased PDGF activity, but is probably more likely to be associated with up-regulated TGF β . Excessive stromal cell proliferation and matrix deposition leads to tissue scarring and progressive loss of organ function. An emerging concept common to most fibrotic diseases is that inflammatory cells such as activated macrophages release PDGFs. The same cells also produce inflammatory cytokines that promote up-regulation of PDGFRs on mesenchymal cells⁴⁹⁶. As a result, PDGF-mediated proliferation of mesenchymal cells, recruitment of fibroblasts and related cells and increased collagen production could all contribute to subsequent fibrosis. A causative role in several animal models of lung and cardiac fibrosis and glomerulosclerosis has been demonstrated by specific loss- and gain-of-function approaches. Also, very recent studies have raised the possibility that PDGFR-activating autoantibodies may participate in autoimmune fibroses.

5.12.3.3.1 Pulmonary fibrosis (PF)

PF often results from different types of environmental exposures leading to acute toxic or chronic inflammatory responses in the lung. Chronic allergic reactions, transplant rejection and auto-immune disorders may also lead to PF, although quite frequently PF the cause of the fibrosis is unknown (idiopathic PF). PF involves the proliferation of myofibroblasts in alveolar walls, pulmonary blood vessels and small airways. Most of the causes of PF have been associated with increased PDGF levels in the lung tissue or bronchoalveolar lavage fluid either in humans or animal models of lung injury and fibrosis⁴⁹⁶. Studies in animal models have also shown that pulmonary fibroblasts respond to PDGFs by increased proliferation and cell migration. Alveolar macrophages appear to be the principal source of PDGF (mainly B) in PF⁴⁹⁶⁻⁴⁹⁹. PDGF-A and PDGF-C expression has also been detected in injured or fibrotic lungs⁵⁰⁰. Up-regulated PDGF ligand expression is thought to occur via inflammatory cytokines such as IFN- γ , IL-1 β and IL-13²⁰. Both PDGFR- α - and PDGFR- β -mediated signals appear to play a role in PF. PDGFR- α expression is markedly up-regulated in the lung mesenchyme in response to inflammatory mediators such as IL-1 β and TGF- β . A similar PDGFR- α response has also been observed in lungs exposed to a number of environmental factors, including asbestos and air pollution particulates⁵⁰¹. PDGFR- β expression in the lung appears constitutive and unaffected by inflammatory stimuli, but it may mediate fibro-proliferative responses alone or in heterodimer configuration with PDGFR- α . With these findings in mind, attenuation of PDGF-induced signals could potentially be a realistic pharmacological target for the prevention and/or treatment of PF. In animal models, three distinct TK inhibitors (SU9518, SU11657, and imatinib), which all potently inhibit PDGFRs, have all been shown to reduce lung fibrosis and increase life span in a model of radiation-induced lung injury⁵⁰². In this model, several different

PDGFs were up-regulated. In another study, mesenchymal cell proliferation and collagen accumulation were reduced by the PDGFR TK inhibitor AG1296 in a model of metal-induced lung fibrosis⁵⁰³.

5.12.3.3.2 Liver fibrosis

Liver fibrosis (and cirrhosis) can be caused by a variety of injuries including drugs, alcohol, infections, metabolic changes and again idiopathic factors. The key fibrogenic cells in the liver are the hepatic stellate cells (of Ito), which are in effect the sinusoidal vessel pericytes. Dormant stellate cells do not express PDGFR- β , and, unlike other pericytes, they do not depend on PDGF-B or PDGFR- β for their development⁵⁰⁴. PDGFR- β expression is up-regulated in activated stellate cells both in vitro and in vivo in animals and humans⁴⁹⁶. This ability to switch on PDGFR- β renders stellate cells highly responsive to PDGF-B and PDGF-D. It appears that TGF β is a major inducer of PDGFR- β in stellate cells and several different cell types produce TGF β in the reactive liver, including stellate cells themselves. Resident hepatic macrophages (Kupffer cells) are a major source of PDGF-B, and when activated they can produce large amounts of PDGF-B, but infiltrating inflammatory cells can also contribute⁵⁰⁵. Increased PDGF-D expression has been detected in the perisinusoidal and periportal areas in experimental liver fibrosis in rats, but the source of the PDGF-D has not yet been established⁵⁰⁶.

5.12.3.3.3 Dermal fibrosis

Scleroderma (also known as systemic sclerosis) is an autoimmune disorder characterized primarily by progressive dermal and vascular fibrosis. Other organs are affected too, including lung, heart, esophagus, intestine, and kidney. Increased expression of both PDGFs and PDGFRs has been demonstrated in scleroderma skin⁴⁹⁶.

A role for PDGF signaling in bleomycin-induced experimental scleroderma in mice was also suggested by the therapeutic effect of imatinib in this model⁵⁰⁷.

As for other fibroses, PDGF-B expression seems to occur in activated macrophages and infiltrating inflammatory cells⁵⁰⁸. In addition, scleroderma myofibroblasts appear to be constitutively phenotypically changed in comparison with normal skin fibroblasts, perhaps indicating that they have a distinct origin. Possibly, they originate from pericytes since the increased PDGFR- β expression was mainly observed around blood vessels in the scleroderma skin⁵⁰⁹. A pericyte origin has also been suggested for myofibroblasts in scarring tissue and tumours^{510,511}. One distinct feature of scleroderma myofibroblasts is that they constitutively express PDGF-A in an autocrine loop driven by $\text{II-1}\alpha$ ⁵¹². This appears to contrast with normal fibroblasts in which transient PDGF-A expression occurs after exposure to mitogens⁵¹³. Another feature that distinguishes scleroderma fibroblasts from their normal counterparts is that $\text{TGF}\beta$ increases the expression of PDGFR- α and enhances the mitogenic effect of PDGF-A in the former but seems to have opposite effects on the latter⁵¹⁴. Down-regulation of Smad7 and up-regulation of Smad3 in scleroderma fibroblasts may account for this difference. Also thrombin and $\text{II-1}\alpha$ have been implicated in the enhanced responsiveness to PDGF stimulation in scleroderma fibroblasts⁵¹⁵.

It was recently reported that autoantibodies directed toward the PDGF receptors are present in the serum from patients with scleroderma⁵¹⁵. Autoantibodies are commonly found in autoimmune disorders and sometimes play direct pathogenic roles. A well-known example is Grave's disease, where agonistic autoantibodies for the thyroid-stimulating hormone receptor activate the thyroid gland, leading to hyperthyroidism. PDGFR autoantibodies were detected in serum from 46 out of 46 scleroderma patients but in no controls. The antibodies activated both PDGFR- α and PDGFR- β

phosphorylation and downstream signaling through the Ras-MAPK pathway. Moreover, the antibodies were shown to convert normal human fibroblasts into collagen type-1-expressing myofibroblasts in vitro. This is an interesting finding with potential openings for new diagnostics. If these antibodies play a direct role in scleroderma pathogenesis, their elimination may also be considered as a putative therapy. Preliminary data suggest that PDGF autoantibodies are present also in patients with chronic graft-versus-host disease⁵¹⁶. The possibility of PDGFR activation through autoantibodies is a novel and provocative concept in autoimmune fibrotic diseases, which will most certainly be extensively explored over coming years.

Furthermore, PDGF has a crucial role in cutaneous wound healing. In fact, recombinant human PDGF-BB (becaplermin) has been trialled as a topical treatment for chronic neuropathic lower-extremity diabetic ulcers⁵¹⁷. In addition, it has been used to prevent and treat pressure sores and ulcers and also to speed up healing after various surgical procedures. The tissue repair mechanisms induced by PDGF-BB involve the processes outlined previously, particularly fibroblast proliferation, collagen production and new vessel formation. Successful treatment may involve direct effects on PDGFR- β -expressing mesenchymal cells and also up-regulation of PDGF-A⁵¹⁸. Clinical efficacy has been demonstrated in several phase III studies⁵¹⁹ and the combined results suggest that topical application of PDGF-BB is safe and well tolerated^{520,521}. A recent study involving more than 900 patients confirmed that topical treatment with PDGF improved healing of chronic full-thickness diabetic foot ulcers⁵²². Local delivery of PDGF-BB has also been tested in patients with severe periodontal disease and found to increase biomarkers for bone metabolism and turnover⁵²³ and provide improved periodontal regeneration⁵²⁴.

5.12.3.3.4 Renal fibrosis

Renal fibrosis is universally present in association with chronic renal failure caused by diabetes, hypertension, renotoxic drugs, autoimmune disorders, immune-complex diseases and idiopathic conditions. The kidney has many components and the fibrosis can involve one or more of these structures. Glomerular fibrosis is generally associated with mesangial cell reaction. In tubulo-Interstitial fibrosis a mixture of mesenchymal cells (some of which are currently unidentified) proliferate and deposit the extracellular matrix material. It has already been outlined that mesangial cell development depends on a PDGF-B–PDGFR- β paracrine loop. On the other hand, tubulointerstitial mesenchyme development depends on combined PDGF-A and PDGF-C signalling via PDGFR- α . Many studies have shown that identical signalling pathways are involved in the development of renal fibrosis. Glomerular disease is frequently accompanied by up-regulated expression of PDGFR- β ⁵²⁵. In experimental animal models of mesangioproliferative disease, neutralizing PDGF antibodies, PDGF-B-binding aptamers, or imatinib can all prevent mesangial cell proliferation and matrix accumulation⁵²⁶⁻⁵²⁸. Furthermore, systemic administration of PDGF-BB promotes resting mesangial cell proliferation, suggesting that these cells are able to respond to PDGF-B independent of prior inflammatory activation⁵²⁹ and increased circulating plasma PDGF-B levels have also been correlated with increased numbers of mesangial cells in mice⁵³⁰. All PDGFs (A–D) have been shown to be up-regulated in situations associated with renal fibrosis. Evidence for a role for PDGF-A and PDGF-C remains somewhat circumstantial, but PDGF-D has been shown to play a role in experimental mesangial proliferative glomerulonephritis in rats by use of a PDGF-D-specific neutralizing antibody⁵³¹. The source(s) of PDGF-B during mesangial proliferative pathology is not entirely clear. Endothelial cells may contribute, as they do during development, but it is likely that PDGF-B is also provided by invading mononuclear

cells. PDGF-D appears to be strongly up-regulated in the mesangial cells themselves during their pathological expansion⁵³² and might act in an autocrine loop.

5.12.3.3.5 Cardiac fibrosis

Cardiac fibrosis occurs after a range of injuries caused by ischaemia/infarction, hypertension, chronic transplant rejection or a variety of endocrine disorders. Cardiac fibrosis is thought to involve the proliferation and excessive collagen deposition by interstitial cardiac fibroblasts. Most of the information suggesting a role for PDGF signalling in cardiac fibrosis originates from gain- and loss-of-function studies in experimental animals. Mice treated with neutralizing PDGFR antibodies following myocardial infarction show attenuated healing responses depending on antibody specificity. PDGFR- β blockade impairs maturation of the new vessels formed in the heart, consistent with the known role of PDGFR- β signalling in smooth muscle cell/pericyte recruitment and vascular development described earlier. PDGFR- α blockade, on the other hand, did not affect the vasculature but decreased collagen deposition, suggesting that this receptor may be more important in fibroblast activation⁵³³. In addition, over-expression of PDGF-C in the heart of transgenic mice has been shown to induce diffuse cardiac fibrosis with extensive hyperplasia of fibroblast-like cells and large collagen deposits⁴⁵³. This was also coupled with dilated cardiomyopathy in females and a hypertrophic response in males. PDGF-D over-expression in the heart also produced diffuse cardiac fibrosis but it was also associated with increased thickness of the medial smooth muscle coat in the coronary vessels⁵³⁴.

PDGF-A, PDGF-C, or PDGF-D genes were introduced into heterotypic rat heart transplant models via adeno-associated virus (AAV)-mediated transfer which led to accelerated cardiac fibrosis and chronic rejection⁵³⁵. PDGF-AA causes potent

proliferation of cardiac fibroblasts in vitro⁵³⁶ but AAV-derived PDGF-B does not seem to. PDGFs have been implicated in the cardiac fibrotic response to angiotensin II⁵³⁷. Angiotensin-II-induced cardiac fibrosis is reduced in mice lacking one copy of the transcription factor KLF5, correlating with reduced cardiac expression of PDGF-A. Evidence for direct binding of KLF5 to the PDGF-A promoter has been described and regulation of expression of PDGF-A by KLF5 occurs in other tissues also⁵³⁸. Overall, these results implicate PDGFR- α stimulation as a major fibrogenic participant in the heart, whereas PDGFR- β stimulation in the heart affects mainly vascular mural cells leading to vessel wall pathology.

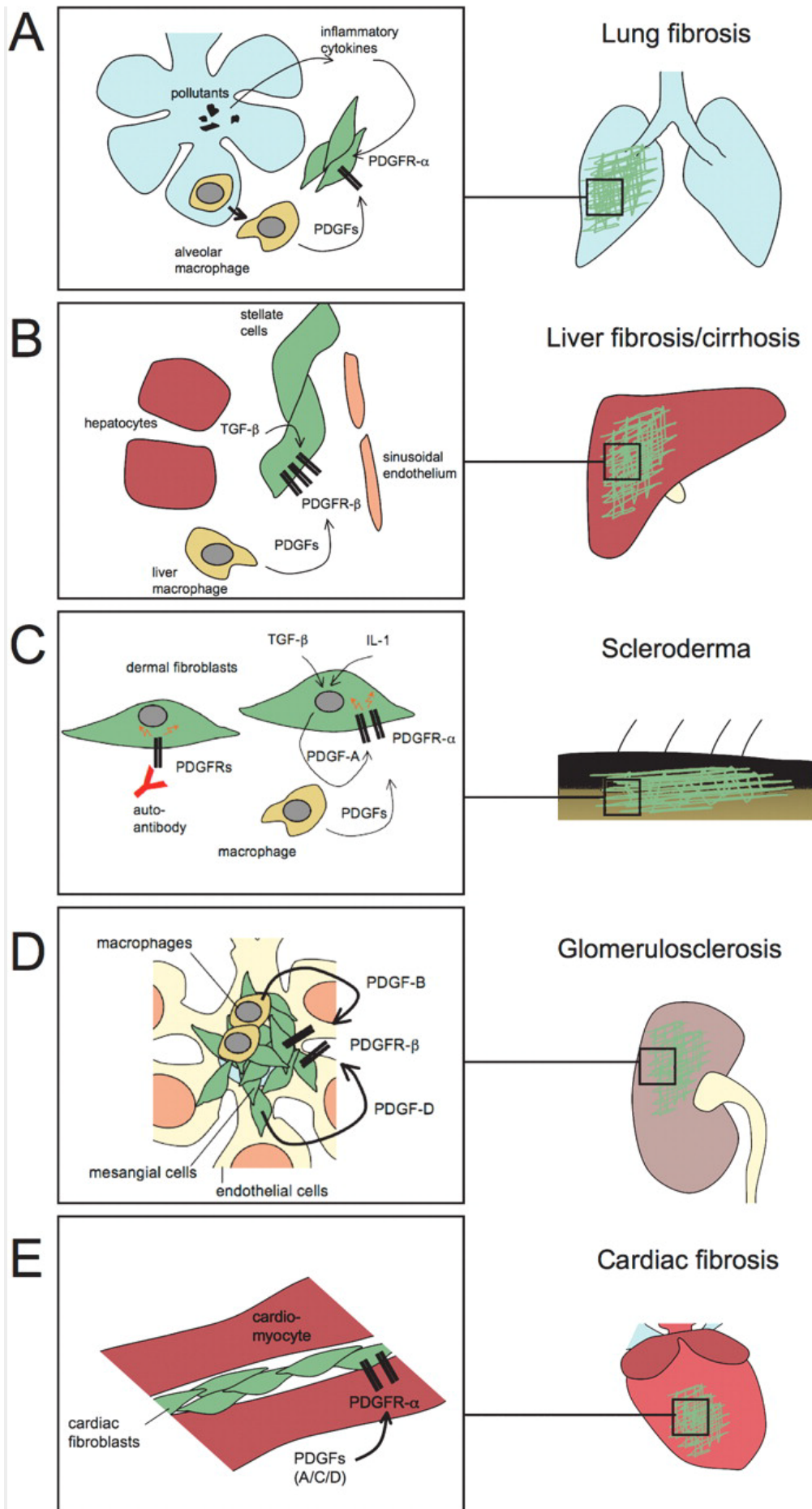


Figure 34. PDGF and tissue fibrosis. In lung fibrosis (A), PDGFs released from alveolar macrophages promote proliferation of alveolar (myo)fibroblasts and fibrogenesis. In liver fibrosis (B), hepatic stellate cells up-regulate PDGFR- β in response to TGF- β . In scleroderma (C), PDGFs released by macrophages promote proliferation of dermal (myo)fibroblasts. In a variety of renal diseases and disease models (D), PDGF-B released from invading macrophages and PDGF-D released from mesangial cells may drive mesangial cell proliferation and matrix deposition, leading to glomerulosclerosis. In transgenic models, the expression of PDGFs in myocardial cells drives proliferative expansion of PDGFR- α -positive cardiac fibroblasts and collagen deposition, leading to severe cardiac fibrosis (E)³⁰²(with permission).

5.13 The relationship between platelet derived growth factor and irradiation

There is potential for a complex bidirectional association between PDGF and radiation. Firstly, PDGF may play a significant role in the formation of some tumours that are subsequently treated by radiotherapy. Secondly, the presence of PDGF may influence the response of tissues to irradiation. Thirdly, PDGF may be a key player in cell and tissue responses to radiation injury especially through its effects on angiogenesis and fibrosis. Finally, PDGF could initiate sequences that lead to the post-radiation tumour development occasionally seen many years after irradiation.

As has been discussed earlier, PDGF can be synthesized by a number of different cell types such as macrophages, epithelial and endothelial cells³⁰². PDGF is also implicated in a range of physiological and developmental systems together with pathological processes, including fibrosis, atherosclerosis, glomerulonephritis and aggressive fibromatosis^{539,540}. Aberrant production of PDGF, with autocrine stimulation, may be an important mechanism in the neoplastic conversion of PDGF receptor-positive cells^{540,541}. Activated RTK phosphorylates numerous signalling molecules that initiate intracellular signalling cascades. The latter includes cellular responses involved in the

activation of the ras/MAPK pathway, which can promote cellular proliferation, migration and differentiation³⁶³, and the PI3K/Akt pathway, which influences cell survival⁵⁴³. Both of these are crucially important for tumour resistance to radiotherapy and chemotherapy. Furthermore, platelet-derived growth factor (PDGF) exerts its potent mitogen and chemotactic effects in a variety of mesenchymal cells such as fibroblasts and vascular smooth muscle cells. PDGF is therefore a potential key molecule for tissue rebuilding in response to radiation injury.

5.13.1 PDGF and tumour formation

This has been discussed previously in some detail. In summary, many studies have suggested that autocrine activation of PDGF is an important pathogenetic mechanism involved in a variety of brain tumours⁵⁴¹⁻⁵⁴³. Growth stimulation of meningioma and neuroblastoma cells in vitro, can be abolished completely by introducing a neutralising antibody against PDGF. DNA synthesis in meningioma cell lines can also be inhibited using a PDGF antagonist³⁹⁷. Adult fibromatoses (desmoid tumours) have been associated with overexpression of PDGF. Inhibition of PDGF signaling by imatinib induced overall 1 year tumour control rate of 36.8% in a phase II clinical study⁴⁴¹. Another important example PDGF's role in oncogenesis is in gastrointestinal stromal tumours (GISTs). Many GISTs have gain-of-function mutations of c-kit receptor tyrosine kinase (KIT) gene. In addition, overexpression of PDGF and c-kit has been observed in Leydig cell tumours. The clinical success of imatinib/gleevec, a triple tyrosine kinase inhibitor of c-kit, PDGF and c-Abl signaling, in chronic myeloid leukemia⁴⁴³ and GISTs⁴⁴⁴ has accelerated the development of molecular targeted cancer therapy.

5.13.2 PDGF and its role in angiogenesis

Angiogenesis is critical for tumour survival and growth. PDGF has at least two distinct functions in pro-angiogenic signalling. Firstly, PDGF increases survival and proliferation of endothelial cells and secondly, it regulates vessel growth via pericyte recruitment and organisation in newly formed vessels. The dual role for PDGF signaling in oncogenesis and angiogenesis suggests the presence of autocrine and paracrine loops in tumour formation. Treatment with imatinib/gleevec may disrupt the PDGF/PDGFR autocrine loop and may act synergistically with ionising radiation in its antitumoral effect (i.e it acts as a radiosensitiser⁴⁵⁷) and it may also inhibit hypoxia-induced angiogenesis and reinforce the anti-angiogenic effect of radiation⁴⁵⁵.

5.14 PDGF and radiation induced fibrosis

Radiation causes acute inflammation and chronic fibrosis in local tissues, as has been described in a previous chapters. This frequently leads to side effects which become a dose-limiting factor for treatment efficacy¹⁸. Fibrosis in particular frequently leads to symptomatic problems.

In the case of lung tumours, the dose tolerance limitation of normal neighbouring tissue often precludes successful radiotherapeutic treatment⁵⁴⁴. The pulmonary fibrosis produced is a progressive condition, characterized by mesenchymal cell proliferation, the subsequent deposition of extracellular matrix proteins and extensive remodeling of the pulmonary parenchyma⁵⁴⁵. In both human and animal model systems, acute pneumonitis and late fibrosis are directly dependent upon total irradiation dose, fraction size, and lung volume irradiated⁵⁴⁶⁻⁵⁴⁸. New precise radiotherapy techniques can spare more normal tissue around the tumour and thus reduce the gravity of the side effects. However a recent study has shown that 14.6 % patients with lung cancer still developed

intermediate grade radiogenic pneumonitis after primary radiotherapy with dose escalation using 3D conformal techniques and 13.8 % patients developed fibrosis⁵⁴⁹.

Prevention and treatment of fibrosis in general and in particular would be a significant break-through in the therapeutic intervention of numerous pathological conditions including radiation induced fibrosis. Such treatment remains elusive, partly because the exact mediators and mechanisms involved in fibrogenesis are not completely understood⁵⁴⁵. A number of investigations have provided clear evidence for increased expression of various cytokines including PDGF, transforming growth factor- β , tumor necrosis factor- α and interleukin-1 in response to ionising radiation⁵⁵⁰⁻⁵⁵². In this regard, some pro-inflammatory cytokines seem to be important for the acute impairment in the pneumonitis phase, for example TNF- α and CD95-ligand^{553,554}, whereas others are involved in the regulation of the fibrotic response. For the development of fibrosis, transforming growth factor- β is a widely accepted key molecule⁵⁵⁵.

Moreover, recent evidence supports an important role of PDGF for the development of lung fibrosis in response to ionising radiation. Firstly, PDGF and PDGFR are expressed at low levels in normal adults, while elevated levels are detected in lungs of patients with radiation-induced pulmonary fibrosis⁵⁵⁶. Augmented expression of PDGF is further observed in asbestos-associated, bleomycin-induced and idiopathic pulmonary fibrosis^{545,557,558}. Increased expression of PDGF in rat lungs by adenoviral delivery or lung-specific over-expression in mice is associated with pronounced lung fibrosis^{559,560}. Moreover, inhibiting the PDGF pathway with neutralising antibodies to PDGF or administration of soluble extracellular region of PDGFR- β can attenuate the development of fibrosis^{561,562}. We suggest that PDGF may also be important in radiation injury to the lower urinary tract (see later in this Chapter) and that similar therapeutic tactics may be helpful at this site too.

The radiation-induced overexpression of PDGF led to phosphorylation and activation of PDGFR in lungs of irradiated mice, while the phosphorylation of PDGFR was strongly inhibited in both irradiated groups treated with RTKIs *in vivo*⁵⁰². Treatment with RTKIs attenuated the development of pulmonary fibrosis with excellent correlation with clinical, histological and computed tomography results, although the acute inflammatory response induced by radiation injury was not completely abrogated. Moreover, all three tyrosine kinase inhibitors reduced lung fibrosis after radiation injury and prolonged animal survival. Thus, there is good evidence to support an important role for PDGFs and their receptors in mesenchymal cell recruitment and stimulation in fibro-proliferative diseases.

There are other potential reasons why PDGF may be important in radiation fibrosis. Fibroblasts are the putative effector cells of this fibrosis, whether they are resident, recruited from local cells or recruited from bone marrow stem cells. Recruitment and stimulation of fibroblasts is clearly an important event in the development of fibrosis. PDGF exerts a profibrotic effect through its mitogenic and chemotactic stimulus to mesenchymal cells, such as fibroblasts, myofibroblasts and smooth muscle cells²⁹⁸. Moreover, PDGF has also been shown to stimulate production of extracellular matrix proteins, such as collagen, hyaluronic acid, fibronectin and proteoglycan⁵⁶³⁻⁵⁶⁶, which may be responsible for the irreversibility of fibrotic lesion. The radiation-induced secretion of PDGF is unlikely to be derived solely from inflammatory cells. Stromal cells, such as fibroblasts and endothelial cells, induce paracrine PDGF in co-culture systems which substantially stimulated the proliferation of non-irradiated fibroblasts³⁰². Endothelial cells are thought to be a potential source of PDGF after radiation *in vitro*⁵⁶⁷ and increased expression of c-sis mRNA (PDGFR β) in epithelial cells has also been observed in several conditions associated with pulmonary fibrosis⁵⁰³.

To support the idea that PDGF is produced by cells other than inflammatory cells, treatment with dexamethasone did not decrease the level of PDGF-BB or the mitogenic activity of bronchial alveolar lavage fluid for fibroblasts in patients with chronic lung disease of prematurity⁵⁶⁸. Cyclosporin A treatment does not appear to inhibit the expression of PDGF ligands and receptors either⁵⁶⁹. Thus, stromal cells, such as endothelial, fibroblasts cells, are likely to be responsible (at least partially) for the release of cytokines, including PDGF, in pulmonary fibrosis.

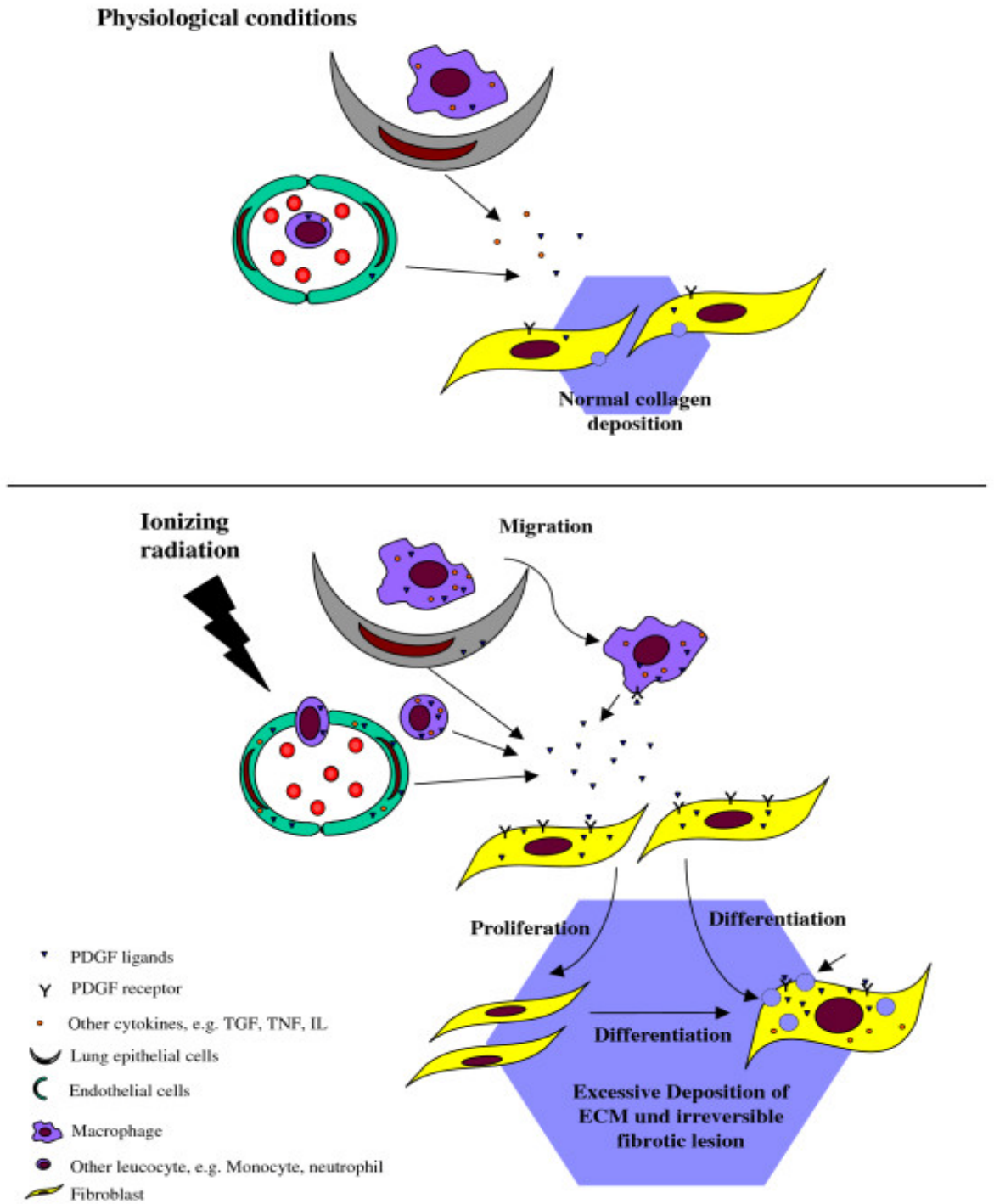


Figure 35. Schematic diagram of the potential role of PDGF signalling in radiation induced fibrosis³⁰⁰

5.15 Platelet-derived growth factor in radiation injury to the bladder

PDGF has been shown to play a role in several human neoplasms and inflammation based, healing and fibrosing conditions including atheroma and fibromatosis. It is thought to play a role in radiation fibrosis in the lung and the gastrointestinal tract but its potential role in lower urinary tract radiation injury is currently unknown. As has been mentioned in the previous sections, radiation is commonly used to treat urothelial and other pelvic malignancies and radiation-induced side effects limit its efficacy. There are different ways to try to minimise the side effects, or treat them, but the possibility of influencing the development of or reversing these effects through PDGF does not appear to have been considered to date. Medical intervention is now available for PDGF and its receptors and it is possible that the use of these compounds may influence effects of radiation. With this in mind, an investigation of PDGF expression was undertaken to establish whether PDGF plays a significant role in radiation injury to the bladder, thereby opening a door for therapeutic amelioration of this dose limiting effect.

5.15.1 Materials and methods

The files of the Pathology Department were searched for all cystectomy specimens following radiation. The search was similar to that described previously for the cases investigated in Chapters 3 and 4. A total of 110 cases were identified which were suitable for inclusion. As before, the patients had received a standard course of radiotherapy and the time between radiation and surgery was known. The expression of PDGF and its receptors was investigated using standard immunohistochemical techniques. For all apart from the PDGF-AB antibody a standard streptavidin-biotin method was used, but vectastain enhancement was required for PDGF-AB. The techniques are provided in the Appendix. The antibodies were all sourced from

commercial companies to ensure that they were well established and likely to work satisfactorily. Western blot and reference data was provided in the datasheets to confirm specificity. The antibodies, sources, dilutions and pre-treatment regimens are detailed in Table 6. For all antibodies, suitable positive and negative controls were used either as recommended by the Company providing the antibody or from previous experience in the laboratory. In fact for the PDGF receptors, normal pituitary gland was used as we had found in an earlier study that there was an interesting pattern of distribution of the receptors in the normal and neoplastic pituitary gland (Fig 36). Furthermore, different antibodies for some antigens were sourced from different companies and the best antibody assessed after optimisation and used for this study. For confirmation of staining the appropriate positive control tissue was run alongside each test according to the manufacturer's recommendations. Negative controls were also performed routinely by omitting the primary antibody.

Antigen	Source	Antibody reference	Monoclonal or polyclonal (M/P)	Dilution	Pretreatment
PDGF-AA	Genzyme	ZP-214	P (rabbit)	1:100	Microwave
PDGF-AB	TCS	06-127	P (goat)	1:1000	Microwave
PDGF-BB	Genzyme	ZP215	P (rabbit)	1:100	Microwave
PDGFR alpha	Genzyme	1264-00	M (mouse)	1:25	Microwave
PDGFR beta	Genzyme	1263-00	M (mouse)	1:50	Microwave

Table 6. PDGF antibodies and their sources and dilutions

The distribution was determined by microscopic examination of the immunohistochemical slides, specifically looking at all areas of the bladder wall and surrounding tissues away from direct tumour involvement. The epithelium, mucosa, muscularis propria, superficial and deep vessels and connective tissue structures were all looked at. The results of the observations were recorded by two examiners, who had scored the first ten cases on a double headed microscope for consistency and in all cases where there was disagreement, a negotiated final result was determined by looking at the slides together.

PDGF and PDGFR expression was scored in a semi-quantitative manner with scores 0, +, ++ and +++ for no staining, weak staining, moderate staining and strong staining respectively. The results from the irradiated group were compared to non-irradiated control groups which consisted of cystectomies for transitional cell carcinomas in patients who had not received radiotherapy, bladders removed for intractable interstitial cystitis and autopsy bladder wall tissue. As the time between radiotherapy and cystectomy was known in all study cases, it was also possible to see if there were any differences in expression of PDGF/R over time since irradiation.

The statistics was analysed using the Statistical Package for the Social Sciences, version 16.0, 2008 (SPSS). The data was fed into the package and tabulated using the cross-tabulation facility. This allowed graphs to be created following which statistical analysis, using the Pearson Chi-squared test was performed. A cut-off level of 0.05 was used for significance as per routine protocol.

5.15.2 Results

PDGF isoforms and PDGF receptors were identified in all post-irradiation cases (Figs. 37-39). An overview of the results is provided here, with an example (Fig.40), but all of

the results are contained in Appendix 3. In summary, there was increased expression of PDGF and its receptors in many components of the bladder wall, including epithelium, superficial and deep endothelial cells, fibroblasts and smooth muscle cells. The results for the irradiated group as a whole were compared with the control groups. The most significant findings were expression of all proteins in the superficial endothelial cells and fibroblasts (mostly $p < 0.001$). PDGFR alpha was also significantly increased in the epithelium.

As a general rule expression appeared to increase in the early phase after radiation, peak within a year to 2 years and then decrease. There was persistent expression for many years in several of the cases.

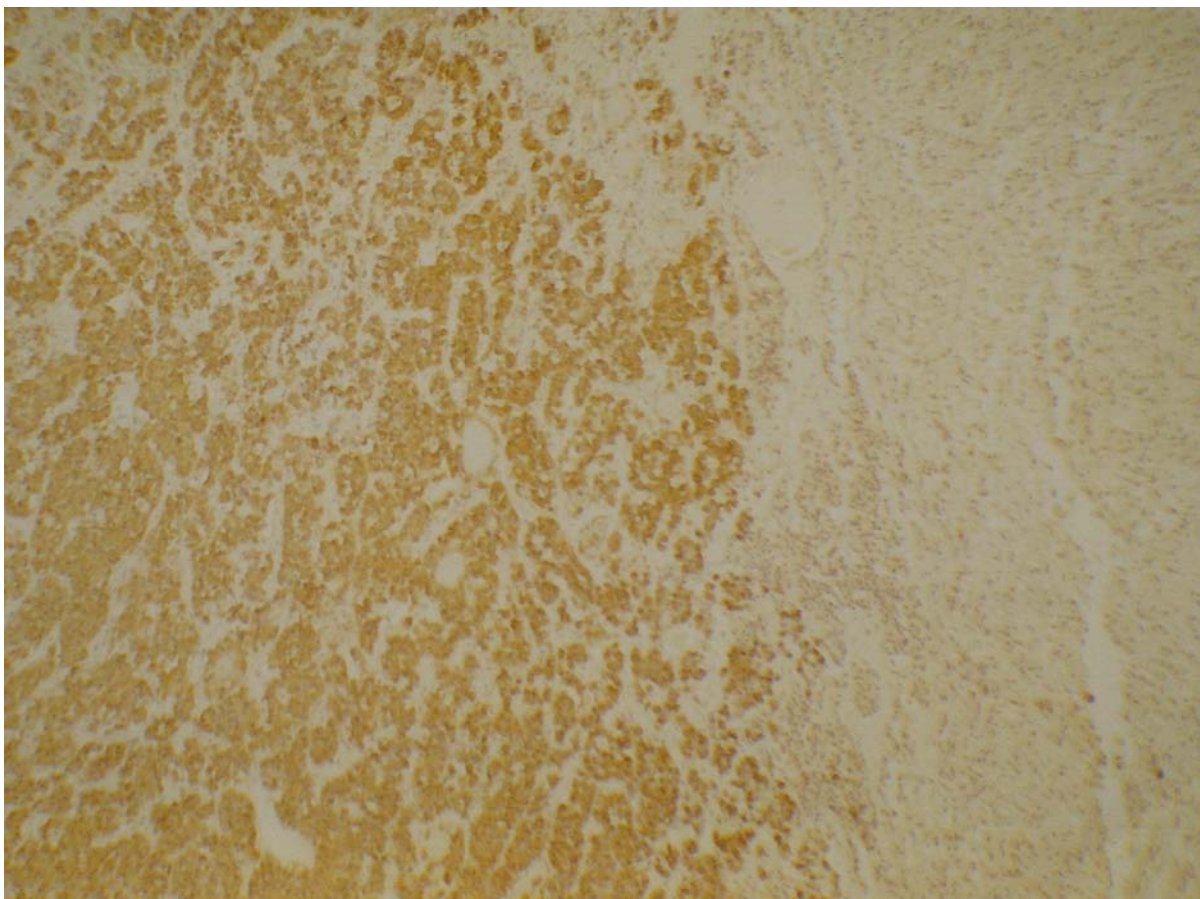


Figure 36. PDGFR alpha control tissue highlights the acidophil wings of the pituitary gland.

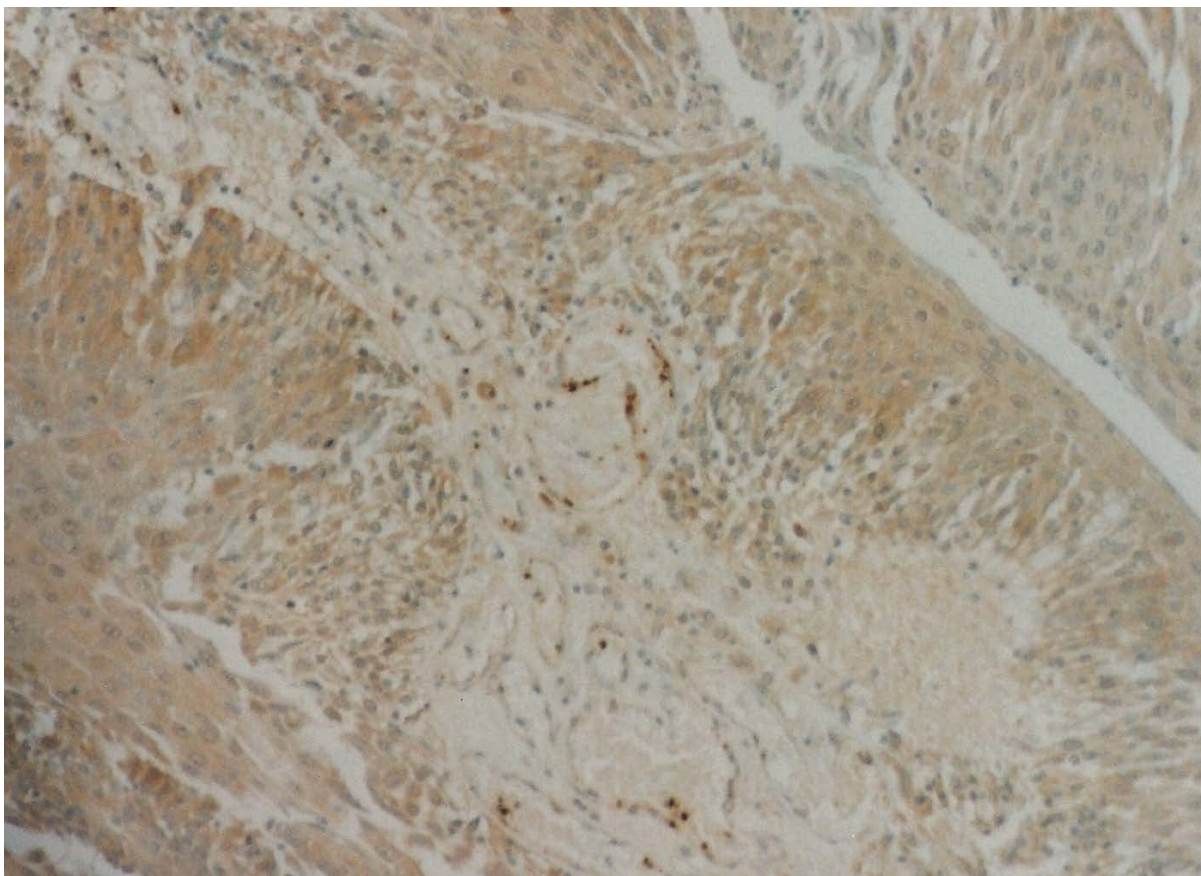


Figure 37. Light generalised epithelial positivity was seen after radiation. Note the positive internal control for platelets.

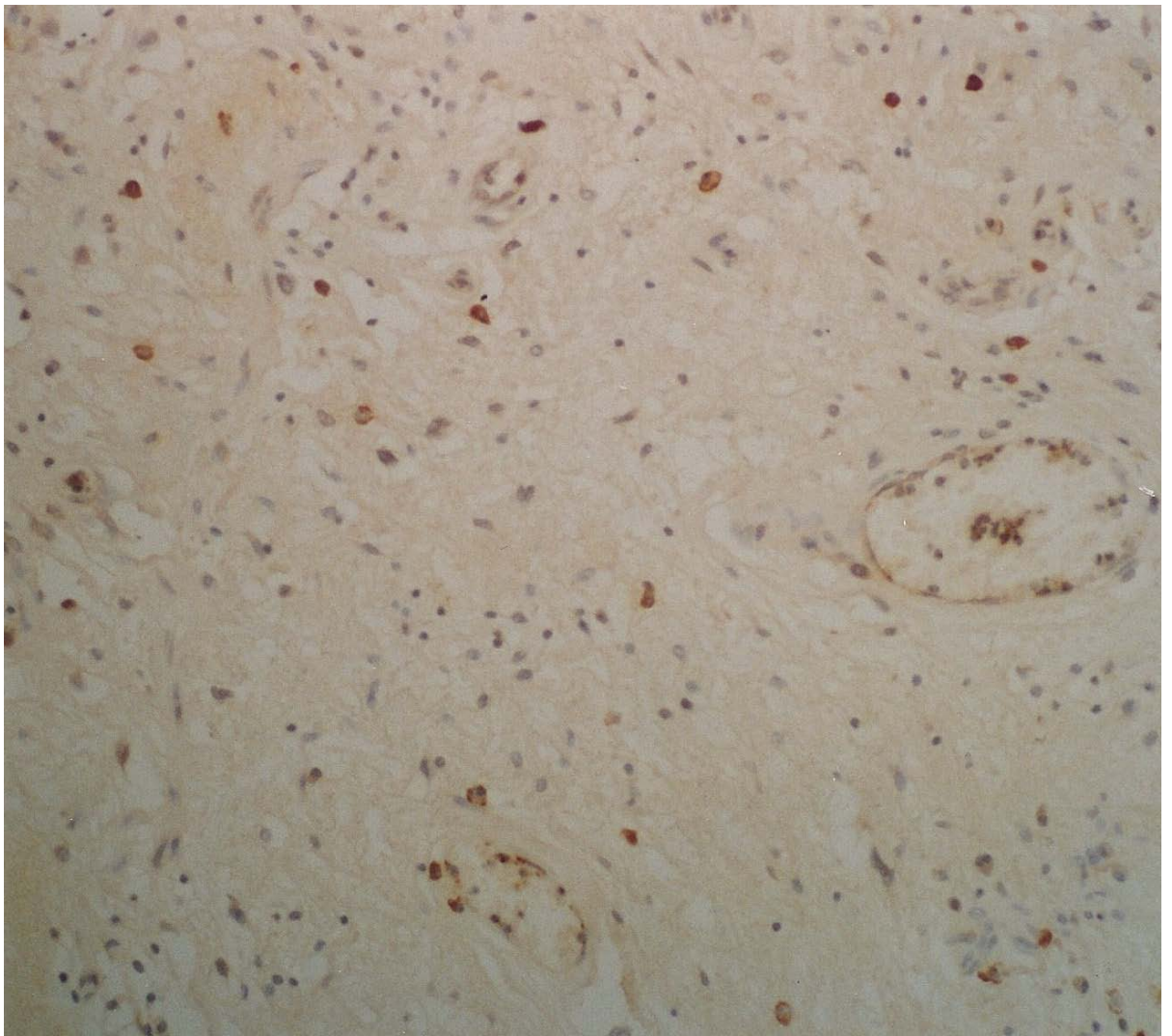


Figure 38. Mast cells cells expressed PDGF AB consistently (again note the internal positive control).

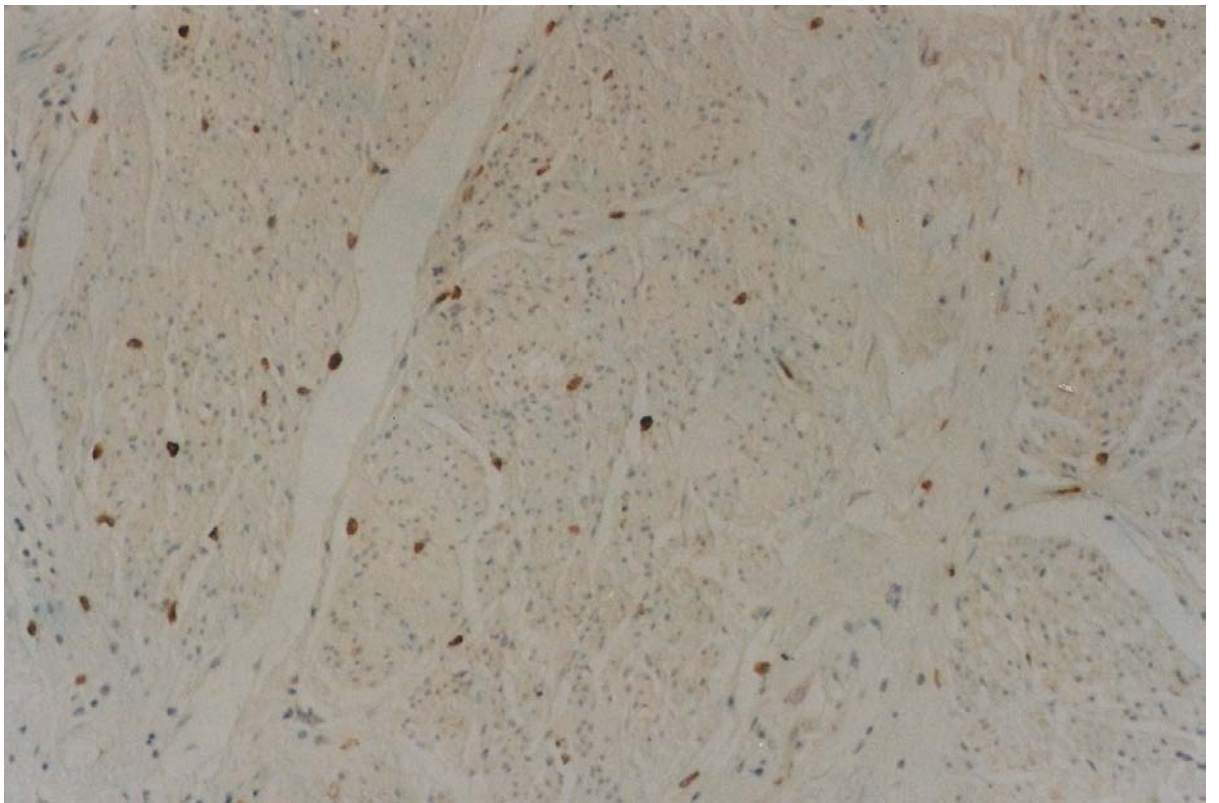


Figure 39. There was limited or no positivity for the smooth muscle with PDGF-AB. Mast cells here also positive.

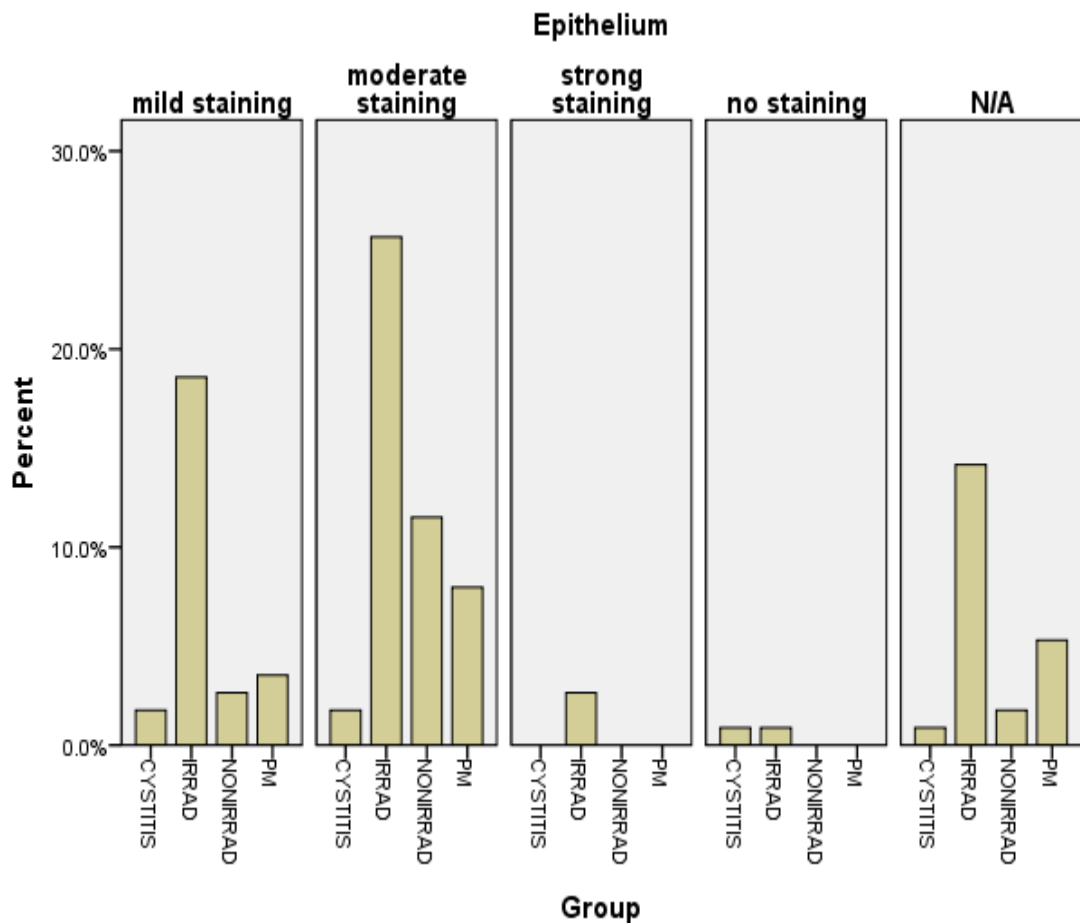


Figure 40. Intensity of staining for PDGF-AB in the test and control bladders (one example- see appendix for the remainder).

5.15.3 Discussion

Radiotherapy causes injury to local normal tissues as well as the tumour at which it is directed. One of the most troublesome side effects is fibrosis, which leads to several of the complications encountered after radiation therapy including pain and functional loss. This is especially prominent in the area of the lower urinary tract, although many organs caught in the radiation field show similar changes⁵⁷⁰. The main pathological feature of such radiation toxicity is transmural fibrosis consisting of marked deposition of extracellular matrix component within the all layers of the bladder wall. Around the microvessels, accumulation of inflammatory cells suggesting an increased vascular

permeability likely caused by endothelial cell damage. The muscle is thickened with zones of complete disruption. In the subepithelial zone, and deeper within the bladder wall, there are dense cords of collagen fibres with few fibroblasts whereas others are edematous or contained fibrosis-related fibroblasts and inflammatory cells located around hyalinized vessels with interlaced fibres. The nerves can also be involved with inflammatory and fibrotic changes⁵⁷¹. The serosa is often abnormal with variable fibrosis containing newly formed microvessels, myofibroblasts, inflammatory cells, and paucicellular zones composed of stromal accumulation.

The pathophysiological mechanisms of acute radiation injury have been well investigated together with the mechanisms underlying delayed radiation-induced complications although the precise sequence of cellular and molecular events that initiates fibrogenesis are not fully known. Classical radiobiological views present radiation-induced tissue injury as the direct consequence of DNA damages and cell death induction in target cells, meaning that the severity of tissue damages would be directly related to cell depletion during the acute phase. However, it is clear that other mechanisms are important since early injury is required for the development of delayed complications in the intestine^{572,573}. These observations led to the idea that increasing the pool of epithelial cells before irradiation might improve acute damage and inhibit the development of late injury⁵⁷⁴. The functional consequences of radiation-induced epithelial depletion are probably far beyond their barrier function. An indirect consequence of the epithelial disruption is the initiation of inflammation, particularly lymphocyte T helper (T_H) involvement. Subsequent production of specific cytokines, most notably the secretion of IFN γ , is associated with resolution of the wound healing process. On the other hand secretion of IL-4, IL-13 and TGF- β 1, triggers a tissue response towards fibrosis, probably mediated predominantly by TGF- β 1⁵⁷⁵⁻⁵⁷⁷. Exposure of intestinal stroma to bacteria can also induce a T_H1 response⁵⁷⁶ but in the lung

persistent exposure to bacterial antigens seems to prefer a T_H2 profile suggesting that chronic epithelial depletion is fibrosis-prone⁵⁷⁷.

However, delayed tissue response to radiation injury depends upon continuous and integrated pathogenic processes, involving cell differentiation and crosstalk between the various cellular components of the tissue and the extracellular matrix⁵⁷⁸. The role of the mesenchymal compartment (smooth muscle cells, submucosal fibroblasts, subepithelial myofibroblasts and the extracellular matrix) is crucial for the development and the maintenance of fibrosis as these cells are responsible of the pathological extracellular matrix accumulation observed in fibrosis.

Radiation-induced urinary tract fibrosis is characterized by the accumulation of extracellular matrix due to a global deregulation of the synthesis/degradation balance^{579,580}. Whether this dynamic remodelling process is a cause or a consequence of the phenotypic alteration of the resident mesenchymal cells is not known⁵⁸¹. After injury, tissue regeneration relies on the differentiation capacity of its resident cells. In the bladder, the mesenchymal compartment is composed of 3 cell types: the subepithelial myofibroblasts, the submucosal fibroblasts and the smooth muscle cells of the muscularis propria. The respective contribution of these three cell types to fibrosis is not clearly defined, but pathological collagen deposition seems mainly to be derived from smooth muscle cells⁵⁸².

In many ways, radiation fibrosis can be viewed as an exaggerated wound healing process. In general, after injury, loss of homeostasis induces mechanical tension in the local wound. This contractile stress added to the secretion of cytokines, such as PDGF, triggers fibroblast recruitment and their phenotypic change⁵⁸³ into proto-myofibroblasts⁵⁸⁴. Then, the mechanical tension associated with fibronectin and TGF- β 1 deposition induces the differentiation of proto-myofibroblasts into myofibroblasts (characterised by altered cytoskeletal filaments; α -smooth muscle actin, myosin,

tropomyosin, α -actinin and filamin). Myofibroblastic differentiation is an intermediate state between fibroblast and smooth muscle cell that ensures the contraction of granulation tissue and the neo-synthesis of the extracellular matrix. After injury, the differentiation of smooth muscle cells is characterized by a phenotypic switch from a contractile role to a secretory function⁵⁸⁵. The plasticity of smooth muscle cells allows significant alterations in their phenotype in response to changes in local environment^{585,586} and in return these differentiated smooth muscle cells controlled tissue response.

It is generally considered that mesenchymal cell differentiation in radiation-induced fibrosis as terminal and thus irreversible^{587,588}. This hypothesis is based on phenotypic characterization of fibroblasts irradiated *in vitro*, which exhibit a premature senescent and pro-secretory phenotype (extracellular matrix secretion). Thus, necrosis or apoptosis of these fibrosis-activated mesenchymal cells could conceivably avoid or reverse fibrosis^{589,590}. On the other hand, it appears that the cells promoting fibrosis after radiation are phenotypically immature and this immaturity has important clinical implications as it suggests that fibrotic tissue has high regenerative potential and that fibrosis might be reversible.

There are many cytokines and other factors that are known or thought to play a role in fibrosis in general and also radiation induced change. These have already been discussed in some detail in Chapter 1. These include TGF- β 1 and CCN2 (also called CTGF). Although most studies focus on these cytokines, it is certainly logical to turn our attention to PDGF and its possible role in radiation injury. PDGF has been implicated in many inflammatory and fibrosing conditions and it is a promoter of TGF. PDGF has also been found to protect against radiation injury *in vivo*. We have shown that PDGF is found more frequently in the bladder after irradiation. It was identified in several cell types including endothelial cells, stromal cells, epithelial cells and incoming

cells including inflammatory cells and platelets. It appears to be expressed at increasing levels over the first year or so, to decrease over the next year or two but continues to be present at relatively low levels after that.

The recognition of PDGF and its receptors using immunohistochemistry does not necessarily mean that there is a functional role for PDGF in radiation injury but the previous chapters have provided extensive background information regarding PDGF and the pathological processes involved in radiation injury and it seems extremely logical that PDGF is implicated in the sequence of events within tissues after irradiation.

The presence of PDGF in mast cells is particularly interesting. Mast cells are often found in conditions characterised by fibrosis such as scleroderma and mast cell proliferations are commonly associated with a degree of local fibrosis^{257,582}. It is attractive to speculate therefore that PDGF production by mast cells is an important feature of such diseases and a similar process might occur in radiation fibrosis. Whether mast cells express PDGF in the resting state, or whether PDGF is only produced after some type of induction process, is unclear. In any event, the mast cell is another potential therapeutic target for the prevention or treatment of radiation fibrosis.

As the PDGF/R family is a mixed group of similar proteins, it is very difficult to tease out which individual isoforms might be more or less involved in radiation injury. It is certainly likely that the PDGFs or the receptors might vary in their relevance to the radiation response. On the other hand, the various PDGF and receptors might be important at different points in the post-radiation cascade, each influencing a number of pathways stimulated after radiation. The first is inflammation and cell recruitment. The second is angiogenesis and wound healing response. The third is fibrosis. The fourth is subsequent malignant transformation. How these impact the various systems is very difficult to figure out. Furthermore, although they may be important, their role might be

indirect (such as via production of another factor such as TGF) or they may have an impact only in combination with any of the other factors involved in the radiation response. Nevertheless, the relevance of PDGF could be determined by using agents that act against PDGFs either by antagonism or inhibition, or receptor blockade. If this is found to be correct, it would also hint that manipulation of the PDGF/R family might provide another means of dampening or reversing the complications of irradiation in the bladder (and more generally). Of course this could apply equally to other conditions involving PDGF/R including post-surgical scarring, wound healing and atherosclerosis.

5.15.4 Conclusion

Radiation injury to the bladder is characterised by an early inflammatory response followed by reactive or atrophic epithelial changes and mural fibrosis. PDGF and receptors are present in many cells involved in the pathogenesis of the radiation response and these appear to be upregulated after radiation exposure. These processes include inflammation, healing, fibrosis, vasculopathic alterations and neoplasia. PDGF/R may therefore play a significant role either directly, indirectly or in combination with other factors in radiation injury to the bladder. Strategies aimed at PDGF family might be useful targets to interfere with the development of post-radiotherapy complications and enable manipulation of treatment regimens, or as a therapeutic agent for complications. If this is the case, it clearly has wider implications for the prevention and treatment of many other general fibrosing conditions which could be exploited clinically.

Future work

The overall conclusion of this study is that PDGF has a role in the effect of irradiation on the urinary tract. It is also likely that it is important in the response of tissues to radiation in general and post-radiation fibrosis in particular. The latter is a potentially severe complication of radiotherapy, which can limit its usefulness and lead to subsequent distressing symptoms. Our findings predict that targeted therapy against PDGFs or their receptors may potentially be beneficial to these patients. However, clearly it is essential to support the findings of this thesis with more sophisticated techniques, before making the above claims. Future work will be aimed at validating these results in order to justify the statements made and confirm the potential of studying PDGF as a target for preventing radiation injury for the future. The work envisioned will centre on studies such as western blotting, in situ hybridization and polymerase chain reaction, together with cell culture work, examining in vitro alterations in endothelial cells, epithelial cells and fibroblasts specifically and how they can be manipulated therapeutically for the potential benefit of the many thousands of patients whose lives may be devastated by a treatment aimed at ridding them of their cancer.

References

1. Mettler FA, Upton AC. The medical effects of ionizing radiation, 3rd edn. WB Saunders Elsevier, Philadelphia, 2008
2. Owen JB, Coia LR, Hanks GE. Recent patterns of growth in radiation therapy facilities in the United States: a patterns of care study report. *Int J Radiat Oncol Biol Phys* 1992;24:983-986
3. Withers HR. Biological basis of radiation therapy for cancer. *Lancet* 1992;339:156-159
4. Doll R. Hazards of ionizing radiation: 100 years of observation on man. *Br J Cancer* 1995;72:1339-1349
5. Kovalchuk O, Baulch JE. Epigenetic changes and nontargeted radiation effects – is there a link? *Environ Mol Mutagen* 2008;49:16-25
6. Dyer O. Study links low dose radiation and down's syndrome. *BMJ* 1995;310:1088-1089
7. Crompton NE. Telomeres, senescence and cellular radiation response. *Cell Mol Life Sci* 1997;53:568-575
8. Jones KR et al. P53-dependent accelerated senescence induced by ionizing radiation in breast tumour cells. *Int J Radiat Biol* 2005;81:445-458
9. Anderson RE, Berthrong M, Fajardo LF. Radiation injury. In: Damjanov I, Linder J (eds) *Anderson's Pathology*, 10th edn. Mosby 1996:484-512
10. Maity A, McKenna WG, Muschel RJ. The molecular basis for cell cycle delays following ionizing radiation: a review. *Radiother Oncol* 1994 ;31 :1-13
11. Villa R, Zaffaroni N, Bearzatto A, Costa A, Sichiolo A, Silvestrini R. Effects of ionizing radiation on cell-cycle progression and cyclin B1 expression in human melanoma cells. *Int J Cancer* 1996 ;66 :104-109
12. Lichter AS, Lawrence TS. Recent advances in radiation oncology. *N Engl J Med* 1995;332 :371-379

13. Sheaff M, Baithun S. Pathological effects of ionizing radiation. *Curr Diagn Pathol* 1997;4:106-115
14. Verheij M, Dewit LG, Boomgaard MN, Brinkman HJ, van Mourik JA. Ionizing radiation enhances platelet adhesion to the extracellular matrix of human endothelial cells by an increase in the release of von Willebrand factor. *Radiat Res* 1994;137 :202-207
15. Yan J, Khanna KK, Lavin MF. Induction of inositol 1,4,5 trisphosphate receptor genes by ionizing radiation. *Int J Radiat Biol* 1996;69 :539-546
16. Magic Z, Matic-Ivanovic S, Savic J, Poznanovic G. Ionizing radiation-induced expression of the genes associated with the acute response to injury in the rat. *Radiat Res* 1995 ;143 :187-193
17. Neta R, Okunieff P. Cytokine-induced radiation protection and sensitization. *Semin Radiat Oncol* 1996;6:306-320
18. Stone HB, Coleman CN, Anscher MS, McBride WH. Effects of radiation on normal tissue: consequences and mechanisms. *Lancet Oncol* 2003; 4:529-536
19. Emami B, Lyman J, Brown A et al. Tolerance of normal tissue to therapeutic irradiation. *Int J Radiat Oncol Biol Phys* 1991; 21:109–122
20. Bentzen SM. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nat Rev Cancer* 2006;6:702-713
21. Delanian S, Lefaix J-L. The radiation-induced fibroatrophic process: therapeutic perspectives via the antioxidant pathway. *Radiother Oncol* 2004;73:119-131
22. Berger ME, Christensen DM, Lowry PC, Jones OW, Wiley AI. Medical management of radiation injuries: current approaches. *Occ Med* 2006;56:162-172
23. Montgomery R, Shivdasani R. Prominin 1 (CD133) as an intestinal stem cell marker: promise and nuance. *Gastroenterology* 2009;136:2051-2054
24. Sheaff MT, Baithun SI. Effects of radiation on the normal prostate gland. *Histopathology* 1997;30:539-549
25. Fajardo LF, Berthrong M. Vascular lesions following irradiation. *Pathol Ann* 1988;23:297-330

26. Wright EG, Coates PJ. Untargeted effects of ionizing radiation: Implications for radiation pathology. *Mut Res* 2006;597:119-132
27. O'Sullivan B, Levin W. Late radiation-related fibrosis: pathogenesis, manifestations, and current management. *Sem Radiat Oncol* 2003;13:274-289
28. Brooks AL. Evidence for 'bystander effect' in vivo. *Hum Exp Toxicol* 2004;23:67-70
29. Dorr W, Bertmann S, Herrmann T. Radiation induced lung reactions in breast cancer therapy. Modulating factors and consequential effects. *Strahlenther Onkol* 2005;181: 567–573
30. Kisseleva T, Brenner DB. Mechanisms of fibrogenesis. *Exp Biol Med* 2008;233:109-122
31. Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. *N Engl J Med* 1994;331:1286–1292
32. Moussad EE, Brigstock DR. Connective tissue growth factor: what's in a name? *Mol Genet Metab* 2000;71, 276–292
33. Leask A, Abraham DJ. TGF signaling and the fibrotic response. *FASEB J* 2004;18, 816–827
34. Hatamochi A, Mori K, Ueki H. Role of cytokines in controlling connective tissue gene expression. *Arch Dermatol Res* 1994;287:115–121
35. Kim JH et al. Natural killer T (NKT) cells attenuate bleomycin-induced pulmonary fibrosis by producing interferon- γ . *Am J Pathol* 2005;167:1231–1241
36. Chen ES, Greenlee BM, Wills-Karp M, Moller DR. Attenuation of lung inflammation and fibrosis in interferon- γ deficient mice after intratracheal bleomycin. *Am J Respir Cell Mol Biol* 2001;24:545–555
37. Gurujeyalakshmi G, Giri SN. Molecular mechanisms of antifibrotic effect of interferon in bleomycin-mouse model of lung fibrosis: downregulation of TGF- β and procollagen I and III gene expression. *Exp Lung Res* 1995;21:791–808

38. Richter KK, Fink LM, Hughes BM et al. Differential effect of radiation on endothelial cell function in rectal cancer and normal rectum. *Am J Surg* 1998;176:642–647
39. Skwarchuk MW, Travis EL. Changes in histology and fibrogenic cytokines in irradiated colorectum of two murine strains. *Int J Radiat Oncol Biol Phys* 1998;42:169–178
40. Wang J, Zheng H, Sung CC et al. Cellular sources of transforming growth factor-beta isoforms in early and chronic radiation enteropathy. *Am J Pathol* 1998;153:1531–1540
41. Chen Y, Williams J, Ding I et al. Radiation pneumonitis and early circulatory cytokine markers. *Semin Radiat Oncol* 2002;12:26–33
42. Hallahan DE, Geng L, Shyr Y. Effects of intercellular adhesion molecule 1 (ICAM-1) null mutation on radiation-induced pulmonary fibrosis and respiratory insufficiency in mice. *J Natl Cancer Inst* 2002;94:733–741
43. Anscher MS, Kong FM, Andrews K et al. Plasma transforming growth factor beta1 as a predictor of radiation pneumonitis. *Int J Radiat Oncol Biol Phys* 1998;41: 1029–1035
44. Fu XL, Huang H, Bentel G et al. Predicting the risk of symptomatic radiation-induced lung injury using both the physical and biologic parameters V(30) and transforming growth factor beta. *Int J Radiat Oncol Biol Phys* 2001;50: 899–908
45. Epperly MW, Travis EL, Sikora C, Greenberger JS. Manganese superoxide dismutase (MnSOD) plasmid/liposome pulmonary radioprotective gene therapy: modulation of irradiation-induced mRNA for IL-1, TNF-alpha, and TGF-beta correlates with delay of organizing alveolitis/fibrosis. *Biol Blood Marrow Transplant* 1999;5: 204–214
46. Johnston CJ, Williams JP, Okunieff P, Finkelstein JN. Radiation-induced pulmonary fibrosis: examination of chemokine and chemokine receptor families. *Radiat Res* 2002;157: 256–265
47. Epperly M, Bray J, Kraeger S et al. Prevention of late effects of irradiation lung damage by manganese superoxide dismutase gene therapy. *Gene Ther* 1998;5:196–208

48. Vozenin-Brotons M-C, Sivan V, Gault N et al. Antifibrotic action of Cu/Zn SOD is mediated by TGF-beta 1 repression and phenotypic reversion of myofibroblasts. *Free Radical Biol Med* 2001;30:30–42
49. Moltenti A, Moulder JE, Cohen EF et al. Control of radiation-induced pneumopathy and lung fibrosis by angiotensin-converting enzyme inhibitors and an angiotensin II type 1 receptor blocker. *Int J Radiat Biol* 2000;76:523–532
50. Wang LW, Fu XL, Clough R et al. Can angiotensin-converting enzyme inhibitors protect against symptomatic radiation pneumonitis? *Radiat Res* 2000;153:405–410
51. Martin M, Lefaix J, Delanian S. TGF-1 and radiation fibrosis: a master switch and a specific therapeutic target? *Int J Radiat Oncol Biol Phys* 2000;47:277–290
52. Dumont N, Arteaga CL. Targeting the TGF signaling network in human neoplasia. *Cancer Cell* 2003;3:531–536
53. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF- β in homeostasis and cancer. *Nature Rev Cancer* 2003;3:807–821
54. Reiss M, Barcellos-Hoff MH. Transforming growth factor- β in breast cancer: a working hypothesis. *Breast Cancer Res Treat* 1997;45:81–95
55. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massague J. Transforming growth factor β signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc Natl Acad Sci USA* 2003;100: 8430–8435
56. Wakefield LM, Roberts AB. TGF- β signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12: 22–29
57. Lawrence DA. Latent-TGF- β : an overview. *Mol Cell Biochem* 2001;219:163–170
58. Ewan KB et al. Transforming growth factor-1 mediates cellular response to DNA damage in situ. *Cancer Res* 2002;62: 5627–5631
59. Ehrhart EJ, Segarini P, Tsang ML, Carroll AG, Barcellos-Hoff MH. Latent transforming growth factor 1 activation in situ: quantitative and functional evidence after low-dose gamma-irradiation. *FASEB J* 1997;11:991–1002
60. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF- β receptor. *Nature* 1994;370:341–347

61. Attisano L, Wrangé JL. Signal transduction by the TGF- β superfamily. *Science* 2002;296:1646–1647
62. Bayreuther K et al. Human skin fibroblasts in vitro differentiate along a terminal cell lineage. *Proc Natl Acad Sci USA* 1988;85:5112–5116
63. Herskind C, Rodemann HP. Spontaneous and radiation-induced differentiation of fibroblasts. *Exp Gerontol* 2000; 5:747–755
64. Martin GM, Sprague CA, Norwood TH, Pendergrass WR. Clonal selection, attenuation and differentiation in an in vitro model of hyperplasia. *Am J Pathol* 1974;74:137–154
65. Rodemann HP, Peterson HP, Schwenke K, von Wangenheim KH. Terminal differentiation of human fibroblasts is induced by radiation. *Scanning Microsc* 1991;5:1135–1142
66. Herskind C et al. Differentiation state of skin fibroblast cultures versus risk of subcutaneous fibrosis after radiotherapy. *Radiother Oncol* 1998;47:263–269
67. Russell NS et al. In vitro differentiation characteristics of human skin fibroblasts: correlations with radiotherapy-induced breast fibrosis in patients. *Int J Radiat Biol* 2000;76: 231–240
68. Kalluri R, Neilson EG. Epithelial–mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003;112:1776–1784
69. Iwano M et al. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002;110:341–350
70. Roberts AB et al. Smad3 is key to TGF-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine Growth Factor Rev* 2006;17:19–27
71. Abe S et al. Cells derived from the circulation contribute to the repair of lung injury. *Am J Respir Crit Care Med* 2004;170:1158–1163
72. Epperly MW, Guo H, Gretton JE, Greenberger JS. Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2003;29:213–224

73. Francois S et al. Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* 2006;24:1020–1029
74. Gervaz P, Philippe M, Vozenin-Brotons M-C. Molecular aspects of intestinal radiation-induced fibrosis. *Curr Mol Med* 2009;9:273-280
75. Vujaskovic Z et al. Radiation-induced hypoxia may perpetuate late normal tissue injury. *Int J Radiat Oncol Biol Phys* 2001;50:851–855
76. Nangaku M. Hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *Nephron Exp Nephrol* 2004;98:e8–e12
77. Urquhart DS, Montgomery H, Jaffe A. Assessment of hypoxia in children with cystic fibrosis. *Arch Dis Child* 2005;90:1138–1143
78. Siegmund SV, Brenner DA. Molecular pathogenesis of alcohol-induced hepatic fibrosis. *Alcohol Clin Exp Res* 2005;29:102S–109S
79. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 2003;22:5734–5754
80. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 2000;29: 222–230
81. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med* 2003;167: 1600–1619
82. Vaziri ND. Oxidative stress in uremia: nature, mechanisms, and potential consequences. *Semin Nephrol* 2004;24:469–473
83. Fubini B, Hubbard A. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. *Free Radic Biol Med* 2003;34:1507–1516
84. Duprez DA. Role of the renin-angiotensin-aldosterone system in vascular remodeling and inflammation: a clinical review. *J Hypertens* 2006;24:983–991
85. Weir MR, Dzau VJ. The renin-angiotensin-aldosterone system: a specific target for hypertension management. *Am J Hypertens* 1999;12:205S–213S

86. Kisseleva T, Brenner DA. Fibrogenesis of parenchymal organs. *Proc Am Thorac Soc* 2008;5:338-342
87. Robbins ME, Diz DI. Pathogenic role of the renin-angiotensin system in modulating radiation-induced late effects. *Int J Radiat Oncol Biol Phys* 2006;64:6–12
88. Dent P, Yacoub A, Contessa J et al. Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res* 2003;159:283-300
89. Rubin P, Johnston CJ, Williams JP et al. A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. *Int J Radiat Oncol Biol Phys* 1995;33: 99–109
90. Madani I, De Ruyck K, Goeminne H, De Neve W, Thierens H, Van Meerbeeck J. Predicting risk of radiation-induced lung injury. *J Thoracic Oncol* 2007;2:864-874
91. Paris F, Fuks Z, Kang A et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* 2001;293:293–297
92. Hauer-Jensen M, Kong FM, Fink LM, Anscher MS. Circulating thrombomodulin during radiation therapy of lung cancer, *Radiat Oncol Investig* 1999;7: 238–242
93. Eifel PJ, Donaldson SS, Thomas PR. Response of growing bone to irradiation: a proposed late effects scoring system. *Int J Radiat Oncol Biol Phys* 1995;31:1301–1307
94. Johansson S, Svensson H, Denekamp J. Timescale of evolution of late radiation injury after postoperative radiotherapy of breast cancer patients. *Int J Radiat Oncol Biol Phys* 2000;48:745–750
95. Barcellos-Hoff MH. How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. *Radiat Res* 1998;150:109–120
96. Hopewell JW. The importance of vascular damage in the development of late radiation effects in normal tissues. In: Meyn RE and Withers HR (eds). *Radiation biology in cancer research*. Raven Press, New York, 1980; pp 449–459
97. Fajardo LF. Morphology of radiation effects on normal tissues. In: Perez CA and Brady LW(eds). *Principles and practice of radiation oncology* (2nd edn). JB Lippincott Company, Philadelphia 1992; pp 114–123

98. Hopewell JW, Young CM. Changes in the microcirculation of normal tissues after irradiation, *Int J Radiat Oncol Biol Phys* 1978;4:53–58
99. Withers HR, Peters LJ, Kogelnik HD. The pathobiology of late effects of irradiation. In: Meyn RE and Withers HR (eds). *Radiation biology in cancer research*. Raven Press, New York 1980; pp 439–448
100. Vujaskovic Z, Anscher MS, Feng Q et al. Radiation-induced hypoxia may perpetuate late normal tissue injury. *Int J Radiat Oncol Biol Phys* 2001;50:851–855
101. Craanen ME, van Triest B, Verheijen RHM, Mulder CJJ. Thalidomide in refractory haemorrhagic radiation induced proctitis. *Gut* 2006;55:1371-1372
102. Pinar B, Lara PC, Lloret M et al. Radiation-induced DNA damage as a predictor of long-term toxicity in locally advanced breast cancer patients treated with high-dose hyperfractionated radical radiotherapy. *Radiat Res* 2007;168:415-422
103. Allan JM, Travis LB. Mechanisms of therapy-related carcinogenesis. *Nat Rev Cancer* 2005;12:943-955
104. www.cancerbackup.org.uk
105. Improving outcomes in Urological cancers. NICE, 2002
106. Gatti RA. The inherited basis of human radiosensitivity. *Acta Oncol* 2001;40: 702–711
107. Taylor AM et al. Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity. *Nature* 1975;258:427–429
108. Abadir R, Hakami N. Ataxia telangiectasia with cancer. An indication for reduced radiotherapy and chemotherapy doses. *Br J Radiol* 1983;56:343–345
109. Hart RM, Kimler BF, Evans RG, Park CH. Radiotherapeutic management of medulloblastoma in a pediatric patient with ataxia telangiectasis. *Int J Radiat Oncol Biol Phys* 1987;13:1237–1240
110. Tamminga RY, Dolsma WV, Leeuw JA, Kampinga HH. Chemo- and radiosensitivity testing in a patient with ataxia telangiectasia and Hodgkin disease. *Pediatr Hematol Oncol* 2002;19:163–171

111. Appleby JM et al. Absence of mutations in the ATM gene in breast cancer patients with severe responses to radiotherapy. *Br J Cancer* 1997;76:1546–1549
112. Ramsay J, Birrell G, Lavin M. Testing for mutations of the ataxia telangiectasia gene in radiosensitive breast cancer patients. *Radiother Oncol* 1998;47:125–128
113. Shayeghi M et al. Heterozygosity for mutations in the ataxia telangiectasia gene is not a major cause of radiotherapy complications in breast cancer patients. *Br J Cancer* 1998;78: 922–927
114. Cortez D, Wang Y, Qin J, Elledge SJ. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* 1999;286:1162–1166
115. Wang HC, Chou WC, Shieh SY, Shen CY. Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining. *Cancer Res* 2006;66:1391–1400
116. Andreassen CN. Can risk of radiotherapy-induced normal tissue complications be predicted from genetic profiles? *Acta Oncol* 2005;44: 801–815
117. Bahlo M et al. Detecting genome wide haplotype sharing using SNP or microsatellite haplotype data. *Hum Genet* 2006;119:38–50
118. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nature Rev Genet* 2005;6:95–108
119. Wang WY, Barratt BJ, Clayton DG, Todd J A. Genome-wide association studies: theoretical and practical concerns. *Nature Rev Genet* 2005;6:109–118
120. Andreassen CN, Alsner J, Overgaard M, Overgaard J. Prediction of normal tissue radiosensitivity from polymorphisms in candidate genes. *Radiother Oncol* 2003;69:127–135
121. Chang-Claude J et al. Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and acute side effects of radiotherapy in breast cancer patients. *Clin Cancer Res* 2005;11:4802–4809

122. Rodningen OK, Overgaard J, Alsner J, Hastie T, Borresen-Dale AL. Microarray analysis of the transcriptional response to single or multiple doses of ionizing radiation in human subcutaneous fibroblasts. *Radiother Oncol* 2005;77:231–240
123. Kruse JJ, te Poele JA, Russell NS, Boersma LJ, Stewart FA. Microarray analysis to identify molecular mechanisms of radiation-induced microvascular damage in normal tissues. *Int J Radiat Oncol Biol Phys* 2004;58:420–426
124. Snyder AR, Morgan WF. Lack of consensus gene expression changes associated with radiation-induced chromosomal instability. *DNA Repair (Amst)* 2005;4:958–970
125. Morgan GW, Breit SN. Radiation and the lung: a re-evaluation of the mechanisms mediating pulmonary injury. *Int J Radiat Oncol Biol Phys* 1995;31:361–369
126. Fajardo LF. Pathology of radiation injury. Masson, New York, 1982
127. McDonald S, Rubin P, Phillips TL, Marks LB. Injury to the lung from cancer therapy: clinical syndromes, measurable endpoints, and potential scoring systems. *Int J Radiat Oncol Biol Phys* 1995;31:1187–1203
128. Anscher MS, Marks LB, Shafman TD et al. Using plasma transforming growth factor beta-1 during radiotherapy to select patients for dose escalation. *J Clin Oncol* 2001;19:3758–3765
129. Boyer AL, Butler EB, DiPetrillo TA et al. Intensity-modulated radiotherapy: current status and issues of interest. *Int J Radiat Oncol Biol Phys* 2001;51: 880–914
130. Ford EC, Mageras GS, Yorke E et al. Evaluation of respiratory movement during gated radiotherapy using film and electronic portal imaging. *Int J Radiat Oncol Biol Phys* 2002;52:522–531
131. Archambeau JO, Pezner R, Wasserman T. Pathophysiology of irradiated skin and breast. *Int J Radiat Oncol Biol Phys* 1995;31:1171–1185
132. Dorr W, Hamilton CS, Boyd T et al. Radiation-induced changes in cellularity and proliferation in human oral mucosa. *Int J Radiat Oncol Biol Phys* 2002;52:911–917
133. Epstein JB, Gorsky M, Guglietta A et al. The correlation between epidermal growth factor levels in saliva and the severity of oral mucositis during oropharyngeal radiation therapy. *Cancer* 2000;89:2258–2265

134. Stephens LC, Schultheiss TE, Price RE et al. Radiation apoptosis of serous acinar cells of salivary and lacrimal glands. *Cancer* 1991;67:1539–1543
135. Cooper JS, Fu K, Marks J, Silverman S. Late effects of radiation therapy in the head and neck region. *Int J Radiat Oncol Biol Phys* 1995;31:1141–1164
136. Taylor SE, Miller EG. Preemptive pharmacologic intervention in radiation-induced salivary dysfunction. *Proc Soc Exp Biol Med* 1999;221:14–26
137. Bras J, de Jonge HK, van Merkesteyn JP. Osteoradionecrosis of the mandible: pathogenesis. *Am J Otolaryngol* 1990;11:244–250
138. Brizel DM, Wasserman TH, Henke M et al. Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. *J Clin Oncol* 2000;18:3339–3345
139. O'Brien PC. Radiation injury of the rectum. *Radiother Oncol* 2001;60:1–14
140. Coia LR, Myerson RJ, Tepper JE. Late effects of radiation therapy on the gastrointestinal tract. *Int J Radiat Oncol Biol Phys* 1995;31:1213–1236
141. Hanks GE, Hanlon AL, Epstein B, Horwitz EM. Dose response in prostate cancer with 8-12 years' follow-up. *Int J Radiat Oncol Biol Phys* 2002;54: 427–435
142. Jackson A, Skwarchuk MW, Zelefsky MJ et al. Late rectal bleeding after conformal radiotherapy of prostate cancer. II. Volume effects and dose-volume histograms. *Int J Radiat Oncol Biol Phys* 2001;49:685–698
143. Valk PE, Dillon WP. Radiation injury to the brain. *AJNR Am J Neuroradiol* 1991;12:45-62
144. Burger PC, Scheithauer BW. Tumors of the central nervous system. Atlas of tumor pathology, fascicle 10. AFIP, Washington DC, 1994
145. Powers BE, Beck ER, Gillette EL, Gould DH, LeCouter RA. Pathology of radiation injury to the canine spinal cord. *Int J Radiat Oncol Biol Phys* 1992;23:539-549
146. Veinot PJ, Edwards WD. Pathology of radiation-induced heart disease. *Hum Pathol* 1996;27:766-773

147. Wenig B. Necrotizing sialometaplasia of the larynx. *Am J Clin Pathol* 1995;103:609-613
148. Hemar P, Kennel P, Piller P, Herman D, Methlin A, Conraux C. Lingual necrosis after neck irradiation (French). *Ann Otolaryngol Chir Cervicofac* 1993 ;110 :351-354
149. Oya M, Yao T, Tsuneyoshi M. Chronic irradiation enteritis. *Hum Pathol* 1996 ;27 :774-781
150. Sedgwick DM, Howard GC, Ferguson A. Pathogenesis of acute radiation injury to the rectum. A prospective study in patients. *Int J Colorectal Dis* 1994;9:23-30
151. Friebe A. Demonstration eines Cancroids des rechten Handrücken, das sich nach langdauernder Einwirkung von Röntgenstrahlen entwickelt hatte. *Fortshr Geb Roengenstr* 1902;6:106
152. Shore RE, Albert RE, Parternach BS. Follow-up study of patients treated by X-ray epilation for tinea capitis: re-survey of post-treatment illness and mortality experience. *Arch Environ Health* 1976;31:17-28
153. Jagic N von, Schwartz G, Siebenrock L. Blutbefunds bei Röntgenologen. *Berl Klin Wochenshr* 1911;48:1220-1222
154. Perrier M, Moeller P. Osteoradionecrosis. A review of the literature. *Schweiz Monatsschr Zahnmed* 1994;104:271-277
155. Enzinger FM, Weiss SW. *Soft tissue tumors*, 5th edn. Mosby 2008
156. Servodidio CA, Abramsom DH. Acute and long-term effects of radiation therapy to the eye in children. *Cancer Nurs* 1993;16 :371-381
157. Adler M, Hawke M, Beregr G, Harwood A. Radiation effects on the external auditory canal. *J Otolaryngol* 1985 ;14 :226-232
158. Estrada J, Boronet M, Mieglo et al. The long-term outcome of pituitary irradiation after unsuccessful trans-sphenoidal surgery in Cushing's disease. *N Engl J Med* 1997 ;336 :172-177
159. Tezelman S, Rodriguez JM, Shen W, Shipperstein AE, Duh QY, Clark OH. Primary hyperparathyroidism in patients who have received radiation therapy and in patients who have not received radiation therapy. *J Am Coll Surg* 1995;180:81-87

160. Ackerman's Surgical Pathology, 9th edn, Mosby 2004 (Rosai J. Thyroid gland)
161. Schnitt SJ, Connolly JL, Harris JR, Cohen RB. Radiation-induced changes in the breast. *Hum Pathol* 1984;15:545-550
162. Tokunaga M, Land CE, Aoki T et al. Proliferative and non-proliferative breast disease in atomic bomb survivors. Results of a histopathologic review of autopsy breast tissue. *Cancer* 1993;72:1657-1665
163. Shield PW. Chronic radiation effects: a correlative study of smears and biopsies from the cervix and vagina. *Diagn Cytopathol* 1995;13:107-119
164. Heptinstall RW. Pathology of the kidney, 6th edn. Little, Brown and Company, Boston 2007
165. Sheaff M, Badenoch D, Baithun SI. Radiation-induced changes in thirty-nine cystectomy specimens. *J Pathol* 1995;175:134A
166. Peterse JL, Thunnissen FB, van Heerde P. Fine needle aspiration cytology of radiation-induced changes in non neoplastic breast lesions. Possible pitfalls in diagnosis. *Acta Cytol* 1989;33:176-180
167. Wojno KJ, Olson JL, Sherman ME. Cytopathology of pleural effusions after radiotherapy. *Acta Cytol* 1994;38:1-8
168. Shield PW, Daunter B, Wright RG. Post-irradiation cytology of cervical cancer patients. *Cytopathology* 1992;3:167-182
169. Trotti A, Byhardt R, Stetz J et al. Common toxicity criteria: version 2.0. An improved reference for grading the acute effects of cancer treatment: impact on radiotherapy. *Int J Radiat Oncol Biol Phys* 2000;47:13-47
170. LENT SOMA scales for all anatomic sites. *Int J Radiat Oncol Biol Phys* 1995;31:1049-1091
171. LENT SOMA tables. *Radiother Oncol* 1995;35:17-60
172. The Nation's progress in cancer research. National Cancer Institute Annual Report, NIH, 2004

173. Bentzen SM, Overgaard M. *Advances in Radiation Biology*, volume 18. Altman KI, Lett JT (eds) Academic Press, San Diego, 1994; pp 25-51
174. Hawkins MM. Long-term survivors of childhood cancers: what knowledge have we gained? *Nat Clin Pract Oncol* 2004;1:26-31
175. Yabroff KR, Lawrence WF, Clauser S, Davis WW, Brown ML. Burden of illness in cancer survivors: findings from a population-based national sample. *J Natl Cancer Inst* 2004;96:1322-1330
176. Moran JM, Elshaikh MA, Lawrence TS. Radiotherapy: what can be achieved by technical improvements in dose delivery? *Lancet Oncol* 2005; 6:51–58
177. Bentzen SM. Radiation therapy: intensity modulated, image guided, biologically optimized and evidence based. *Radiother Oncol* 2005;77:227–230
178. Brizel DM et al. Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. *J Clin Oncol* 2000;18:3339–3345
179. Lindegaard JC, Grau C. Has the outlook improved for amifostine as a clinical radioprotector? *Radiother Oncol* 2000;57:113–118
180. Delanian S et al. Successful treatment of radiation-induced fibrosis using liposomal Cu/Zn superoxide-dismutase-clinical-trial. *Radiother Oncol* 1994;32:12–20
181. Stone HB, McBride WH, Coleman CN. Modifying normal tissue damage postirradiation. Report of a workshop sponsored by the Radiation Research Program, National Cancer Institute, Bethesda, Maryland, September 6–8, 2000. *Radiat Res* 2002;157:204–223
182. Anscher MS et al. Recent progress in defining mechanisms and potential targets for prevention of normal tissue injury after radiation therapy. *Int J Radiat Oncol Biol Phys* 2005;62:255–259
183. Iyer S, Wang ZG, Akhtari M, Zhao W, Seth P. Targeting TGF signaling for cancer therapy. *Cancer Biol Ther* 2005;4:261–266
184. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF- β signalling inhibitors for cancer therapy. *Nature Rev Drug Discov* 2004;3:1011–1022

185. Giri SN, Hyde DM, Hollinger M. A. Effect of antibody to transforming growth factor on bleomycin induced accumulation of lung collagen in mice. *Thorax* 1993;48: 959–966
186. Rabbani ZN et al. Soluble TGF type II receptor gene therapy ameliorates acute radiation-induced pulmonary injury in rats. *Int J Radiat Oncol Biol Phys* 2003;57:563–572
187. Wang Q, Hyde DM, Gotwals PJ, Giri SN. Effects of delayed treatment with transforming growth factor-beta soluble receptor in a three-dose bleomycin model of lung fibrosis in hamsters. *Exp Lung Res* 2002;28:405–417
188. Roberts AB et al. Is Smad3 a major player in signal transduction pathways leading to fibrogenesis? *Chest* 2001;120:43S–47S
189. Ishida W et al. Intracellular TGF- receptor blockade abrogates Smad-dependent fibroblast activation in vitro and in vivo. *J Invest Dermatol* 2006;126:1733–1744
190. Xavier S et al. Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor- signaling by halofuginone. *J Biol Chem* 2004;279:15167–15176
191. Prosser CC, Yen RD, Wu J. Molecular therapy for hepatic injury and fibrosis: where are we? *World J Gastroenterol* 2006;12:509–515
192. Epperly MW et al. Intratracheal injection of adenovirus containing the human SOD transgene protects athymic nude mice from irradiation-induced organizing alveolitis. *Int J Radiat Oncol Biol Phys* 1999;43:169–181
193. Giri SN, Biring I, Nguyen T, Wang Q, Hyde DM. Abrogation of bleomycin-induced lung fibrosis by nitric oxide synthase inhibitor, aminoguanidine in mice. *Nitric Oxide* 2002;7:109–118
194. Gurujeyalakshmi G, Wang Y, Giri SN. Suppression of bleomycin-induced nitric oxide production in mice by taurine and niacin. *Nitric Oxide* 2000;4:399–411
195. Delanian S, Porcher R, Rudant J, Lefaix JL. Kinetics of response to long-term treatment combining pentoxifylline and tocopherol in patients with superficial radiation-induced fibrosis. *J Clin Oncol* 2005;23:8570–8579

196. Delanian S, Porcher R, Balla-Mekias S, Lefaix JL. Randomized, placebo-controlled trial of combined pentoxifylline and tocopherol for regression of superficial radiation-induced fibrosis. *J Clin Oncol* 2003;21:2545–2550
197. Ha H, Lee HB. Reactive oxygen species and matrix remodeling in diabetic kidney. *J Am Soc Nephrol* 2003;14:S246–S249
198. Moulder JE. Report on an interagency workshop on the radiobiology of nuclear terrorism. *Radiat Res* 2002;158:118–124
199. Hammerick KH, Ayala GE, Wheeler TM. Anatomy of the prostate gland and surgical pathology of prostate cancer. In: Prostate cancer. Hricak H, Scardino PT (eds), Cambridge University Press, 2009
200. Cancer facts and figures 2007. American Cancer Society, NCI
201. Mortality statistics: Cause 2007, 2008. Office for National Statistics, 2009
202. Cheng L, Cheville JC, Bostwick DG. Diagnosis of prostate cancer in needle biopsies after radiation therapy. *Am J Surg Pathol* 1999;6:541-551
203. Cheng L, Cheville JC, Pisansky TM et al. Prevalence and distribution of prostatic intraepithelial neoplasia in salvage radical prostatectomy specimens after radiation therapy. *Am J Surg Pathol* 1999;23:803-808
204. Arakawa A, Song S, Scardino PT et al. High grade intraepithelial neoplasia in prostates removed following irradiation failure in the treatment of prostatic adenocarcinoma. *Pathol Res Pract* 1995;191:868-872
205. Crook JM, Bahadur YA, Robertson SJ et al. Evaluation of radiation effect, tumor differentiation, and prostate specific antigen staining in sequential prostate biopsies after external beam radiotherapy for patients with prostate carcinoma. *Cancer* 1997;79:81-89
206. Goldstein NS, Martinez A, Vincini F et al. The histology of radiation therapy effect on prostate adenocarcinoma as assessed by needle biopsy after brachytherapy boost: correlation with biochemical failure. *Am J Clin Pathol* 1998;110:765-775
207. Siders DB, Lee F. Histologic changes of irradiated prostatic carcinoma diagnosed by transrectal ultrasound. *Hum Pathol* 1992;147:900-902

208. Bostwick DG, Egbert BM, Fajardo LF. Radiation injury of the normal and neoplastic prostate gland. *Am J Surg Pathol* 1982;6:541-551
209. <http://training.seer.cancer.gov/prostate/anatomy>
210. Boyer CW Jr, Brwon VC, DeMare PA, Huynh TV, Yamashiro CH. Radiation therapy for carcinoma of the prostate gland: experience at a community hospital. *Hawaii Med J* 1990;49:185-194
211. Bittner N, Merrick GS, Wallner KE, Butler WM. Interstitial brachytherapy should be standard of care for treatment of high-risk prostate cancer. *Oncol (Wiliston Park)* 2008;22:995-1004
212. Ganswindt U, Stenzl A, Bamberg M, Belka C. Adjuvant radiotherapy for patients with locally advanced prostate cancer – a new standard ? *Eur Urol* 2008 ;54 :528-542
213. Eichler TJ. Radiotherapy for localized prostate cancer. *Can J Urol* 2007;14(S1):19-23
214. Nguyen PL, Zeitmen AL. High-dose external beam radiation for localized prostate cancer: current status and future challenges. *Cancer J* 2007;13:295-301
215. Schwarz R. Curative radiotherapy of localized prostate cancer. Treatment methods and results. *Urologe A* 2003;42:1212-1220
216. Fitzpatrick JM, Anderson J, Sternberg CN et al. Optimizing treatment for men with advanced prostate cancer: expert recommendations and the multidisciplinary approach. *Crit Rev Oncol Hematol* 2008;68(S1):9-22
217. Gaudin PB, Zelefsky MJ, Leibel SA et al. Histopathologic effects of three-dimensional conformal external beam radiation therapy on benign and malignant prostate tissue. *Am J Surg Pathol* 1999;23:1021-1031
218. Nilsson S, Norlen BJ, Widmark A. A systematic overview of radiation therapy effects in prostate cancer. *Acta Oncol* 2004;43:316-381
219. Bostwick DG, Meiers I. Diagnosis of prostatic carcinoma after therapy. *Arch Pathol Lab Med* 2007;131:360-371

220. Roznovanu SL, Radulescu D, Novac C, Stolnicu S. The morphologic changes induced by hormone and radiation therapy on prostate carcinoma. *Rev Med Chir Soc Med Nat Iasi* 2005;109:337-342
221. Magi-Galluzzi C, Sanderson H, Epstein JI. Atypia in nonneoplastic prostate glands after radiotherapy for prostate cancer. *Am J Surg Pathol* 2003;27:206-212
222. Asai A, Matsutani M, Kohno T et al. Subacute brain atrophy after radiation therapy for malignant brain tumor. *Cancer* 1989;63:1962-1974
223. Schultz-Hecotr S. Radiation-induced heart disease: review of experimental data on dose response and pathogenesis. *Radiat Biol* 1992;61:149-160
224. Eldor A, Fuks Z, Matzner Y, Witte LD, Vlodaysky I. Perturbation of endothelial functions by ionizing irradiation: effects on prostaglandins, chemoattractants and mitogens. *Semin Thromb Hemostasis* 1989;15:215-225
225. Sufrin G, Heston WD, Hazra T, Coffey DS. The effect of irradiation on prostatic growth. *Invest Uroil* 1976;13:418-423
226. Gillies NE. Effects of radiation on cells. *BMJ* 1987;295:1390-1391
227. Adamson IY. Radiation enhances silica translocation to the pulmonary interstitium and increases fibrosis in mice. *Environ Health Perspect* 1994;97:233-238
228. Thornton SC, Walsh BJ, Bennett S et al. Both in vitro and in vivo irradiation are associated with induction of macrophage-derived fibroblast growth factors. *Clin Exp Immunol* 1996;103:67-73
229. Witte L, Fuks Z, Haimovitz-Friedman A, Vlodaysky I, Goodman DS, Eldor A. Effects of irradiation on the release of growth factors from cultured bovine, porcine and human endothelial cells. *Cancer Res* 1989;49:5066-5072
230. Langberg CW, Hauer-Jensen M, Sung C-C, Kane CJM. Expression of fibrogenic cytokines in rat small intestine after fractionated irradiation. *Radiother Oncol* 1994;32:29-36
231. Suresh UR, Smith VJ, Lupton EW, Haboubi NY. Radiation disease of the urinary tract: Histological features of 18 cases. *J Clin Pathol* 1993;46:228-231

232. Gikas PD, Hanna SA, Aston Will et al. Post-radiation sciatic neuropathy: a case report and review of the literature. *World J Surgery* 2008;6:130
233. Blandy JP, Fowler C. *Urology*, 2nd edn. Blackwell, Oxford, 1996
234. Lynch WJ, Jenkins BJ, Fowler CG, Hope-Stone HF, Blandy JP. The quality of life after radical radiotherapy for bladder cancer. *Br J Urol* 1992;70:1013-1020
235. Borgaonkar S, Jain A, Bollina P, McLaren DB, Tulloch D, Kerr GR, Howard GC. Radical radiotherapy and salvage cystectomy as the primary management of transitional cell carcinoma of the bladder. Results following the introduction of a CT planning technique. *Clin Oncol (R Coll Radiol)* 2002;14:141-147
236. Milosevic M, Gospodarowicz M, Zietmen A, Abbas F, Haustermans K, Moonon L, Rodel C, Scoenberg M, Shipley W. Radiotherapy for bladder cancer. *Urology* 2007;69(S1):80-92
237. Tomic R, Grnafos T, Modig H. Morbidity after preoperative radiotherapy and cystectomy in patients with bladder cancer. *Scand J Uroil Nephrol* 1997;31:149-153
238. http://academic.kellogg.edu/herbrandsonc/bio201_McKinley/f27-9a_bladder_c.jpg
239. Widmark A, Flodgren P, Damber JE, Hellsten S, Cavllin-Stahl E. A systematic overview of radiation therapy effects in urinary bladder cancer. *Acta Oncol* 2003;42:567-581
240. Moonen L. Indications and outcome of radiotherapy for invasive bladder cancer: review of developments during 2002. *Curr Opin Urol* 2003;13:385-387
241. Marks LB, Carroll PR, Dugan TC, Anscher MS. The response of the urinary bladder, urethra, and ureter to radiation and chemotherapy. *Int J Radiat Oncol Biol Phys* 1995;31:1257-1280
242. Zelefsky MJ, Levin EJ, Hunt M et al. Incidence of late rectal and urinary toxicities after three-dimensional conformal radiotherapy and intensity-modulated radiotherapy for localized prostate cancer. *Int J Radiat Oncol Biol Phys* 2008;70:1124-1129
243. Tonoli S, Bertoni F, De Stefani A et al. Radical radiotherapy for bladder cancer: retrospective analysis of a series of 459 patients treated in an Italian institution. *Clin Oncol (R Coll Radiol)* 2006 ;18 :52-59

244. Suzuki K, Kurokawa K Suzuki T, Okazaki H, Otake N, Imai K, Yamanaka H. Successful treatment of radiation cystitis with hyperbaric oxygen therapy : resolution of bleeding event and changes of histopathological findings of the bladder mucosa. *Int Urol Nephrol* 1998 ;30 :267-271
245. Arthur JJ, Kleiter MM, Thrall DE, Pruitt AF. Characterization of normal tissue complications in 51 dogs undergoing definitive pelvic region irradiation. *Vet Radiol Ultrasound* 2008;49:85-89
246. Kohler M, Michel C, Zimmerman A. Histological changes after fractionated whole or partial irradiation of the rabbit urinary bladder. *Acta Oncol* 1995;24:199-204
247. Hietala SO, Winblad B, Hassler O. Vascular and morphological changes in the urinary bladder wall after irradiation. *Int J Urol Nephrol* 1975;7:119-129
248. Anatonakopoulos GN, Hicks RM, Hamilton E, Berry RJ. Early and late changes (including carcinoma of the urothelium) induced by irradiation of the rat urinary bladder. *Br J Cancer* 1982 ;46 :403-416
249. Vale JA, Bowsher WG, Liu K, Tomlinson A, Whitfield HN, Trott KR. Post-irradiation bladder dysfunction: development of a rat model. *Urol Res* 1993;21:383-388
250. Dorr W, Eckhardt M, Ehme A, Koi S. Pathogenesis of acute radiation effects in the urinary bladder. Experimental results. *Stralenter Onkol* 1998;174(S3):93-95
251. Hatcher S, Arroll B. Assessment and management of medically unexplained symptoms. *BMJ* 2008; 336:1124-1128
252. Chan TY, Epstein JI. Radiation or chemotherapy cystitis with 'pseudocarcinomatous' features. *Am J Surg Pathol* 2004;28:909-913
253. Lane Z, Epstein JI. Polypoid/papillary cystitis : a series of 41 cases misdiagnosed as papillary urothelial neoplasia. *Am J Surg Pathol* 2008;32:758-764
254. Marons MM, Rubio BJ, Arribas AL et al. Radiation induced infiltrating transitional cell carcinoma. *Actas Urol Esp* 2005;29:562-566
255. Romanenko A, Morimura K, Wei M, Zaparin W, Vozianov A, Fukushima S. DNA damage repair in bladder urothelium after the Chernobyl accident in Ukraine. *J Urol* 2002;168:973-977

256. Lagrange JL. Late effects of radiations on the bladder. *Cancer Radiother* 1997;1:764-769
257. Artuc M, Hermes B, Steckelings UM, Grutzkas A, Henz BM. Mast cells and their mediators in cutaneous wound healing – active participants or innocent bystanders? *Exp Dermatol* 1999;8:1-16
258. Howard PS, Refrow D, Schechter NM, Kucich U. Mast cell chymase as a possible mediator or neurogenic bladder fibrosis. *Neurourol Urodyn* 2004;23:372-382
259. Withers HR, Peters LJ and Kogelnick HD. The pathobiology of late effects of irradiation. In Meyn RE and Withers HR eds. *Radiation Biology in Cancer Research*. Raven Press. 1980; 439-448
260. Reinhold HS. The influence of radiation on blood vessels and circulation. Chapter IV. Structural changes in blood vessels. *Curr Top Radiat Res Q* 1974;10:58-74
261. Kamiryo T et al. Histological changes in the normal rat brain after gamma irradiation. *Acta Neurochir* 1996; 138(4); 451-459.
262. Zelefsky MJ, Fuks Z, Hunt M et al. High dose radiation delivered by intensity modulated conformal radiotherapy improves the outcome of localised prostate cancer. *J Urol* 2001;166:876–881
263. Anthony PP, Ishak KG, Nayak NC, Poulsen HE, Scheuer PJ, Sobin LH. The morphology of cirrhosis. Recommendations on definition, nomenclature, and classification by a working group sponsored by the World Health Organization. *J Clin Pathol* 1978; 31: 395-414
264. Hovdenak N, Wang J, Sung CC, Kelly T, Fajardo LF, Hauer-Jensen M. Clinical significance of increased gelatinolytic activity in the rectal mucosa during external beam radiation therapy of prostate cancer. *Int J Radiat Oncol Biol Phys* 2002;53:919-927
265. Strup-Perrot C, Mathe D, Linard C, Violot D, Milliat F, Francois A, Bourhis J, Vozenin-Brotons MC. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol* 2004; 287: G875-G885

266. Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med* 2006; 10: 76-99
267. Ffrench-Constant C et al. Reappearance of embryonic pattern of fibronectin splicing during wound healing in adult rat. *J Cell Biol* 1989;109:903-914
268. Tavian D et al. RT-PCR detection of fibronectin EDA+ and EDB+ mRNA isoforms: molecular markers for hepatocellular carcinoma. *Int J Cancer* 1994;56:820-825
269. Halliday NL et al. Distribution of ED-A and ED-B fibronectin isoforms in Dupuytren's disease. *Am J Hand Surg* 1994;19: 428-434
270. Yoshida T et al. Co-expression of tenascin and fibronectin in epithelial and stromal cells of benign lesions and ductal carcinomas in the human breast. *J Pathol* 1997;182: 421-428
271. Castellani P et al. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int J Cancer* 1994;59: 612-618
272. Cordes N, Beinke C. Fibronectin alters cell survival and intracellular signalling of confluent A549 cultures after irradiation. *Cancer Biol Ther* 2004;3:47-53
273. Dimitrijevic-Bussod M, Balzaretta-Maggi VS, Gadbois DM. Extracellular matrix and radiation G1 cell cycle arrest in human fibroblasts. *Cancer Res* 1999; 59: 4983-4987
274. Cordes N, Blaese MA, Plasswilm L, Rodmann HP, Van Beuningen D. Fibronectin and laminin increase resistance to ionizing radiation and the cytotoxic drug Ukrain in human tumour and normal mcells in vitro. *Int J Radiat Biol* 2003; 79:709-720
275. Cordes N, van Beuningen D. Cell adhesion to the extracellular matrix protein fibronectin modulates radiation G2 phase arrest involving integrin-linked (ILK) and glycogen synthase kinase-3beta (GSK-3beta) in vitro. *Br J Cancer* 2003;88:1470-1479
276. van der Kuip H, Goetz AW, Miething C, Duyster J, Aulitzky WE. Adhesion to fibronectin selectively protects Bcr-Abl+ cells from DNA damage-induced apoptosis. *Blood* 2001;98:1532-1541

277. Antonakopoulos GN, Hicks RM, Berry RJ. The subcellular base of damage to the human urinary bladder induced by irradiation. *J Pathol* 1984;143:103-116
278. Lundbeck F, Nielsen K, Stewart F. Late changes in the normal mouse bladder after irradiation alone or in combination with cis-DDP or cyclophosphamide, assessed by stereological analysis. *APMIS* 1993;101:275-280
279. James J et al. Histophotometric estimation of volume density of collagen as an indication of fibrosis in rat liver. *Histochemistry* 1986; 85:129-133
280. Lopez-de Leon A and Rojkind M. A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J Histochem Cytochem* 1985;33:737-743
281. Wynn TA. Common and unique mechanisms in various fibroproliferative disease. *J Clin Invest* 2007;117:524-529
282. Kumar V, Abbas AK, Fausto N. Tissue renewal and repair: Regeneration, healing, and fibrosis. Elsevier Saunders, Philadelphia, 2005
283. Verrecchia F, Mauviel A. Transforming growth factor-beta and fibrosis. *World J Gastroenterol* 2007;13:3056-3062
284. Yoshida Y, Kang K, Chen G, Gilliam AC, Cooper KD. Cellular fibronectin is induced in ultraviolet-exposed human skin and induces IL-10 production by monocytes/macrophages. *J Invest Dermatol* 1999;113:49-55
285. Norton PA, Reis HM, Prince S, Larkin J, Pan J, Liu J et al. Activation of fibronectin gene expression by hepatitis B virus x antigen. *J Viral Hepat* 2004;11:332-341
286. Sclaf G, Schmitz M, Heine I, Demberg T, Schieferdecker HL and Gotze O. Upregulation of fibronectin but not of entactin, collagen IV and smooth muscle actin by anaphylatoxin C5a in rat hepatic stellate cells. *Histol Histopathol* 2004;19:1165-1974
287. Friedrich RE, Bartel-Friedrich S, Holzhausen HJ, Lautenschlager C. The effect of external fractionated irradiation on the distribution pattern of extracellular matrix proteins in submandibular salivary glands of the rat. *J Craniomaxillofac Surg* 2002; 30:246-254

288. Bartel-Friedrich S, Friedrich RE, Lautenschlager C, Moll R. Immunohistochemical detection of extracellular matrix proteins in the irradiated rat submandibular gland. *Laryngorhinootologie* 1999;78:500-507
289. Anscher MS et al. Changes in plasma TGF- β levels during pulmonary radiotherapy as a predictor of the risk of developing radiation pneumonitis. *Int J Radiat Oncol Biol Phys* 1994; 30:671-676
290. Knowlton et al. Rapid expression of fibronectin in the rabbit heart after myocardial infarction with and without reperfusion. *J Clin Invest* 1992;89:1060-1068
291. Willems IEMG, Arends JW and Daemen MJAP. Tenascin and fibronectin expression in healing human myocardial scars. *J Pathol* 1996;179:321-325
292. Rubin P, Johnson CJ, Williams JP, McDonald S, Finkelstein JN. A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. *Int J Radiat Oncol Biol Phys* 1995;33:99-109
293. Fajardo LF. The pathology of ionizing radiation as defined by morphological patterns. *Acta Oncol* 2005;44:13-22
294. Rubio CA, Jalnas M. Dose-time-dependent histological changes following irradiation of the small intestine of rats. *Dig Dis Sci* 1996;41:392-401
295. True LD. Morphometric applications in anatomic pathology. *Hum Pathol* 1996; 27:450-467
296. Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells. *Proc Natl Acad Sci* 1974;71:1207–1210
297. Westermark B, Wasteson A. A platelet factor stimulating human normal glial cells. *Exp Cell Res* 1976;98:170–174
298. Heldin C, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 1999;79:1283-1316
299. Antoniades H N, Scher CD, Stiles CD. Purification of human platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* 1979;76: 1809-1812

300. Kohler N, Lipton A. Platelets as a source of fibroblast growth-promoting activity. *Exp Cell Res* 1974;87:297–301
301. Betsholtz C, Johnsson A, Heldin CH, Westermark B, Lind P, Urdea MS, Eddy R, Shows TB, Philpott K, Mellor AL. cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines. *Nature* 1986;320:695–699
302. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes and development* 2008;22:1276-1312
303. Li X, Pontén A, Aase K, Karlsson L, Abramsson A, Uutela M, Bäckström G, Hellström M, Boström H, Li H et al. PDGF-C is a new protease-activated ligand for the PDGF α -receptor. *Nat Cell Biol* 2000;2:302–309
304. Aase K, Abramsson A., Karlsson L, Betsholtz C, Eriksson U. Expression analysis of PDGF-C in adult and developing mouse tissues. *Mech Dev* 2002;110:187–191
305. Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U. PDGF-D is a specific, protease-activated ligand for the PDGF β -receptor. *Nat Cell Biol.* 2001;3:512–516
306. Li M, Jendrossek V, Belka C. The role of PDGF in radiation oncology. *Rad Oncol* 2007;2:5
307. Swan D C, McBride OW, Robbins KC, Keithley DA, Reddy EP, Aaronson SA. Chromosomal mapping of the simian sarcoma virus oncogene analogue in human cells. *Proc Natl Acad Sci USA* 1982;79: 4691-4695
308. Bonthron DT, Morton CC, Orkin SH, Collins T. Platelet-derived growth factor A chain: gene structure, chromosomal location, and basis for alternative mRNA splicing. *Proc Natl Acad Sci USA* 1988;85: 1492-1496
309. Kourembanas S, Morita T, Liu Y, Christou H. Mechanisms by which oxygen regulates gene expression and cell-cell interaction in the vasculature. *Kidney Int* 1997;51: 438-443
310. Daniel TO, Gibbs VC, Milfay DF, Garovoy MR, Williams LT. Thrombin stimulates c-sis gene expression in microvascular endothelial cells. *J Biol Chem* 1986;261: 9579-9582

311. Harlan JM, Thompson PJ, Ross R, Bowen-Pope DF. α -Thrombin induces release of platelet-derived growth factor-like molecule(s) by cultured human endothelial cells. *J Cell Biol* 1986;103:1129-1133
312. Betscholtz C. In: *The PDGF Genes and Their Regulation*. Westermark B and Sorg C (eds). Karger, Basel, 1993, volume 5, pp 11-30
313. Mendoza AE, Young R, Orkin SH, Collins T. Increased platelet-derived growth factor A-chain expression in human uterine smooth muscle cells during the physiologic hypertrophy of pregnancy. *Proc Natl Acad Sci USA* 1990;87: 2177-2181
314. Demayo F, Minoo P, Plopper CG, Schuger L, Shannon J, Torday JS. Mesenchymal-epithelial interactions in lung development and repair: are modeling and remodeling the same process? *Am J Physiol Lung Cell Mol Physiol* 2002;283:L510-L517
315. Zhang S, Smartt H, Holgate ST, Roche WR. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. *Lab Invest* 1999;79:395-405
316. Mondy JS, Lindner V, Miyashiro JK, Berk BC, Dean RH, Geary RL: Platelet-derived growth factor ligand and receptor expression in response to altered blood flow in vivo. *Circ Res* 1997, 81:320-327
317. Lindroos PM, Coin PG, Badgett A, Morgan DL, Bonner JC. Alveolar macrophages stimulated with titanium dioxide, chrysotile asbestos, and residual oil fly ash upregulate the PDGF receptor-alpha on lung fibroblasts through an IL-1beta-dependent mechanism. *Am J Respir Cell Mol Biol* 1997;16:283-292
318. Fredriksson L, Li H, Eriksson U. The PDGF family: Four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev* 2004;15:197-204
319. Reigstad LJ, Varhaug JE, Lillehaug JR. Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. *FEBS J* 2005;272:5723-5741
320. Fredriksson L, Li H, Fieber C, Li X, Eriksson U. Tissue plasminogen activator is a potent activator of PDGF-CC. *EMBO J* 2004;23:3793-3802

321. Lin X. Functions of heparan sulfate proteoglycans in cell signaling during development. *Development* 2004;131:6009–6021
322. Häcker U, Nybakken K, Perrimon N. Heparan sulphate proteoglycans: The sweet side of development. *Nat Rev Mol Cell Biol* 2005;6:530–541
323. Abramsson A, Kurup S, Busse M, Yamada S, Lindblom P, Schallmeiner E, Stenzel D, Sauvaget D, Ledin J, Ringvall M et al. Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. *Genes and Development* 2007;21:316–331
324. Bonner JC, Osornio-Vargas AR. Differential binding and regulation of platelet-derived growth factor A and B chain isoforms by α 2-macroglobulin. *J Biol Chem* 1995;270:16236–16242
325. Bonner JC, Badgett A, Hoffman M, Lindroos PM. Inhibition of platelet-derived growth factor-BB-induced fibroblast proliferation by plasmin-activated α 2-macroglobulin is mediated via an α 2-macroglobulin receptor/low density lipoprotein receptor-related protein-dependent mechanism. *J Biol Chem* 1995;270:6389–6395
326. Kelly JL, Sánchez A, Brown GS, Chesterman CN, Sleigh MJ. Accumulation of PDGF B and cell-binding forms of PDGF A in the extracellular matrix. *J. Cell Biol* 1993;121:1153–1163
327. Claesson-Welsh L, Eriksson A, Westermark B, Heldin C-H. cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. *Proc Natl Acad Sci USA* 1989 ;86: 4917-4921
328. Matsui T, Heidaran M, Miki T, Toru M, Popescu N, La Rochelle W, Kraus M, Pierce J, Aaronson SA. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* 1989;243: 800-803
329. Yarden Y, Escobedo JA, Kuang W-J, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Friend VA, Ullrich A, Williams LT. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* 1986;323: 226-232

330. Coussens L, van Beveren LC, Smith D, Chen E, Mitchell RL, Isacke C, Verma IA, Ullrich A. Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. *Nature* 1986;320: 277-280
331. Yarden Y, Kuang W-J, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A. Human proto-oncogene *c-kit*: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J* 1987;6:3341-3351
332. Spritz RA, Strunk KM, Lee ST, Lu-Kuo JM, Ward DC, Le Paslier D, Altherr MR, Dorman TE, Moir DT. A YAC contig spanning a cluster of human type III receptor protein tyrosine kinase genes (PDGFRA-KIT-KDR) in chromosome segment 4q12. *Genomics* 1994;22:431-436
333. Roberts WM, Look AT, Roussel MF, Sherr CJ. Tandem linkage of human CSF-1 receptor (*c-fms*) and PDGF receptor genes. *Cell* 1988;55:655-661
334. Bishayee S, Majumdar S, Khire J, Das M. Ligand-induced dimerization of the platelet-derived growth factor receptor. Monomer-dimer interconversion occurs independent of receptor phosphorylation. *J Biol Chem* 1989;264:11699-11705
335. Heldin C-H, Ernlund A, Rorsman C, Ronnstrand L. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J Biol Chem* 1989;264:8905-8912
336. Seifert RA, Hart CE, Philips PE, Forstrom JW, Ross R, Murray M, Bowen-Pope DF. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. *J Biol Chem* 1989;264:8771-8778
337. Hammacher A, Mellstrom K, Heldin C-H, Westermark B. Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer. *EMBO J* 1989;8:2489-2495
338. Kanakaraj P, Raj S, Khan SA, Bishayee S. Ligand-induced interaction between α - and β -type platelet derived growth factor (PDGF) receptors: role of receptor heterodimers in kinase activation. *Biochemistry* 1991;30:1761-1767
339. Liu P, Ying Y, Ko Y-G, Anderson RGW. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J Biol Chem* 1996;271:10299-10303

340. Souza P, Tanswell AK, Post M. Different roles for PDGF- α and - β receptors in embryonic lung development. *Am J Respir Cell Mol Biol* 1996;15: 551-562
341. Mori S, Heldin C-H, Claesson-Welsh L. Ligand-induced polyubiquitination of the platelet-derived growth factor β -receptor. *J Biol Chem* 1992;267:6429-6434
342. Mori S, Tanaka K, Omura S, Saito Y. Degradation process of ligand-stimulated platelet-derived growth factor β -receptor involves ubiquitin-proteasome proteolytic pathway. *J Biol Chem* 1995;270:29447-29452
343. Sorkin A, Westermark B, Heldin C-H, Claesson-Welsh L. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGF β -receptor. *J Cell Biol* 1991;112:469-478
344. Joly M, Kazlauskas A, Fay FS, Corvera S. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. *Science* 1994;263:684-687
345. Mori S, Ronnstrand L, Claesson-Welsh L, Heldin C-H. A tyrosine residue in the juxtamembrane segment of the platelet-derived growth factor β -receptor is critical for ligand-mediated endocytosis. *J Biol Chem* 1994;269:4917-4921
346. Hossain MZ, Ao P, Boynton AL. Rapid disruption of gap junctional communication and phosphorylation of connexin43 by platelet-derived growth factor in T51B rat liver epithelial cells expressing platelet-derived growth factor receptor. *J Cell Physiol* 1998;174:66-77
347. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* 1995;267:2003-2006
348. Shure D, Senior RM, Griffin GL, Deuel TF. PDGF AA homodimers are potent chemoattractants for fibroblasts and neutrophils, and for monocytes activated by lymphocytes or cytokines. *Biochem Biophys Res Commun* 1992;186:1510-1514
349. Bar-Sagi D, Feramisco JR. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science* 1986;233:1061-1068
350. Seger R, Krebs EG. The MAPK signaling cascade. *FASEB J* 1995;9:726-735

351. Hu Q, Klippel A, Muslin AJ, Fantl WJ, Williams LT. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science* 1995;268:100–102
352. Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature* 1993;361:315–325
353. Kundra V, Escobedo JA, Kazlauskas A, Kim HK, Rhee SG, Williams LT, Zetter BR Regulation of chemotaxis by the platelet-derived growth factor receptor- β . *Nature* 1994;367:474–476
354. Erpel T, Courtneidge SA. Src family protein tyrosine kinases and cellular signal transduction pathways. *Curr Opin Cell Biol* 1995;7:176–182
355. Su YC, Han J, Xu S, Cobb M, Skolnik EY. NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. *EMBO J* 1997;16:1279–1290
356. Yokote K, Margolis B, Heldin C-H, Claesson-Welsh L. Grb7 is a downstream signaling component of platelet-derived growth factor α - and β -receptors. *J Biol Chem* 1996;271:30942–30949
357. Darnell JE. STATs and gene regulation. *Science* 1997;277:1630–1635
358. Assoian RK. Anchorage-dependent cell cycle progression. *J Cell Biol* 1997;136:1–4
359. Clark EA, Brugge JS. Integrins and signal transduction pathways: The road taken. *Science* 1995;268:233–239
360. James MF, Beauchamp RL, Manchanda N, Kazlauskas A, Ramesh V. A NHERF binding site links the β PDGFR to the cytoskeleton and regulates cell spreading and migration. *J Cell Sci* 2004;117:2951–2961
361. Theisen CS, Wahl JK, Johnson KR, Wheelock MJ. NHERF links the N-cadherin/catenin complex to the platelet-derived growth factor receptor to modulate the actin cytoskeleton and regulate cell motility. *Mol Biol Cell* 2007;18:1220–1232

362. Takahashi Y, Morales FC, Kreimann EL, Georgescu MM. PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. *EMBO J* 2006;25:910–920
363. Schlessinger J. How receptor tyrosine kinases activate Ras. *Trends Biochem Sci* 1993;18:273-275
364. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 1995;81:727-736
365. Yagi M, Kato S, Kobayashi Y, Kobayashi N, Inuma N, Nakamura K, Kubo K, Ohyama SI, Murooka H, Shimizu T, Nishitoba T, Osawa T, Nagano N. Beneficial effects of a novel inhibitor of platelet-derived growth factor receptor autophosphorylation in the rat with mesangial proliferative glomerulonephritis. *Gen Pharmacol* 1998;31:765-773
366. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 1999;277:C1-C9
367. Siegbahn A, Hammacher A, Westermark B, Heldin C-H. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *J Clin Invest* 1990;85:916-920
368. Shih AH, Holland EC. Platelet-derived growth factor (PDGF) and glial tumorigenesis. *Cancer Lett* 2006;232:139-147
369. Lechleider RJ, Sugimoto S, Bennett AM, Kashishian AS, Cooper JA, Shoelson SE, Walsh CT, Neel BG. Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor. *J Biol Chem* 1993;268:21478–21481
370. Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, Williams LT. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 1992;69:413–423

371. Lennartsson J, Wardega P, Engström U, Hellman U, Heldin C-H. Alix facilitates the interaction between c-Cbl and platelet-derived growth factor β -receptor and thereby modulates receptor down-regulation. *J Biol Chem* 2006;281:39152–39158
372. Fatatis A, Miller RJ. Platelet-derived growth factor (PDGF)-induced Ca^{2+} signaling in the CG4 oligodendroglial cell line and in transformed oligodendrocytes expressing the β -PDGF receptor. *J Biol Chem* 1997;272:4351–4358
373. Hossain MZ, Ao, P, Boynton AL. Platelet-derived growth factor-induced disruption of gap junctional communication and phosphorylation of connexin43 involves protein kinase C and mitogen-activated protein kinase. *J Cell Physiol* 1998;176:332–341
374. Cochran BH, Reffel AC, Stiles CD. Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* 1983;33:939–947
375. Heuchel R, Berg A, Tallquist M, Ahlén K, Reed RK, Rubin K, Claesson-Welsh L, Heldin CH, Soriano P. Platelet-derived growth factor β receptor regulates interstitial fluid homeostasis through phosphatidylinositol-3' kinase signaling. *Proc Natl Acad Sci* 1999;96:11410–11415
376. Klinghoffer RA, Hamilton TG, Hoch R, Soriano P. An allelic series at the PDGF α R locus indicates unequal contributions of distinct signaling pathways during development. *Dev Cell* 2002;2:103–113
377. Klinghoffer RA, Muetting-Nelsen PF, Faerman A, Shani M, Soriano P. The two PDGF receptors maintain conserved signaling in vivo despite divergent embryological functions. *Mol Cell* 2001;7:343–354
378. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 1997;277:242–245
379. Boström H, Willetts K, Pekny M, Levéen P, Lindahl P, Hedstrand H, Pekna M, Hellström M, Gebre-Medhin S, Schalling M et al. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 1996;85:863–873
380. Karlsson L, Lindahl P, Heath JK, Betsholtz C. Abnormal gastrointestinal development in PDGF-A and PDGFR- α deficient mice implicates a novel mesenchymal

structure with putative instructive properties in villus morphogenesis. *Development* 2000;127:3457–3466

381. Abramsson A, Lindblom P, Betsholtz C. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J Clin Invest* 2003;112:1142-1151

382. Enge M, Bjarnegård M, Gerhardt H, Gustafsson E, Kalén M, Asker N, Hammes HP, Shani M, Fässler R, Betsholtz C. Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *EMBO J* 2002;21:4307–4316

383. Hoch RV, Soriano P. Roles of PDGF in animal development. *Development* 2003;130:4769–4784

384. Reneker LW, Overbeek PA. Lens-specific expression of PDGF-A alters lens growth and development. *Dev Biol* 1996;180:554–565

385. Soriano P. The PDGF α receptor is required for neural crest cell development and for normal patterning of the somites. *Development* 1997;124:2691–2700

386. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70

387. Johnsson A, Betsholtz C, Heldin C-H, Westermark B. Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus. *Nature* 1985;317:438–440

388. Uhrbom L, Hesselager G, Nistér M, Westermark B. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. *Cancer Res* 1998;58:5275–5279

389. Guha A, Dashner K, Black PM, Wagner JA, Stiles CD. Expression of PDGF and PDGF receptors in human astrocytoma operation specimens supports the existence of an autocrine loop. *Int J Cancer* 1995;60:168-173

390. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin C-H, Westermark B, Nistér M. Platelet-derived growth factor and its receptors in human glioma tissue: Expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res* 1992;52:3213–3219

391. Nistér M, Libermann TA, Betsholtz C, Pettersson M, Claesson-Welsh L, Heldin C-H, Schlessinger J, Westermark B. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor- α and their receptors in human malignant glioma cell lines. *Cancer Res* 1988;48:3910–3918
392. Johansson FK, Brodd J, Eklof C et al. Identification of candidate cancer-causing genes in mouse brain tumours by retroviral tagging. *PNAS* 2004;101:11334-11337
393. Dai C, Celestino JC, Okada Y, Louis DN, Fuller GN, Holland EC. PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. *Genes and Dev* 2001;15:1913–1925
394. Servidei T, Riccardi A, Sanguinetti M, Dominici C, Riccardi R. Increased sensitivity to the platelet-derived growth factor (PDGF) receptor inhibitor STI571 in chemoresistant glioma cells is associated with enhanced PDGF-BB-mediated signaling and STI571-induced Akt inactivation. *J Cell Physiol* 2006;208:220–228
395. Uhrbom L, Hesselager G, Ostman A, Nistér M, Westermark B. Dependence of autocrine growth factor stimulation in platelet-derived growth factor-B-induced mouse brain tumor cells. *Int J Cancer* 2000;85:398–406
396. Newton HB. Molecular neuro-oncology and development of targeted therapeutic strategies for brain tumors. Part 1: Growth factor and Ras signaling pathways. *Expert Rev Anticancer Ther* 2003;3:595–614
397. Todo T, Adams EF, Fahlbusch R. Inhibitory effect of trapidil on human meningioma cell proliferation via interruption of autocrine growth stimulation. *J Neurosurg* 1993;78:463-469
398. Adams EF, Todo T, Schrell UM, Thierauf P, White MC, Fahlbusch R. Autocrine control of human meningioma proliferation: secretion of platelet-derived growth-factor-like molecules. *Int J Cancer* 1991;49:398-402
399. Bruna A, Darken RS, Rojo F, Ocaña A, Peñuelas S, Arias A, Paris R, Tortosa A, Mora J, Baselga J et al. High TGF β -Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. *Cancer Cell* 2007;11:147–160

400. Jennings MT, Hart CE, Commers PA, Whitlock JA, Martincic D, Maciunas RJ, Moots PL, Shehab TM. Transforming growth factor β as a potential tumor progression factor among hyperdiploid glioblastoma cultures: Evidence for the role of platelet-derived growth factor. *J Neurooncol* 1997;31:233–254
401. Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, Roy M, Quinones-Hinojosa A, Vandenberg S, Alvarez-Buylla A. PDGFR α -positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron* 2006;51:187–199
402. Fleming TP, Saxena A, Clark WC, Robertson JT, Oldfield EH, Aaronson SA, Ali IU. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. *Cancer Res* 1992;52:4550–4553
403. Smith JS, Wang XY, Qian J, Hosek SM, Scheithauer BW, Jenkins RB, James .D. Amplification of the platelet-derived growth factor receptor-A (PDGFRA) gene occurs in oligodendrogliomas with grade IV anaplastic features. *J Neuropathol Exp Neurol* 2000;59:495–503
404. Arai H, Ueno T, Tangoku A, Yoshino S, Abe T, Kawauchi S, Oga A, Furuya T, Oka M, Sasaki K. Detection of amplified oncogenes by genome DNA microarrays in human primary esophageal squamous cell carcinoma: Comparison with conventional comparative genomic hybridization analysis. *Cancer Genet Cytogenet* 2003;146:16–21
405. Zhao J, Roth J, Bode-Lesniewska B, Pfaltz M, Heitz PU, Komminoth P. Combined comparative genomic hybridization and genomic microarray for detection of gene amplifications in pulmonary artery intimal sarcomas and adrenocortical tumors. *Genes Chromosomes Cancer* 2002;34:48–57
406. Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A et al. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* 2003;299:708–710
407. Hirota S, Ohashi A, Nishida T, Isozaki K, Kinoshita K, Shinomura Y, Kitamura Y. Gain-of-function mutations of platelet-derived growth factor receptor α gene in gastrointestinal stromal tumors. *Gastroenterology* 2003;125:660–667

408. Ali Y, Lin Y, Gharibo MM, Gounder MK, Stein MN, Lagattuta TF, Egorin MJ, Rubin EH, Poplin EA. Phase I and pharmacokinetic study of imatinib mesylate (Gleevec) and gemcitabine in patients with refractory solid tumors. *Clin. Cancer Res* 2007;13:5876–5882
409. Simon MP, Pedeutour F, Sirvent N, Grosgeorge J, Minoletti F, Coindre JM, Terrier-Lacombe MJ, Mandahl N, Craver RD, Blin N et al. Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. *Nat Genet* 1997;15:95–98
410. Sjöblom T, Shimizu A, O'Brien KP, Pietras K, Dal Cin P, Buchdunger E, Dumanski JP, Ostman A, Heldin CH. Growth inhibition of dermatofibrosarcoma protuberans tumors by the platelet-derived growth factor receptor antagonist STI571 through induction of apoptosis. *Cancer Res* 2001;61:5778–5783
411. Sirvent N, Maire G, Pedeutour F. Genetics of dermatofibrosarcoma protuberans family of tumors: From ring chromosomes to tyrosine kinase inhibitor treatment. *Genes Chromosomes Cancer* 2003;37:1–19
412. Griffin JH, Leung J, Bruner RJ, Caligiuri MA, Briesewitz R. (2003) Discovery of a fusion kinase in EOL-1 cells and idiopathic hypereosinophilic syndrome. *Proc Natl Acad Sci* 2003;100:7830–7835
413. Cools J, DeAngelo DJ, Gotlib J, Stover EH, Legare RD, Cortes J, Kutok J, Clark J, Galinsky I, Griffin JD et al. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003;348:1201–1214
414. Pardanani A, Ketterling RP, Brockman SR, Flynn HC, Paternoster SF, Shearer BM, Reeder TL, Li CY, Cross NC, Cools J et al. CHIC2 deletion, a surrogate for FIP1L1–PDGFRA fusion, occurs in systemic mastocytosis associated with eosinophilia and predicts response to imatinib mesylate therapy. *Blood* 2003;102:3093–3096
415. Pietras K, Sjöblom T, Rubin K, Heldin CH, Ostman A. PDGF receptors as cancer drug targets. *Cancer Cell* 2003;3:439–443
416. Furuhashi M, Sjöblom T, Abramsson A, Ellingsen J, Micke P, Li H, Bergsten-Folestad E, Eriksson U, Heuchel R, Betsholtz C et al. Platelet-derived growth factor

production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. *Cancer Res* 2004;64:2725–2733

417. Heldin NE, Gustavsson B, Claesson-Welsh L, Hammacher A, Mark J, Heldin CH, Westermark B. Aberrant expression of receptors for platelet-derived growth factor in an anaplastic thyroid carcinoma cell line. *Proc Natl Acad Sci USA* 1998;85:9302–9306

418. Seymour L, Dajee D, Bezwoda WR. Tissue platelet derived-growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Res Treat* 1993;26:247–252

419. Jechlinger M, Grünert S, Tamir IH, Janda E, Lüdemann S, Waerner T, Seither P, Weith A, Beug H, Kraut N. Expression profiling of epithelial plasticity in tumor progression. *Oncogene* 2003;22:7155–7169

420. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, Donovan M, Cordon-Cardo C, Beug H, Grünert S. Autocrine PDGFR signaling promotes mammary cancer metastasis. *J Clin Invest* 2006;116:1561–1570

421. Ostman A. PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine Growth Factor Rev* 2004;15:275–286

422. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 2003;111:1287–1295

423. Schlingemann RO, Rietveld FJ, Kwaspen F, de van Kerkhof PC, de Waal RM, Ruiter DJ. Differential expression of markers for endothelial cells, pericytes, and basal lamina in the microvasculature of tumors and granulation tissue. *Am J Pathol* 1991;138:1335–1347

424. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol* 2000;156:1363–1380

425. Abramsson A, Berlin O, Papayan H, Paulin D, Shani M, Betsholtz C. Analysis of mural cell recruitment to tumor vessels. *Circulation* 2002;105:112–117

426. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003;33:49–54

427. Gerhardt H, Semb H. Pericytes: Gatekeepers in tumour cell metastasis? *J Mol Med* 2008;86:135–144
428. Skobe M, Fusenig NE. Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. *Proc Natl Acad Sci USA* 1998;95:1050–1055
429. Lederle W, Stark HJ, Skobe M, Fusenig NE, Mueller MM. Platelet-derived growth factor-BB controls epithelial tumor phenotype by differential growth factor regulation in stromal cells. *Am J Pathol* 2006;169:1767–1783
430. Dong J, Grunstein J, Tejada M, Peale F, Frantz G, Liang WC, Bai W, Yu L, Kowalski J, Liang X et al. VEGF-null cells require PDGFR α signaling-mediated stromal fibroblast recruitment for tumorigenesis. *EMBO J* 2004;23:2800–2810
431. Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M, Westermark B. Platelet-derived growth factor (PDGF) in oncogenesis: Development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proc Natl Acad Sci* 1993;90:393–397
432. Tejada ML, Yu L, Dong J, Jung K, Meng G, Peale FV, Frantz GD, Hall L, Liang X, Gerber HP et al. Tumor-driven paracrine platelet-derived growth factor receptor α signaling is a key determinant of stromal cell recruitment in a model of human lung carcinoma. *Clin Cancer Res* 2006;12:2676–2688
433. Pietras K, Pahler J, Bergers G, Hanahan D. Functions of paracrine PDGF signaling in the pro-angiogenic tumor stroma revealed by pharmacological targeting. *PloS Med* 2007;5:e19
434. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121:335–348
435. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;449:557–563

436. Pietras K, Ostman A, Sjöquist M, Buchdunger E, Reed RK, Heldin CH, Rubin K. Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. *Cancer Res* 2001;61:2929–2934
437. Pietras K, Rubin K, Sjöblom T, Buchdunger E, Sjöquist M, Heldin CH, Ostman A. Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. *Cancer Res* 2002;62:5476–5484
438. Pietras K, Stumm M, Hubert M, Buchdunger E, Rubin K, Heldin CH, McSheehy P, Wartmann M, Ostman A. STI571 enhances the therapeutic index of epothilone B by a tumor-selective increase of drug uptake. *Clin Cancer Res* 2003;9:3779–3787
439. Hwang RF, Yokoi K, Bucana CD, Tsan R, Killion JJ, Evans DB, Fidler IJ. Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. *Clin Cancer Res* 2003;9:6534–6544
440. Heldin CH, Rubin K, Pietras K, Ostman A. High interstitial fluid pressure—An obstacle in cancer therapy. *Nat Rev Cancer* 2004;4:806–813
441. Heinrich MC, McArthur GA, Demetri GD, Joensuu H, Bono P, Herrmann R, Hirte H, Cresta S, Koslin DB, Corless CL, Dirnhofer S, van Oosterom AT, Nikolova Z, Dimitrijevic S, Fletcher JA. Clinical and molecular studies of the effect of imatinib on advanced aggressive fibromatosis (desmoid tumor). *J Clin Oncol* 2006;24:1195-1203
442. Basciani S, Brama M, Mariani S, De Luca G, Arizzi M, Vesci L, Pisano C, Dolci S, Spera G, Gnessi L. Imatinib mesylate inhibits Leydig cell tumor growth: evidence for in vitro and in vivo activity. *Cancer Res* 2005;65:1897-1903
443. Kantarjian HM, Cortes JE, O'Brien S, Luthra R, Giles F, Verstovsek S, Faderl S, Thomas D, Garcia-Manero G, Rios MB, Shan J, Jones D, Talpaz M. Long-term survival benefit and improved complete cytogenetic and molecular response rates with imatinib mesylate in Philadelphia chromosome-positive chronic-phase chronic myeloid leukemia after failure of interferon-alpha. *Blood* 2004;104:1979-1988
444. Melichar B, Voboril Z, Nozicka J, Ryska A, Urminska H, Vanecek T, Michal M. Pathological complete response in advanced gastrointestinal stromal tumor after imatinib therapy. *Intern Med* 2005;44:1163-1168

445. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249-257
446. Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, Menger MD, Ullrich A, Vajkoczy P. Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB J* 2004;18:338-340
447. Risau W, Drexler H, Mironov V, Smits A, Siegbahn A, Funa K, Heldin CH. Platelet-derived growth factor is angiogenic in vivo. *Growth Factors* 1992;7:261-266
448. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 2003;111:1287-1295
449. Abdollahi A, Lipson KE, Han X, Krempien R, Trinh T, Weber KJ, Hahnfeldt P, Hlatky L, Debus J, Howlett AR, Huber PE. SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. *Cancer Res* 2003;63:3755-3763
450. Motzer RJ, Rini BI, Bukowski RM, Curti BD, George DJ, Hudes GR, Redman BG, Margolin KA, Merchan JR, Wilding G, Ginsberg MS, Bacik J, Kim ST, Baum CM, Michaelson MD. Sunitinib in patients with metastatic renal cell carcinoma. *JAMA* 2006;295:2516-2524
451. Strumberg D. Preclinical and clinical development of the oral multikinase inhibitor sorafenib in cancer treatment. *Drugs Today (Barc)* 2005;41:773-784
452. Budach W, Taghian A, Freeman J, Gioioso D, Suit HD. Impact of stromal sensitivity on radiation response of tumors. *J Natl Cancer Inst* 1993;85:988-993
453. Ponten A, Li X, Thoren P, Aase K, Sjoblom T, Ostman A, Eriksson U. Transgenic overexpression of platelet-derived growth factor-C in the mouse heart induces cardiac fibrosis, hypertrophy, and dilated cardiomyopathy. *Am J Pathol* 2003;163:673-682
454. Li M, Ping G, Plathow C, Trinh T, Lipson KE, Hauser K, Krempien R, Debus J, Abdollahi A, Huber PE. Small molecule receptor tyrosine kinase inhibitor of platelet-derived growth factor signaling (SU9518) modifies radiation response in fibroblasts and endothelial cells. *BMC Cancer* 2006;6:79

455. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002;2:727-739
456. Geng L, Donnelly E, McMahon G, Lin PC, Sierra-Rivera E, Oshinka H, Hallahan DE. Inhibition of vascular endothelial growth factor receptor signaling leads to reversal of tumor resistance to radiotherapy. *Cancer Res* 2001;61:2413-2419
457. Holdhoff M, Kreuzer KA, Appelt C, Scholz R, Na IK, Hildebrandt B, Riess H, Jordan A, Schmidt CA, Van Etten RA, Dorken B, le Coutre P. Imatinib mesylate radiosensitizes human glioblastoma cells through inhibition of platelet-derived growth factor receptor. *Blood Cells Mol Dis* 2005;34:181-185
458. Plathow C, Li M, Gong P, Zieher H, Kiessling F, Peschke P, Kauczor HU, Abdollahi A, Huber PE. Computed tomography monitoring of radiation-induced lung fibrosis in mice. *Invest Radiol* 2004;39:600-609
459. Raines EW. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev* 2004;15:237-254
460. Majesky MW, Daemen MJ, Schwartz SM. α 1-adrenergic stimulation of platelet-derived growth factor A-chain gene expression in rat aorta. *J Biol Chem* 1990;265:1082-1088
461. Negoro N, Kanayama Y, Haraguchi M, Umetani N, Nishimura M, Konishi Y, Iwai J, Okamura M, Inoue T, Takeda T. Blood pressure regulates platelet-derived growth factor A-chain gene expression in vascular smooth muscle cells in vivo. An autocrine mechanism promoting hypertensive vascular hypertrophy. *J Clin Invest* 1995;95:1140-1150
462. Mondy JS, Lindner V, Miyashiro JK, Berk BC, Dean RH, Geary RL. Platelet-derived growth factor ligand and receptor expression in response to altered blood flow in vivo. *Circ Res* 1997;81:320-327
463. Ross R, Glomset JA. The pathogenesis of atherosclerosis. *N Engl J Med* 1976;295:369-377
464. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* 1993;362:801-809

465. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685–1695
466. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 1991;253:1129–1132
467. Banai S, Wolf Y, Golomb G, Pearle A, Waltenberger J, Fishbein I, Schneider A, Gazit A, Perez L, Huber R et al. PDGF-receptor tyrosine kinase blocker AG1295 selectively attenuates smooth muscle cell growth in vitro and reduces neointimal formation after balloon angioplasty in swine. *Circulation* 1998;97:1960–1969
468. Yamasaki Y, Miyoshi K, Oda N, Watanabe M, Miyake H, Chan J, Wang X, Sun L, Tang C, McMahon G. Weekly dosing with the platelet-derived growth factor receptor tyrosine kinase inhibitor SU9518 significantly inhibits arterial stenosis. *Circ Res* 2001;88:630–636
469. Giese NA, Marijianowski MM, McCook O, Hancock A, Ramakrishnan V, Fretto LJ, Chen C, Kelly AB, Koziol JA, Wilcox JN et al. The role of α and β platelet-derived growth factor receptor in the vascular response to injury in nonhuman primates. *Arterioscler Thromb Vasc Biol* 1999;19:900–909
470. Sano H, Sudo T, Yokode M, Murayama T, Kataoka H, Takakura N, Nishikawa S, Nishikawa SI, Kita T. Functional blockade of platelet-derived growth factor receptor- β but not of receptor- α prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. *Circulation* 2001;103:2955–2960
471. Kozaki K, Kaminski WE, Tang J, Hollenbach S, Lindahl P, Sullivan C, Yu JC, Abe K, Martin PJ, Ross R et al. Blockade of platelet-derived growth factor or its receptors transiently delays but does not prevent fibrous cap formation in ApoE null mice. *Am J Pathol* 2002;161:1395–1407
472. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J Clin Invest* 1992;89:507–511
473. Wagaster D, Zhu C, Bjorck HM, Eriksson P. Effects of PDGF-C and PDGF-D on monocyte migration and MMP-2 and MMP-9 expression. *Atherosclerosis* 2009;202:415–423

474. Buetow BS, Tappan KA, Crosby JR, Seifert RA, Bowen-Pope DF. Chimera analysis supports a predominant role of PDGFR β in promoting smooth-muscle cell chemotaxis after arterial injury. *Am J Pathol* 2003;163:979–984
475. Tang J, Kozaki K, Farr AG, Martin PJ, Lindahl P, Betsholtz C, Raines EW. The absence of platelet-derived growth factor-B in circulating cells promotes immune and inflammatory responses in atherosclerosis-prone ApoE^{-/-} mice. *Am J Pathol* 2005;167:901–912
476. Newton CS, Loukinova E, Mikhailenko I, Ranganathan S, Gao Y, Haudenschild C, Strickland DK. Platelet-derived growth factor receptor- β (PDGFR- β) activation promotes its association with the low density lipoprotein receptor-related protein (LRP). Evidence for co-receptor function. *J Biol Chem* 2005;280:27872–27878
477. Boucher P, Liu P, Gotthardt M, Hiesberger T, Anderson RG, Herz J. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. *J Biol Chem* 2002;277:15507–15513
478. Loukinova E, Ranganathan S, Kuznetsov S, Gorlatova N, Migliorini MM, Loukinov D, Ulery PG, Mikhailenko I, Lawrence DA, Strickland DK. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function between LRP and the PDGF. *J Biol Chem* 2002;277:15499–15506
479. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: Role in vascular wall integrity and protection from atherosclerosis. *Science* 2003;300:329–332
480. Boucher P, Li WP, Matz RL, Takayama Y, Auwerx J, Anderson RG, Herz J. LRP1 functions as an atheroprotective integrator of TGF β and PDFG signals in the vascular wall: Implications for Marfan syndrome. *PLoS ONE* 2007;2:e448
481. Takayama Y, May P, Anderson RG, Herz J. Low density lipoprotein receptor-related protein 1 (LRP1) controls endocytosis and c-CBL-mediated ubiquitination of the platelet-derived growth factor receptor β (PDGFR β) *J Biol Chem* 2005;280:18504–18510
482. Humbert M, Monti G, Fartoukh M, Magnan A, Brenot F, Rain B, Capron F, Galanaud P, Duroux P, Simonneau G et al. Platelet-derived growth factor expression in

primary pulmonary hypertension: Comparison of HIV seropositive and HIV seronegative patients. *Eur Respir J* 1998;11:554–559

483. Balasubramaniam V, Le Cras TD, Ivy DD, Grover TR, Kinsella JP, Abman SH. Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L826–L833

484. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W et al. (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 2005;115:2811–2821

485. Hansmann G, Wagner RA, Schellong S, Perez VA, Urashima T, Wang L, Sheikh AY, Suen RS, Stewart DJ, Rabinovitch M. Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor- γ activation. *Circulation* 2007;115:1275–1284

486. Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M et al. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 2002;105:2893–2898

487. Wang Y, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A. Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J Biol Chem* 2005;280:18341–18347

488. Jones R, Capen D, Jacobson M. PDGF and microvessel wall remodeling in adult lung: Imaging PDGF-R β and PDGF-BB molecules in progenitor smooth muscle cells developing in pulmonary hypertension. *Ultrastruct Pathol* 2006;30:267–281

489. Ghofrani HA, Seeger W, Grimminger F. Imatinib for the treatment of pulmonary arterial hypertension. *N Engl J Med* 2005;353:1412–1413

490. Akiyama H, Kachi S, Silva RL, Umeda N, Hackett SF, McCauley D, McCauley T, Zoltoski A, Epstein DM, Campochiaro PA. Intraocular injection of an aptamer that binds PDGF-B: A potential treatment for proliferative retinopathies. *J Cell Physiol* 2006;207:407–412

491. Jo N, Mailhos C, Ju M, Cheung E, Bradley J, Nishijima K, Robinson GS, Adamis AP, Shima DT. Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. *Am J Pathol* 2006;168:2036–2053
492. Enge M, Bjarnegard M, Gerhardt H, Gustafsson E, Kalen M, Asker N, Hammes HP, Shani M, Fassler R, Betsholtz C. Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *EMBO J* 2002;21:4307-4316
493. Lindblom P, Gerhardt H, Liebner S, Abramsson A, Enge M, Hellström M, Backstrom G, Fredriksson S, Landegren U, Nyström HC et al. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes and Dev* 2003;17:1835–1840
494. Hammes, H.P., Lin, J., Renner, O., Shani, M., Lundqvist, A., Betsholtz, C., Brownlee, M., Deutsch, U. (2002) Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 51:3107–3112
495. Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine Growth Factor Rev* 2004;15:215–228
496. Bonner JC. Regulation of PDGF and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev* 2004;15:255–273
497. Martinet Y, Rom WN, Grotendorst GR, Martin GR, Crystal RG. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. *N Engl J Med* 1987;317:202–209
498. Antoniades HN, Bravo MA, Avila RE, Galanopoulos T, Neville-Golden J, Maxwell M, Selman M. Platelet-derived growth factor in idiopathic pulmonary fibrosis. *J Clin Invest* 1990;86:1055-1064
499. Homma S, Nagaoka I, Abe H, Takahashi K, Seyama K, Nukiwa T, Kira S. Localization of platelet-derived growth factor and insulin-like growth factor I in the fibrotic lung. *Am J Respir Crit Care Med* 1995;152:2084–2089
500. Zhuo Y, Zhang J, Laboy M, Lasky JA. Modulation of PDGF-C and PDGF-D expression during bleomycin-induced lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L182–L188

501. Bonner JC, Goodell AL, Coin PG, Brody AR. Chrysotile asbestos upregulates gene expression and production of α -receptors for platelet-derived growth factor (PDGF-AA) on rat lung fibroblasts. *J Clin Invest* 1993;92:425–430
502. Abdollahi A, Li M, Ping G, Plathow C, Domhan S, Kiessling F, Lee LB, McMahon G, Gröne HJ, Lipson KE et al. Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. *J Exp Med* 2005;201:925–935
503. Rice AB, Moomaw, CR, Morgan DL, Bonner JC. Specific inhibitors of platelet-derived growth factor or epidermal growth factor receptor tyrosine kinase reduce pulmonary fibrosis in rats. *Am J Pathol* 1999;155:213–221
504. Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR- β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 1999 ;126:3047–3055
505. Pinzani M, Milani S, Herbst H, DeFranco R, Grappone C, Gentilini A, Caligiuri A, Pellegrini G, Ngo DV, Romanelli RG et al. Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. *Am J Pathol* 1996;148:785–800
506. Borkham-Kamphorst E, van Roeyen CR, Ostendorf T, Floege J, Gressner AM, Weiskirchen R. Pro-fibrogenic potential of PDGF-D in liver fibrosis. *J Hepatol* 2007;46:1064–1074
507. Distler JH, Jüngel A, Huber LC, Schulze-Horsel U, Zwerina J, Gay RE, Michel BA, Hauser T, Schett G, Gay S et al. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. *Arthritis Rheum* 2007;56:311–322
508. Gay S, Jones RE, Huang GQ, Gay RE. Immunohistologic demonstration of platelet-derived growth factor (PDGF) and sis-oncogene expression in scleroderma. *J Invest Dermatol* 1989;92:301–303
509. Klareskog L, Gustafsson R, Scheynius A, Hällgren R. Increased expression of platelet-derived growth factor type B receptors in the skin of patients with systemic sclerosis. *Arthritis Rheum* 1990;33:1534–1541

510. Lindmark G, Sundberg C, Glimelius B, Pählman L, Rubin K, Gerdin B. Stromal expression of platelet-derived growth factor β -receptor and platelet-derived growth factor B-chain in colorectal cancer. *Lab Invest* 1993;69:682–689
511. Reuterdahl C, Sundberg C, Rubin K, Funa K, Gerdin B. Tissue localization of β receptors for platelet-derived growth factor and platelet-derived growth factor B chain during wound repair in humans. *J Clin Invest* 1993;91:2065–2075
512. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1 α from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. *J Clin Invest* 1999;103:1253–1260
513. Paulsson Y, Hammacher A, Heldin CH, Westermark B. Possible positive autocrine feedback in the prereplicative phase of human fibroblasts. *Nature* 1987;328:715–717
514. Yamakage A, Kikuchi K, Smith EA, LeRoy EC, Trojanowska M. Selective upregulation of platelet-derived growth factor α receptors by transforming growth factor β in scleroderma fibroblasts. *J Exp Med* 1992;175:1227–1234
515. Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, Fraticelli P, Sambo P, Funaro A, Kazlauskas A et al. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med* 2006;354:2667–2676
516. Svegliati S, Olivieri A, Campelli N, Luchetti M, Poloni A, Trappolini S, Moroncini G, Bacigalupo A, Leoni P, Avvedimento EV et al. Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood* 2007;110:237–241
517. Robson MC, Phillips LG, Thomason A, Robson LE, Pierce GF. Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. *Lancet* 1992;339:23–25
518. Pierce GF, Tarpley JE, Tseng J, Bready J, Chang D, Kenney WC, Rudolph R, Robson MC, Vande Berg J, Reid P. Detection of platelet-derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic nonhealing wounds. *J Clin Invest* 1995;96:1336–1350
519. Smiell JM, Wieman TJ, Steed DL, Perry BH, Sampson AR, Schwab BH. Efficacy and safety of becaplermin (recombinant human platelet-derived growth factor-BB) in patients with nonhealing, lower extremity diabetic ulcers: A combined analysis of four randomized studies. *Wound Repair Regen* 1999;7:335–346

520. Edmonds M, Bates M, Doxford M, Gough A, Foster A. (2000) New treatments in ulcer healing and wound infection. *Diabetes Metab Res Rev* 2000;16(Suppl1) S51–S54
521. Perry BH, Sampson AR, Schwab BH, Karim MR, Smiell JM. A meta-analytic approach to an integrated summary of efficacy: A case study of becaplermin gel. *Control Clin Trials* 2002;23:389–408
522. Steed DL. Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity ulcers. *Plast Reconstr Surg* 2006;117:143S–149S
523. Sarment DP, Cooke JW, Miller SE, Jin Q, McGuire MK, Kao RT, McClain PK, McAllister BS, Lynch SE, Giannobile WV. Effect of rhPDGF-BB on bone turnover during periodontal repair. *J Clin Periodontol* 2006;33:135–140
524. Nevins M, Camelo M, Nevins ML, Schenk RK, Lynch SE. Periodontal regeneration in humans using recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and allogenic bone. *J Periodontol* 2003;74:1282–1292
525. Fellström B, Klareskog L, Heldin CH, Larsson E, Rönstrand L, Terracio L, Tufveson G, Wahlberg J, Rubin K. Platelet-derived growth factor receptors in the kidney-upregulated expression in inflammation. *Kidney Int* 1989;36:1099–1102
526. Johnson RJ, Raines EW, Floege J, Yoshimura A, Pritzl P, Alpers C, Ross R. Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. *J Exp Med* 1992;175:1413–1416
527. Floege J, Ostendorf T, Janssen U, Burg M, Radeke HH, Vargeese C, Gill SC, Green LS, Janjic N. Novel approach to specific growth factor inhibition in vivo: antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. *Am J Pathol* 1999;154:169–179
528. Gilbert RE, Kelly DJ, McKay T, Chadban S, Hill PA, Cooper ME, Atkins RC, Nikolic-Paterson DJ. PDGF signal transduction inhibition ameliorates experimental mesangial proliferative glomerulonephritis. *Kidney Int* 2001;59:1324–1332
529. Tang WW, Ulich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, Van GY, Tarpley JE, Yee JS. Platelet-derived growth factor-BB induces renal tubulointerstitial myofibroblast formation and tubulointerstitial fibrosis. *Am J Pathol* 1996;148:1169–1180

530. Shimoda HK, Yamamoto M, Shide K, Kamezaki K, Matsuda T, Ogawa K, Harada M, Shimoda K. Chronic thrombopoietin overexpression induces mesangioproliferative glomerulopathy in mice. *Am J Hematol* 2007;82:802–806
531. Ostendorf T, Rong S, Boor P, Wiedemann S, Kunter U, Haubold U, van Roeyen CR, Eitner F, Kawachi H, Starling G et al. Antagonism of PDGF-D by human antibody CR002 prevents renal scarring in experimental glomerulonephritis. *J Am Soc Nephrol* 2006;17:1054–1062
532. Ostendorf T, van Roeyen CR, Peterson JD, Kunter U, Eitner F, Hamad AJ, Chan G, Jia XC, Macaluso J, Gazit-Bornstein G et al. A fully human monoclonal antibody (CR002) identifies PDGF-D as a novel mediator of mesangioproliferative glomerulonephritis. *J Am Soc Nephrol* 2003;14:2237–2247
533. Zymek P, Bujak M, Chatila K, Cieslak A, Thakker G, Entman ML, Frangogiannis NG. The role of platelet-derived growth factor signaling in healing myocardial infarcts. *J Am Coll Cardiol* 2006;48:2315–2323
534. Pontén A, Folestad EB, Pietras K, Eriksson U. Platelet-derived growth factor D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice. *Circ Res* 2005;97:1036–1045
535. Tuuminen R, Nykänen A, Keränen MA, Krebs R, Alitalo K, Koskinen PK, Lemström KB. The effect of platelet-derived growth factor ligands in rat cardiac allograft vasculopathy and fibrosis. *Transplant Proc* 2006;38:3271–3273
536. Simm A, Nestler M, Hoppe V. Mitogenic effect of PDGF-AA on cardiac fibroblasts. *Basic Res Cardiol* 1998;93:(Suppl 3)40–43
537. Nishioka T, Suzuki M, Onishi K, Takakura N, Inada H, Yoshida T, Hiroe M, Imanaka-Yoshida K. Eplerenone attenuates myocardial fibrosis in the angiotensin II-induced hypertensive mouse: Involvement of tenascin-C induced by aldosterone-mediated inflammation. *J Cardiovasc Pharmacol* 2007;49:261–268
538. Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H et al. Krüppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med* 2002;8:856–863

539. Iida H, Seifert R, Alpers CE, Gronwald RG, Phillips PE, Pritzl P, Gordon K, Gown AM, Ross R, Bowen-Pope DF. Platelet-derived growth factor (PDGF) and PDGF receptor are induced in mesangial proliferative nephritis in the rat. *Proc Natl Acad Sci USA* 1991;88:6560-6564
540. Wilcox JN, Smith KM, Williams LT, Schwartz SM, Gordon D. Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. *J Clin Invest* 1988;82:1134-1143
541. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin CH, Westermark B, Nister M. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res* 1992;52:3213-3219
542. Nister M, Libermann TA, Betsholtz C, Pettersson M, Claesson-Welsh L, Heldin CH, Schlessinger J, Westermark B. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. *Cancer Res* 1988;48:3910-3918
543. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. *Cancer Res* 2002;62:3729-3735
544. Abratt RP, Morgan GW, Silvestri G, Willcox P. Pulmonary complications of radiation therapy. *Clin Chest Med* 2004;25:167-177
545. Trott KR, Herrmann T, Kasper M. Target cells in radiation pneumopathy. *Int J Radiat Oncol Biol Phys* 2004;58:463-469
546. Chen ES, Greenlee BM, Wills-Karp M, Moller DR. Attenuation of lung inflammation and fibrosis in interferon-gamma-deficient mice after intratracheal bleomycin. *Am J Respir Cell Mol Biol* 2001;24:545-555
547. Sunyach MP, Falchero L, Pommier P, Perol M, Arpin D, Vincent M, Boutry D, Rebatu P, Ginestet C, Martel-Lafay I, Perol D, Carrie C. Prospective evaluation of early lung toxicity following three-dimensional conformal radiation therapy in non-small-cell lung cancer: preliminary results. *Int J Radiat Oncol Biol Phys* 2000;48:459-463

548. Rosenzweig KE, Mychalczak B, Fuks Z, Hanley J, Burman C, Ling CC, Armstrong J, Ginsberg R, Kris MG, Raben A, Leibel S. Final report of the 70.2-Gy and 75.6-Gy dose levels of a phase I dose escalation study using three-dimensional conformal radiotherapy in the treatment of inoperable non-small cell lung cancer. *Cancer J* 2000;6:82-87
549. Kong FM, Hayman JA, Griffith KA, Kalemkerian GP, Arenberg D, Lyons S, Turrisi A, Lichter A, Fraass B, Eisbruch A, Lawrence TS, Ten Haken RK. Final toxicity results of a radiation-dose escalation study in patients with non-small-cell lung cancer (NSCLC): Predictors for radiation pneumonitis and fibrosis. *Int J Radiat Oncol Biol Phys* 2006;65:1075-1086
550. Heldin CH. Simultaneous induction of stimulatory and inhibitory signals by PDGF. *FEBS Lett* 1997; 410:17-21
551. Broekelmann TJ, Limper AH, Colby TV, McDonald JA. Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci USA* 1991;88:6642-6646
552. Johnston CJ, Piedboeuf B, Rubin P, Williams JP, Baggs R, Finkelstein JN. Early and persistent alterations in the expression of interleukin-1 alpha, interleukin-1 beta and tumor necrosis factor alpha mRNA levels in fibrosis-resistant and sensitive mice after thoracic irradiation. *Radiat Res* 1996;145:762-767
553. Lindroos PM, Coin PG, Osornio-Vargas AR, Bonner JC. Interleukin 1 beta (IL-1 beta) and the IL-1 beta-alpha 2-macroglobulin complex upregulate the platelet-derived growth factor alpha-receptor on rat pulmonary fibroblasts. *Am J Respir Cell Mol Biol* 1995;13:455-465
554. Heinzelmann F, Jendrossek V, Lauber K, Nowak K, Eldh T, Boras R, Handrick R, Henkel M, Martin C, Uhlig S, Kohler D, Eltzhig HK, Wehrmann M, Budach W, Belka C. Irradiation-induced pneumonitis mediated by the CD95/CD95-ligand system. *J Natl Cancer Inst* 2006;98:1248-1251
555. Hill RP, Rodemann HP, Hendry JH, Roberts SA, Anscher MS. Normal tissue radiobiology: from the laboratory to the clinic. *Int J Radiat Oncol Biol Phys* 2001;49:353-365

556. Tada H, Ogushi F, Tani K, Nishioka Y, Miyata J, Sato K, Asano T, Sone S. Increased binding and chemotactic capacities of PDGF-BB on fibroblasts in radiation pneumonitis. *Radiat Res* 2003;159:805-811
557. Gurujeyalakshmi G, Hollinger MA, Giri SN. Inhibitory effect of interferon gamma, interleukin-1, interleukin-6 and platelet-derived growth factor-A mRNA expression in bleomycin-mouse model of lung fibrosis. *Res Commun Pharmacol Toxicol* 1996;1-15
558. Liu JY, Morris GF, Lei WH, Hart CE, Lasky JA, Brody AR. Rapid activation of PDGF-A and -B expression at sites of lung injury in asbestos-exposed rats. *Am J Respir Cell Mol Biol* 1997;17:129-140
559. Hoyle GW, Li J, Finkelstein JB, Eisenberg T, Liu JY, Lasky JA, Athas G, Morris GF, Brody AR. Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor. *Am J Pathol* 1999;154:1763-1775
560. Yoshida M, Sakuma J, Hayashi S, Abe K, Saito I, Harada S, Sakatani M, Yamamoto S, Matsumoto N, Kaneda Y. A histologically distinctive interstitial pneumonia induced by overexpression of the interleukin 6, transforming growth factor beta 1, or platelet-derived growth factor B gene. *Proc Natl Acad Sci USA* 1995;92:9570-9574
561. Duan DS, Pazin MJ, Fretto LJ, Williams LT. A functional soluble extracellular region of the platelet-derived growth factor (PDGF) beta-receptor antagonizes PDGF-stimulated responses. *J Biol Chem* 1991;266:413-418
562. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 1991;253:1129-1132
563. Blatti SP, Foster DN, Ranganathan G, Moses HL, Getz MJ. Induction of fibronectin gene transcription and mRNA is a primary response to growth-factor stimulation of AKR-2B cells. *Proc Natl Acad Sci USA* 1988;85:1119-1123
564. Heldin P, Laurent TC, Heldin CH. Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. *Biochem J* 1989;258:919-922

565. Canalis E. Effect of platelet-derived growth factor on DNA and protein synthesis in cultured rat calvaria. *Metabolism* 1981;30:970-975
566. Schonherr E, Jarvelainen HT, Sandell LJ, Wight TN. Effects of platelet-derived growth factor and transforming growth factor-beta 1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J Biol Chem* 1991;266:17640-17647
567. Zerwes HG, Risau W. Polarized secretion of a platelet-derived growth factor-like chemotactic factor by endothelial cells in vitro. *J Cell Biol* 1987;105:2037-2041
568. Dik WA, Versnel MA, Naber BA, Janssen DJ, van Kaam AH, Zimmermann LJ. Dexamethasone treatment does not inhibit fibroproliferation in chronic lung disease of prematurity. *Eur Respir J* 2003;21:842-847
569. Savikko J, Taskinen E, Von Willebrand E. Chronic allograft nephropathy is prevented by inhibition of platelet-derived growth factor receptor: tyrosine kinase inhibitors as a potential therapy. *Transplantation* 2003;75:1147-1153
570. Haydont V, Vozenin-Brotans M-C. Maintenance of radiation-induced intestinal fibrosis: Cellular and molecular features. *World J Gastroenterol* 2007;13:2675-2683
571. Sheaff M, Nicholls C, Hopster D, Martin J. Platelet-derived growth factor in radiation injury. *J Pathol* 1996;179S1:39A
572. Sartor RB. Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. *Gastroenterol Clin North Am* 1995; 24: 475-507
573. Denham JW, Hauer-Jensen M, Kron T, Langberg CW. Treatment-time-dependence models of early and delayed radiation injury in rat small intestine. *Int J Radiat Oncol Biol Phys* 2000; 48: 871-887
574. Dorr W, Hendry JH. Consequential late effects in normal tissues. *Radiother Oncol* 2001; 61: 223-231
575. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* 2004;4: 583-594

576. Bamias G, Martin C, Mishina M, Ross WG, Rivera-Nieves J, Marini M, Cominelli F. Proinflammatory effects of TH2 cytokines in a murine model of chronic small intestinal inflammation. *Gastroenterology* 2005; 128: 654-666
577. Strieter RM, Keane MP. Innate immunity dictates cytokine polarization relevant to the development of pulmonary fibrosis. *J Clin Invest* 2004; 114: 165-168
578. Barcellos-Hoff MH, Costes SV. A systems biology approach to multicellular and multi-generational radiation responses. *Mutat Res* 2006; 597: 32-38
579. Hovdenak N, Wang J, Sung CC et al. Clinical significance of increased gelatinolytic activity in the rectal mucosa during external beam radiation therapy of prostate cancer. *Int J Radiat Oncol Biol Phys* 2002;53:919-927
580. Strup-Perrot C, Mathe D, Linard C et al. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G875-885
581. Vozenin-Brotons MC, Milliat F, Sabourin JC, de Gouville AC, Francois A, Lasser P, Morice P, Haie-Meder C, Lusinchi A, Antoun S, Bourhis J, Mathe D, Girinsky T, Aigueperse J. Fibrogenic signals in patients with radiation enteritis are associated with increased connective tissue growth factor expression. *Int J Radiat Oncol Biol Phys* 2003; 56: 561-572
582. Zheng H, Wang J, Hauer-Jensen M. Role of mast cells in early and delayed radiation injury in the rat intestine. *Radiat Res* 2000;153:533-539
583. Martin P. Wound healing--aiming for perfect skin regeneration. *Science* 1997; 276: 75- 81
584. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002; 3: 349-363
585. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004; 84: 767-801
586. Hendrix JA, Wamhoff BR, McDonald OG, Sinha S, Yoshida T, Owens GK. 5' CArG degeneracy in smooth muscle alpha-actin is required for injury-induced gene suppression in vivo. *J Clin Invest* 2005; 115: 418-427

587. Bayreuther K, Rodemann HP, Hommel R, Dittmann K, Albiez M, Francz PI. Human skin fibroblasts in vitro differentiate along a terminal cell lineage. *Proc Natl Acad Sci USA* 1988; 85: 5112-5116
588. Rodemann HP, Bamberg M. Cellular basis of radiation-induced fibrosis. *Radiother Oncol* 1995; 35: 83-90
589. Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med* 2006;10:76-99
590. Desmouliere A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 1995; 146: 56-66

Appendix 1. Immunohistochemical methods for PDGF and receptor studies and risk assessment

Procedure

1. The following controls were used for each run performed.

Positive control section for each primary antibody used.
Negative control section [omitting primary antibody].
2. The sections were placed into a black plastic staining rack.
3. They were dewaxed in xylene for 2 changes of 2 minutes each.
4. The xylene was removed in alcohol for 2 changes of 2 minutes each.
5. The sections were placed into the sink and washed in tap water for 5 minutes.

For antigen retrieval:

- a. Take a microwave container and fill it with 1000ml of the appropriate antigen unmasking solution. (See Section 4 for the available solutions)
- b. Place the rack of slides into this and put on the perforated lid on.
- c. Place the container into the microwave oven and close the door.
- d. The default of the microwave is maximum power, so this need not be altered.
- e. Set the length of time required. Each press of the “Hour” button will increment by 10 minutes. Each press of the “Min” button will increment by 1 minute.

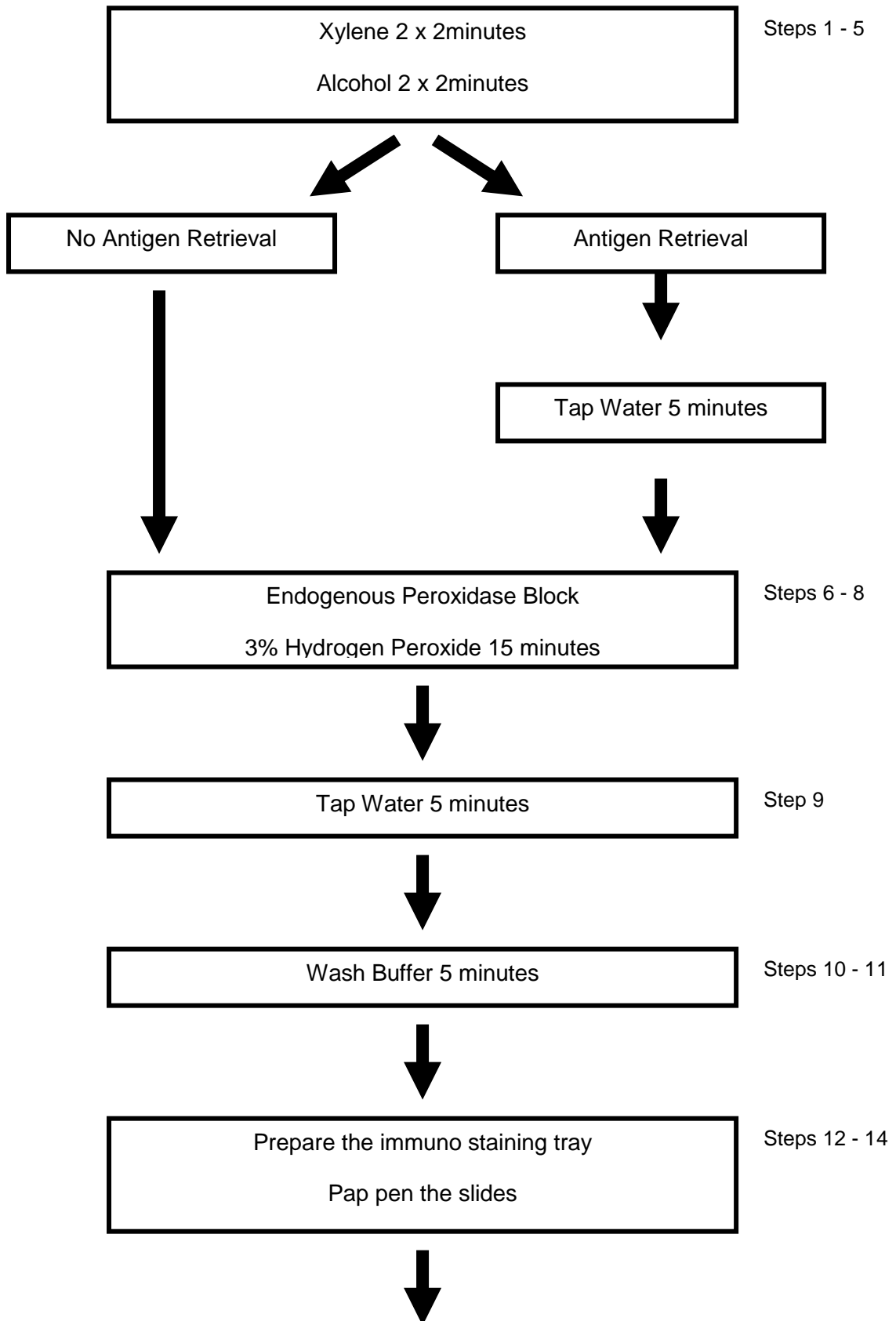
- f. When the time has been set, press the “Start” button to begin microwaving.
 - g. When the time has elapsed, the microwave oven will emit a ping to indicate that it has finished.
 - h. Using heat resistant gloves carefully remove the trough from the oven and place into the staining tray of running water in the sink beside the microwave oven.
 - i. Allow cool for 5 minutes.
 - j. Remove the rack and place into the sink and wash in tap water for 5 minutes.
 - k. Return to the staining procedure and continue with step 6.
6. At this stage avidin/biotin blocking was performed (if required)
7. The the endogenous peroxidase blocking solution was prepared by adding 6ml. of hydrogen peroxide to 194ml. of distilled water in a black staining trough.
8. The rack of sections was placed into the endogenous peroxidise blocking solution for 15 minutes.
9. The rack was removed and placed into the sink and washed in tap water for 5 minutes.
10. The black plastic staining trough previously used for the endogenous block was washed out with distilled water and filled with wash buffer obtained from the aspirator.

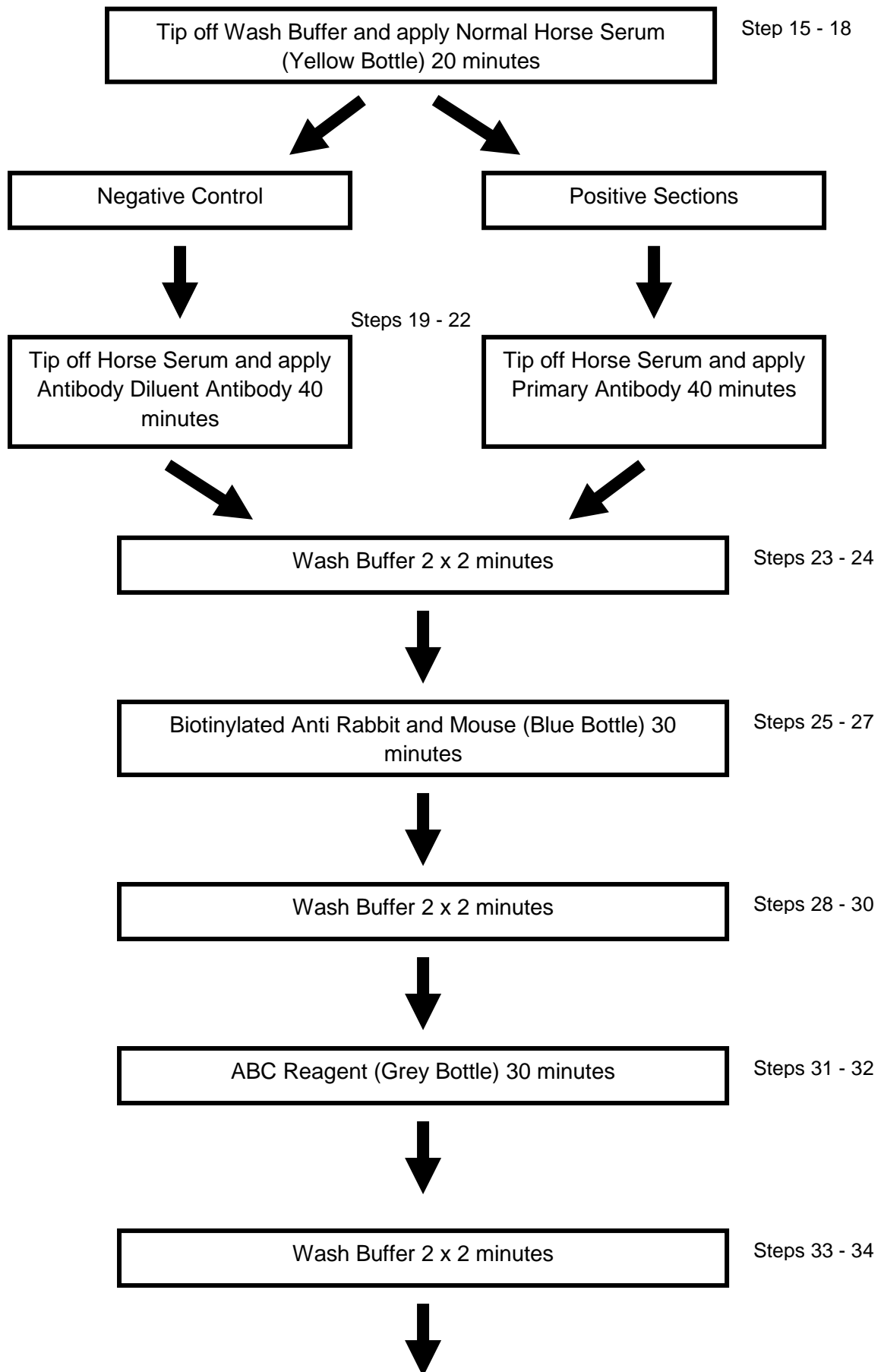
11. The rack of slides was placed into the wash buffer and left to soak for 5 minutes.
12. The immuno staining tray was prepared by adding a few drops of wash buffer to create a moist chamber.
13. Taking each slide in turn, the sections were wiped around to remove excess wash buffer and with a PAP pen a line was drawn across the slide above and below the section.
14. The sections were then placed onto the rack in the immuno staining tray and covered with wash buffer, being careful not to allow the sections to dry out.
15. When all of the sections were on the rack the wash buffer was tipped off.
16. Sufficient drops of normal blocking serum was applied to cover each section.
17. The lid was placed on the immuno staining tray and left for 20 minutes.
18. The primary antibody was prepared at the appropriate dilution using antibody diluent.
19. The normal blocking serum was tipped off.
20. The primary antibody was applied to each test section and the positive control section.
21. Only the antibody diluent was applied to the negative control section.
22. The immuno staining tray lid was replaced and left for 40 minutes.

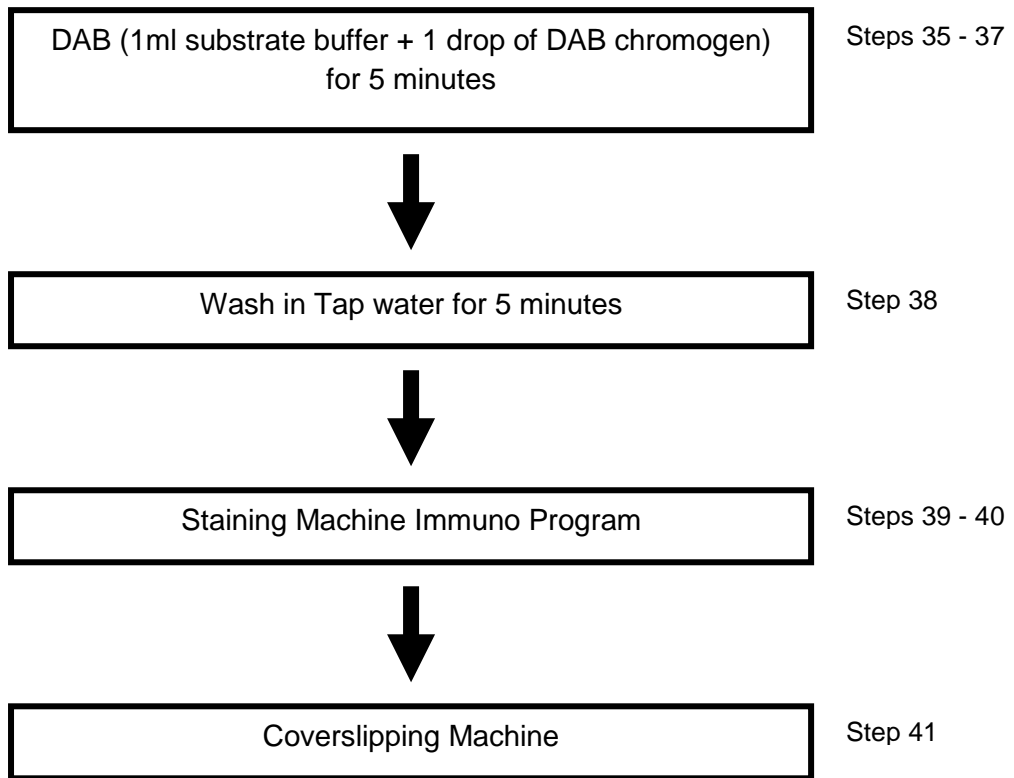
23. The primary antibody was rinsed off with wash buffer.
24. Each section was covered with wash buffer and the immuno staining tray lid was replaced and left for 2 minutes. The sections were rinse with wash buffer and covered again and left for a further 2 minutes.
25. The wash buffer was then tipped off.
26. The sections were all covered by drops of the universal biotinylated secondary antibody.
27. The immuno staining tray lid was replaced and left for 30 minutes.
28. The secondary antibody was rinsed off with wash buffer.
29. Each section was covered with wash buffer and the immuno staining tray lid was replaced and left for 2 minutes. The sections were rinsed again with wash buffer, covered and left for a further 2 minutes.
30. The wash buffer was tipped off.
31. Sufficient drops of the Vectastain elite ABC reagent were added to cover each section.
32. The immuno staining tray lid was replaced and left for 30 minutes.
33. The ABC reagent was rinsed off with wash buffer.
34. Each section was covered with wash buffer and the immuno staining tray lid was replaced and left for 2 minutes. The sections were rinsed with wash buffer and covered again and left for a further 2 minutes.

35. 1ml of substrate buffer from the Biogenex two component DAB kit was placed into a yellow topped tube.
36. One drop of the DAB chromogen was added from the kit and mixed well.
37. The wash buffer was tipped from the slides and the DAB solution was applied to each of the slides for 5 minutes.
38. The slides were washed in tap water for 5 minutes.
39. The slides were loaded into a machine staining rack (ensuring that all of the sections were facing in the direction of the arrow on the rack).
40. The remainder of the technique was automated, staining the sections with the haematoxylin, dehydrating in alcohol to remove water and washing in xylene before coverslipping.

Flow Chart







Safety Measures

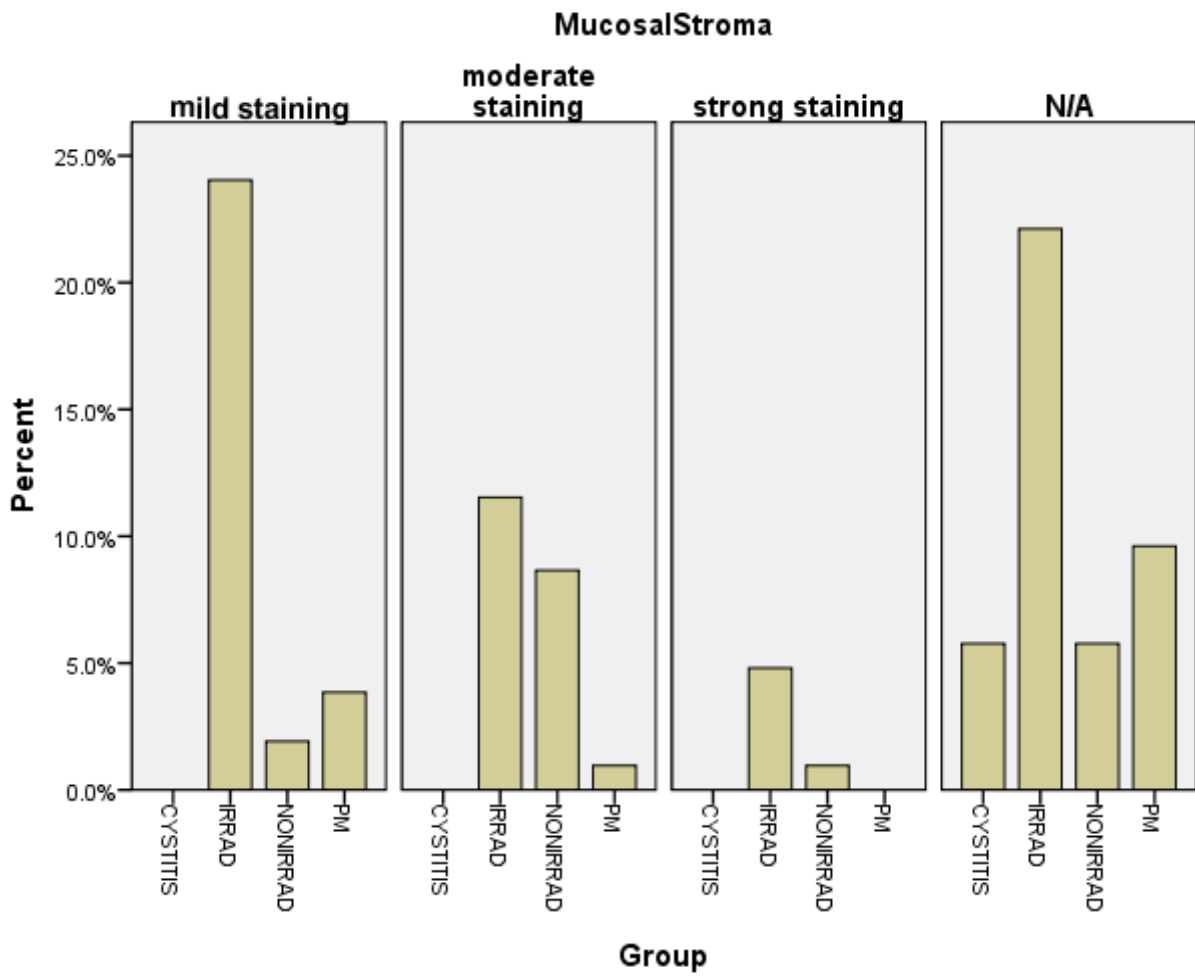
1. Wear personal protective clothing especially safety glasses at all times.
2. Do not undertake of activities likely to cause any reagents to come into contact with skin, eyes and mucosal surfaces.
3. Avoid skin contact with any chemical where possible by the use of forceps to carry out manipulations.
4. Ensure that there is adequate ventilation of the work area. All waste reagents must be disposed of in accordance with the manufacturer's instruction or local by laws.
5. All flammable reagents must be stored in the laboratory in fire cabinets where the volumes are kept as low as possible consistent with their frequency of use.
6. Bulk staining troughs must be used on a spillage containment tray and where possible within a fume hood.
7. Spillage containment kits are available to limit the extent of any spillage.
8. All reagent bottles must be marked with risk phrase labels and a departmental COSHH number, which indicates the page in the COSHH files where safety information can be found.
9. All hand mounting must be carried out in the mounting hood with extraction through a filter system.
10. Hydrogen peroxide must be stored in loosely capped black containers at 4°C.
11. Open hydrogen peroxide containers with caution if pressure has built up in the container it can spray out.

12. Wear gloves and discharge static electricity if weighing out diaminobenzidine tetrahydrochloride (DAB).
13. All weighing of solid chemicals must be carried out within balance extraction hoods.
14. All immunochemicals are measured using automatic pipettes with disposable tips.
15. Large bottles of chemicals must be transported in bottle carriers, unless a trolley is used.
16. Pipette tips and other plasticware must be discarded into the yellow plastic bins on each bench before placing in an orange clinical waste sack for disposal.
17. Sharps must be discarded into the sharps bin and not into the plasticware bins on the benches.
18. Dispose of each category of waste in the approved manner.
19. Under no circumstances should any attempt be made to operate the microwave oven with the door open or to tamper with the safety interlocks (door latches) or to insert anything into the safety interlock holes.
20. Do not place any object between the oven door and front face, or allow residues to accumulate on sealing surfaces.
21. Ensure that the door and door sealing surfaces are kept clean by wiping after use with first a damp cloth and then a final wipe with a soft dry cloth.
22. The microwave oven should not be operated if it has sustained damage to the door or door sealing area.
23. Do not use any metal containers or instruments in the microwave oven.

24. Only use microwave compatible containers.
25. Do not place any sealed containers in the microwave oven.
26. Use heat resistant gloves when removing items from the microwave oven.
27. Do not operate the microwave oven if it is empty.
28. Liquids should be left in the microwave oven for at least 20 seconds after it has stopped to allow the temperature to equalize.
29. Only use cling film suitable for use in a microwave.
30. Stand away from the oven when opening the microwave oven door. The hot air or steam which escapes can cause burns.
31. Keep the microwave oven vent ducts unobstructed.
32. Never use flammable materials in the microwave oven.
33. If materials inside the microwave oven should ignite, keep the oven door closed, turn oven off, and disconnect the power cord.
34. Do not touch or allow any object to cover the openings on the top of the outer case of the microwave oven during operation.
35. Cracked and damaged glassware should not be used.
36. Use heat resistant gloves when removing solutions from the microwave oven.

37. A visor, heat protective gloves and heavy-duty apron are available for wearing when using the pressure cooker.
38. The pressure cooker must be visually checked for damage before use.
39. Do not leave the pressure cooker unattended during use.
40. Do not lean over the cooker whilst in operation in case an explosive release of steam occurs

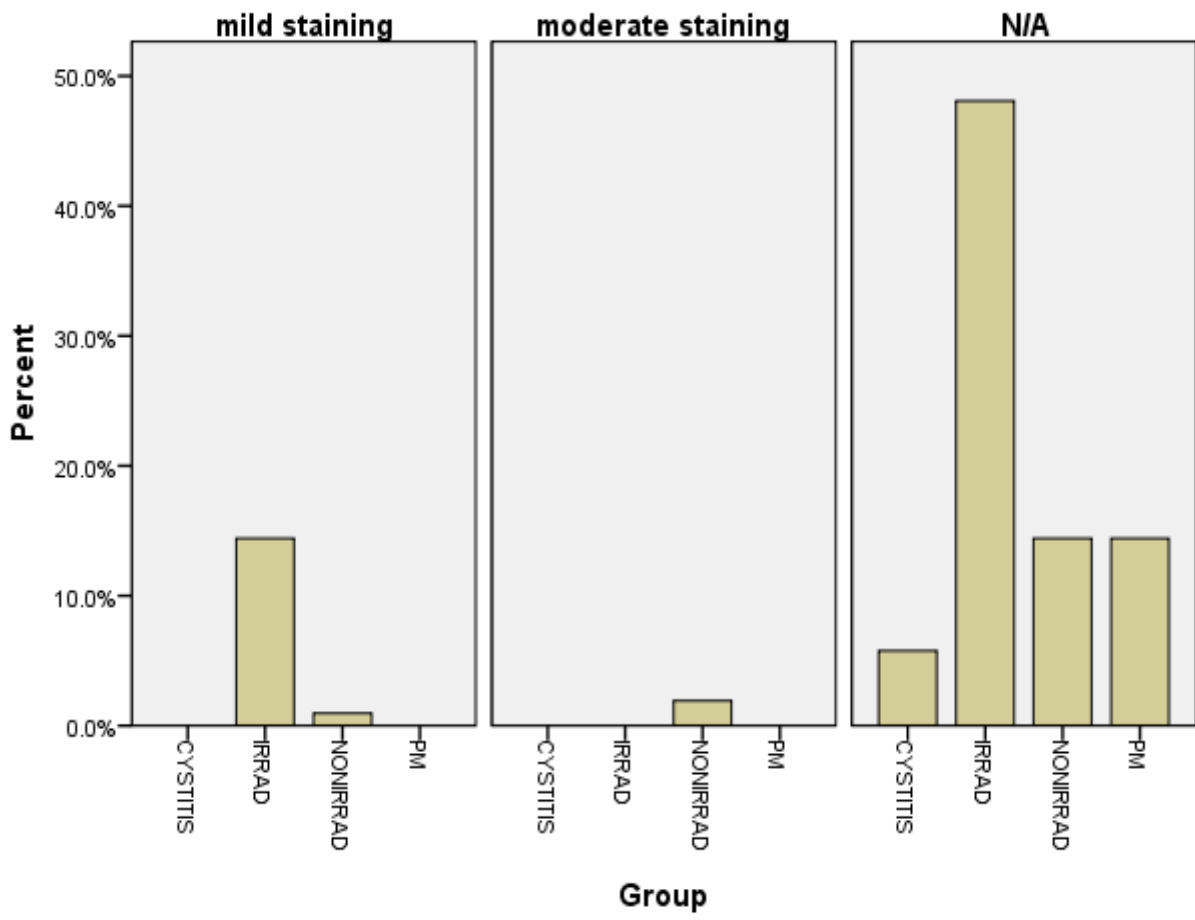
Appendix 2. Fibronectin data and statistics (N/A = negative/absent)



Mucosal Stroma Crosstabulation					
Group	Mucosal Stroma				
	mild staining	moderate staining	strong staining	N/A	Total
CYSTITIS	0	0	0	6	6
IRRAD	25	12	5	23	65
NONIRRAD	2	9	1	6	18
PM	4	1	0	10	15
	31	22	6	45	104

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	24.977^a	9	.003
Likelihood Ratio	26.929	9	.001
N of Valid Cases	104		

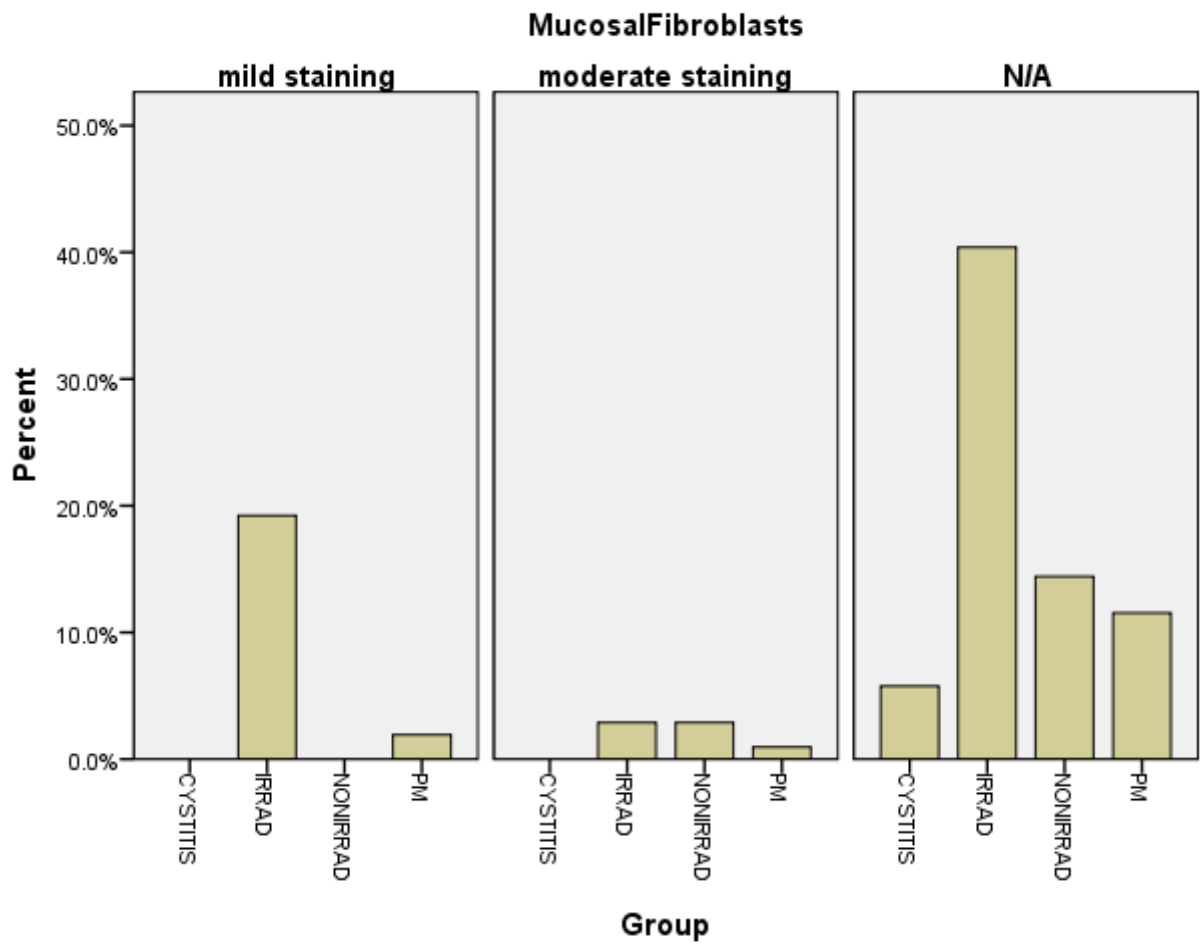
MucosalBasementMembrane



Mucosal Basement Membrane Crosstabulation

Group	Mucosal Basement Membrane			
	mild staining	moderate staining	N/A	Total
CYSTITIS	0	0	6	6
IRRAD	15	0	50	65
NONIRRAD	1	2	15	18
PM	0	0	15	15
	16	2	86	104

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	17.440^a	6	.008
Likelihood Ratio	18.124	6	.006
N of Valid Cases	104		



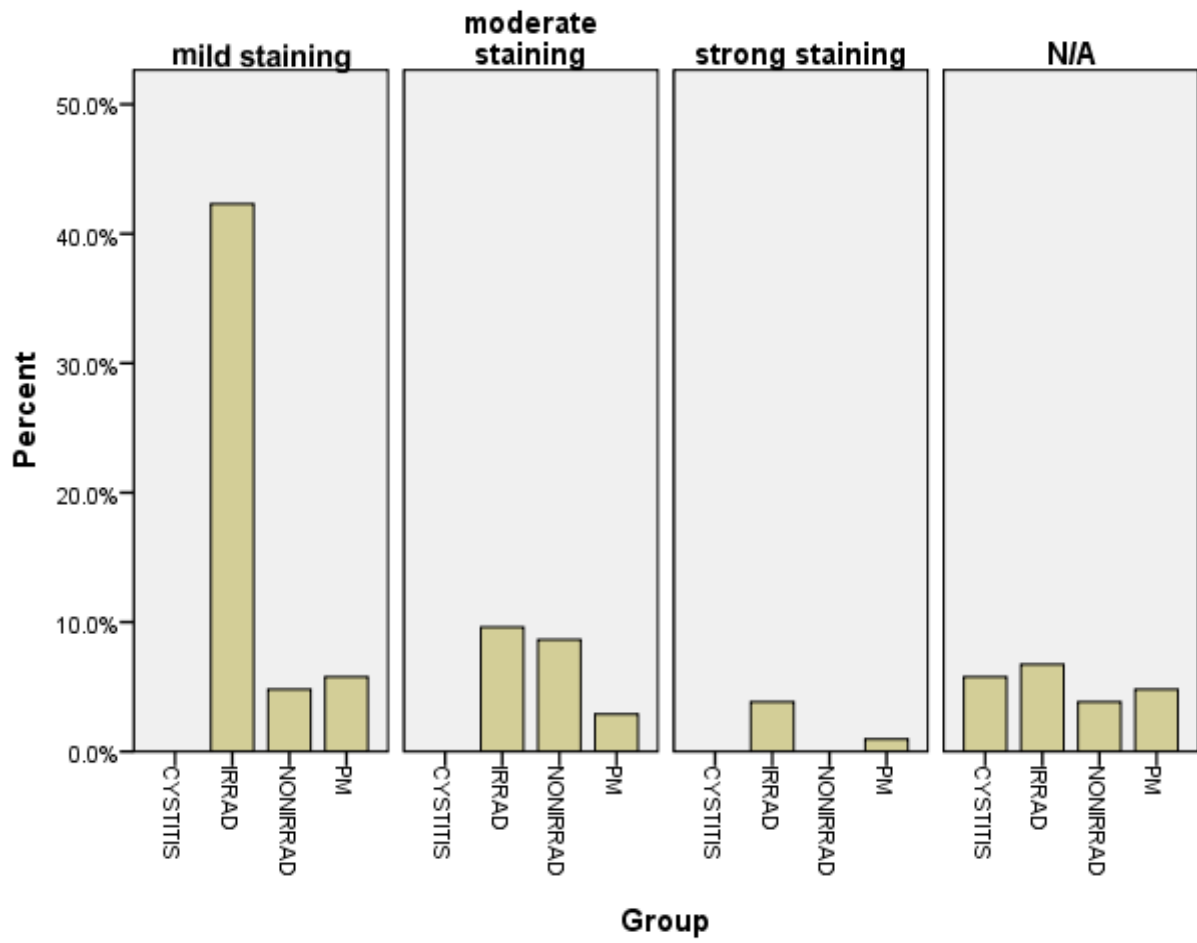
Mucosal Fibroblasts Crosstabulation

Group	Mucosal Fibroblasts			
	mild staining	moderate staining	N/A	Total
CYSTITIS	0	0	6	6
IRRAD	20	3	42	65
NONIRRAD	0	3	15	18
PM	2	1	12	15
	22	7	75	104

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	13.425^a	6	.037
Likelihood Ratio	17.825	6	.007
N of Valid Cases	104		

MucosalEndothelium



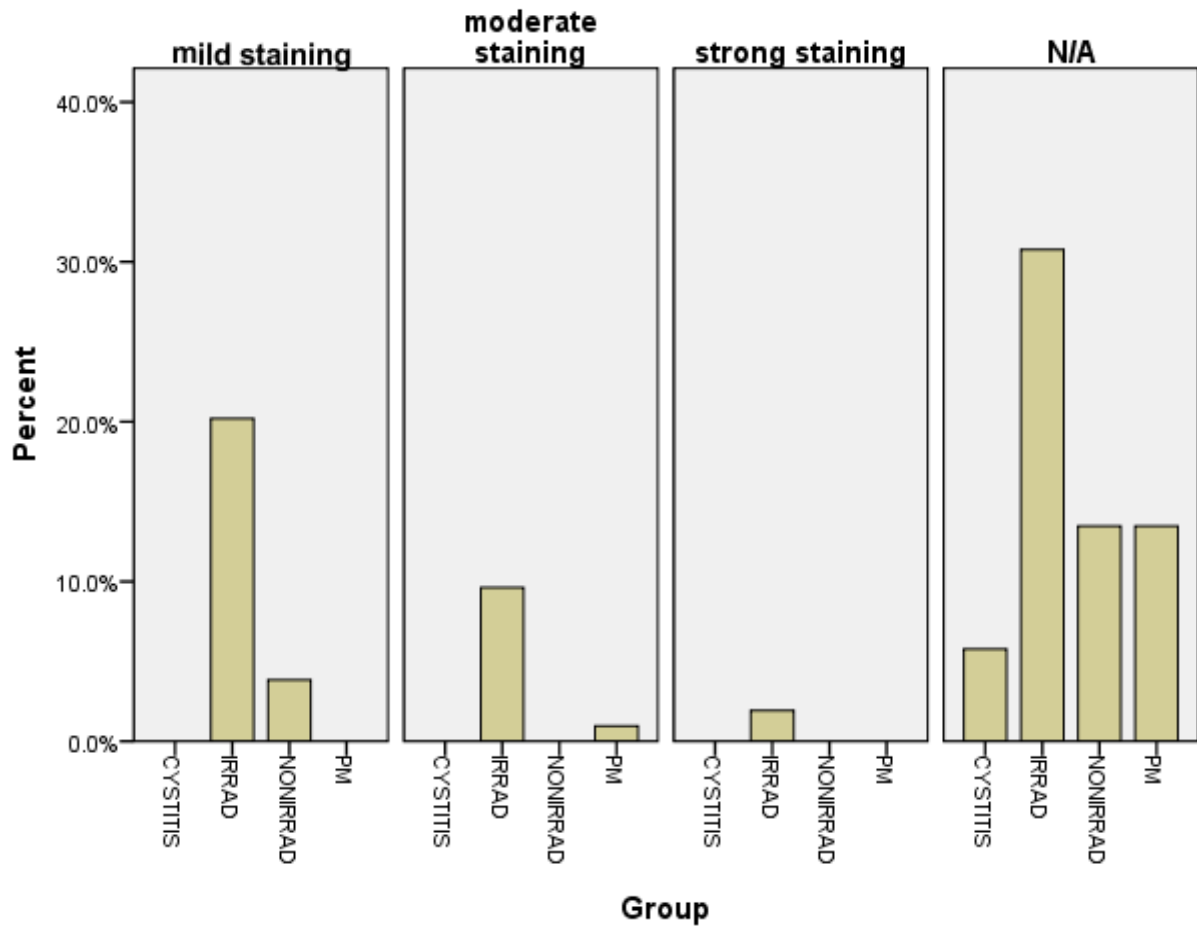
Mucosal Endothelium Crosstabulation

Group	Mucosal Endothelium				Total
	mild staining	moderate staining	strong staining	N/A	
CYSTITIS	0	0	0	6	6
IRRAD	44	10	4	7	65
NONIRRAD	5	9	0	4	18
PM	6	3	1	5	15
	55	22	5	22	104

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	41.381^a	9	.000
Likelihood Ratio	37.471	9	.000
N of Valid Cases	104		

IntermuscularStroma



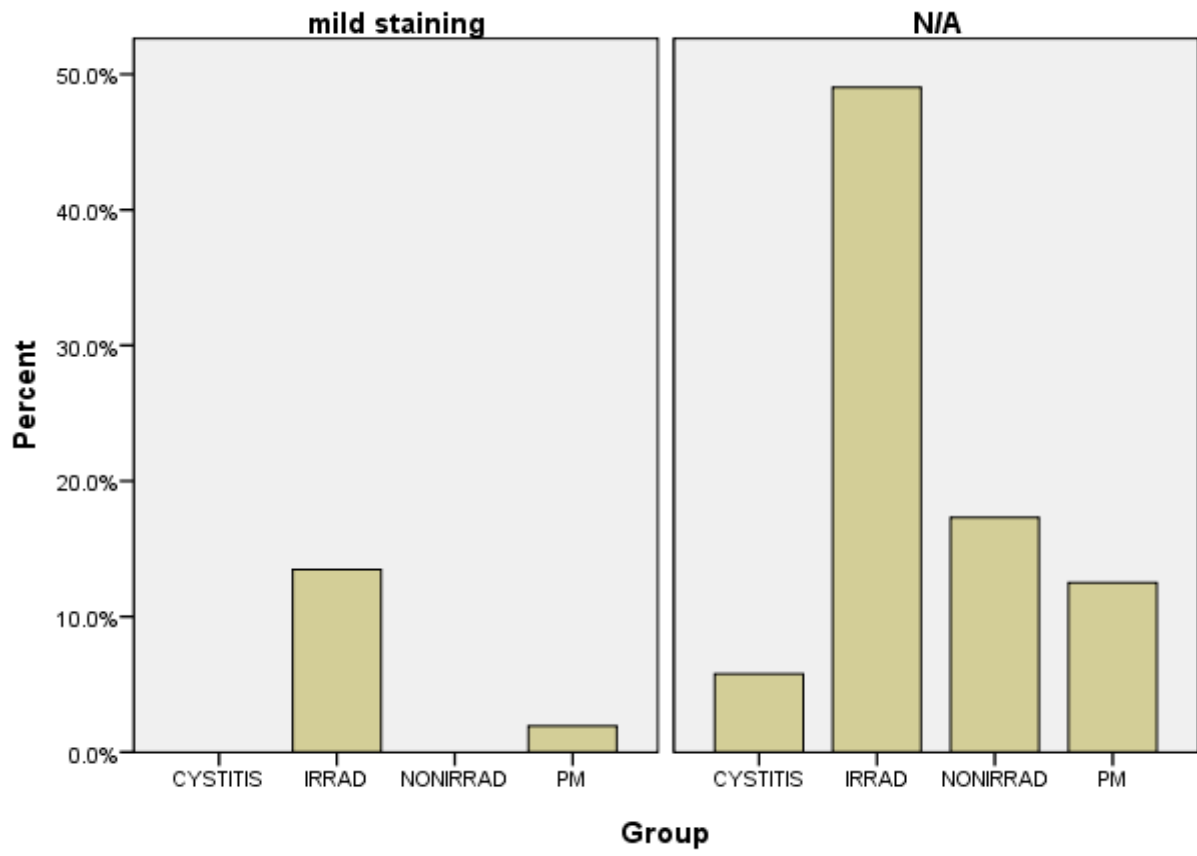
Intermuscular Stroma Crosstabulation

Group	Intermuscular Stroma				
	mild staining	moderate staining	strong staining	N/A	Total
CYSTITIS	0	0	0	6	6
IRRAD	21	10	2	32	65
NONIRRAD	4	0	0	14	18
PM	0	1	0	14	15
	25	11	2	66	104

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	18.324^a	9	.032
Likelihood Ratio	25.942	9	.002
N of Valid Cases	104		

IntermuscularFibroblasts



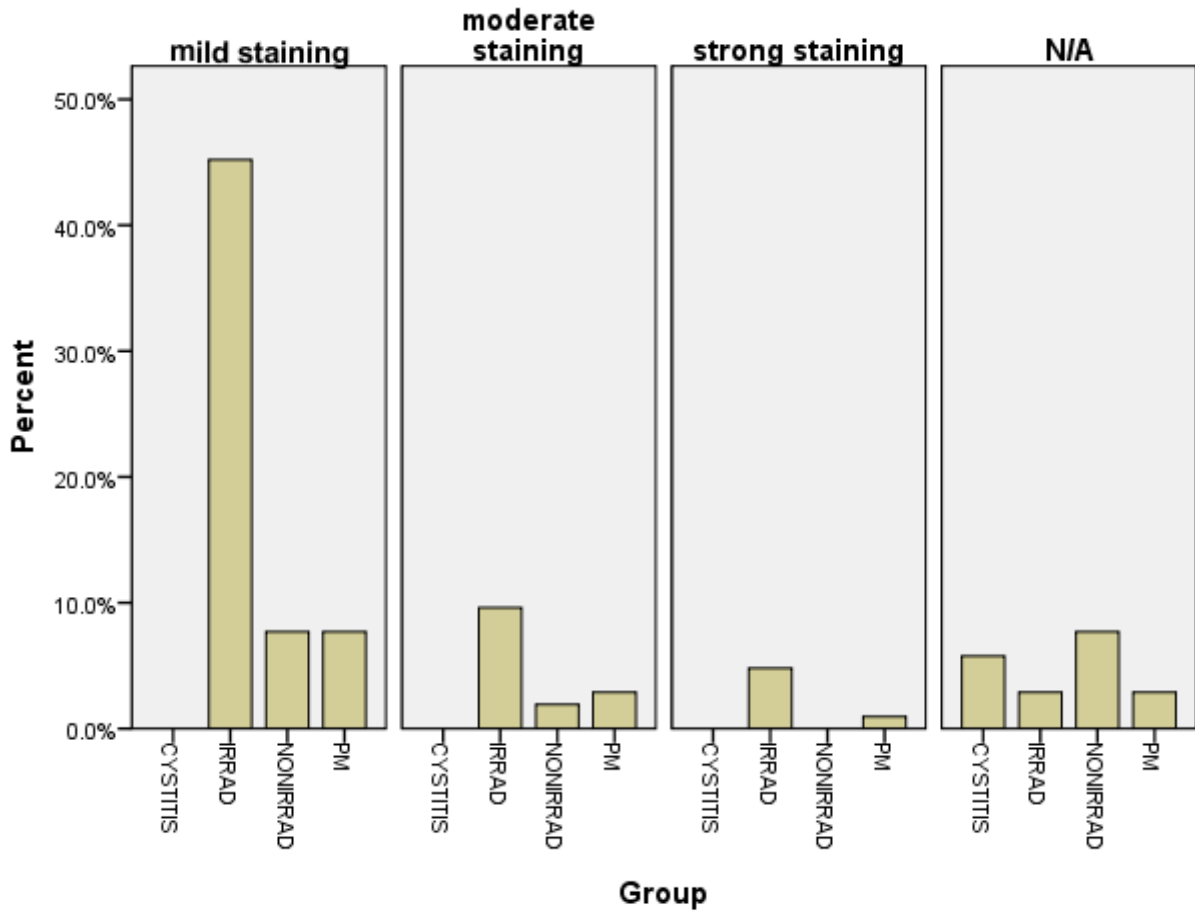
Intermuscular Fibroblasts Crosstabulation

Group	Intermuscular Fibroblasts		
	mild staining	N/A	Total
CYSTITIS	0	6	6
IRRAD	14	51	65
NONIRRAD	0	18	18
PM	2	13	15
	16	88	104

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.303^a	3	.098
Likelihood Ratio	9.788	3	.020
N of Valid Cases	104		

IntermuscularEndothelium

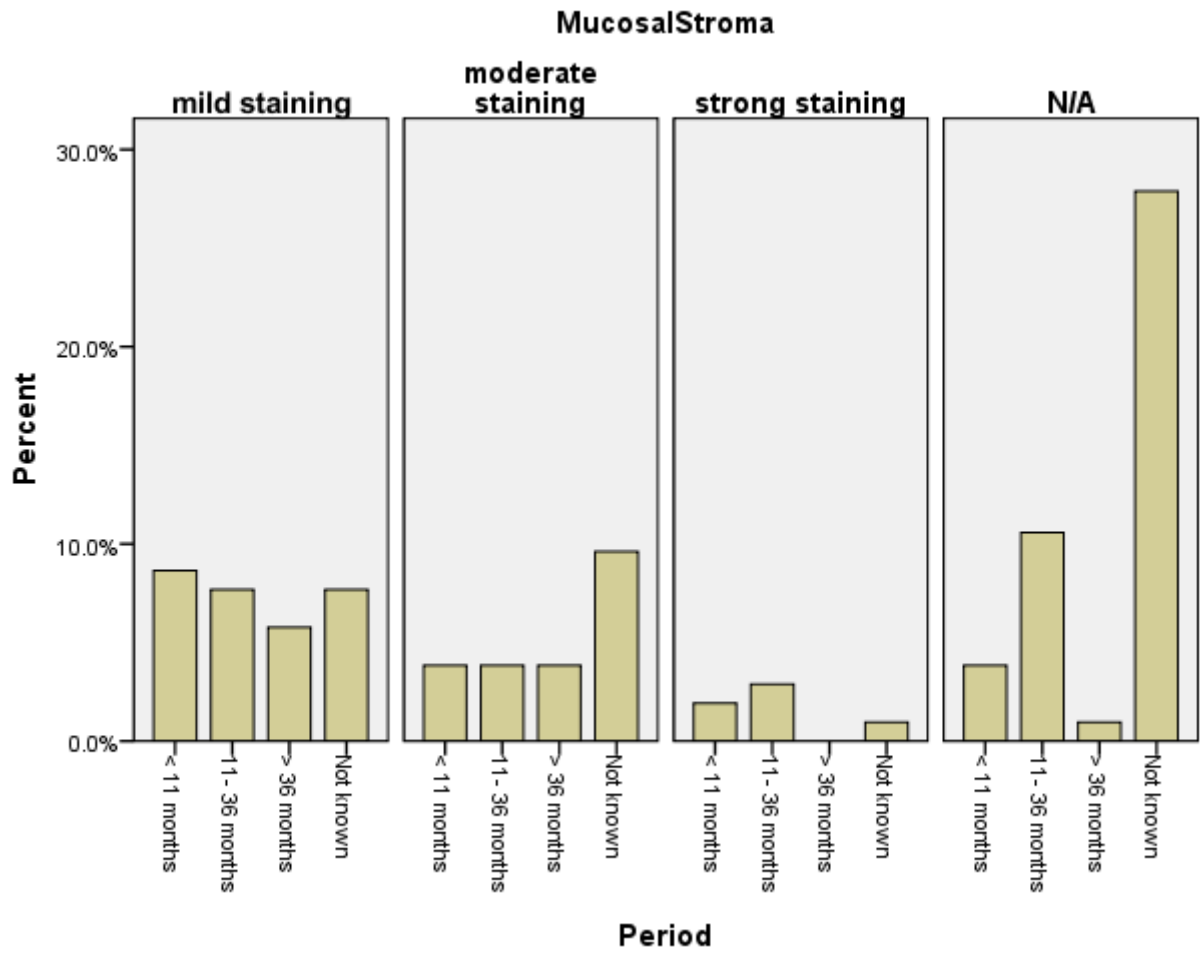


Intermuscular Endothelium Crosstabulation

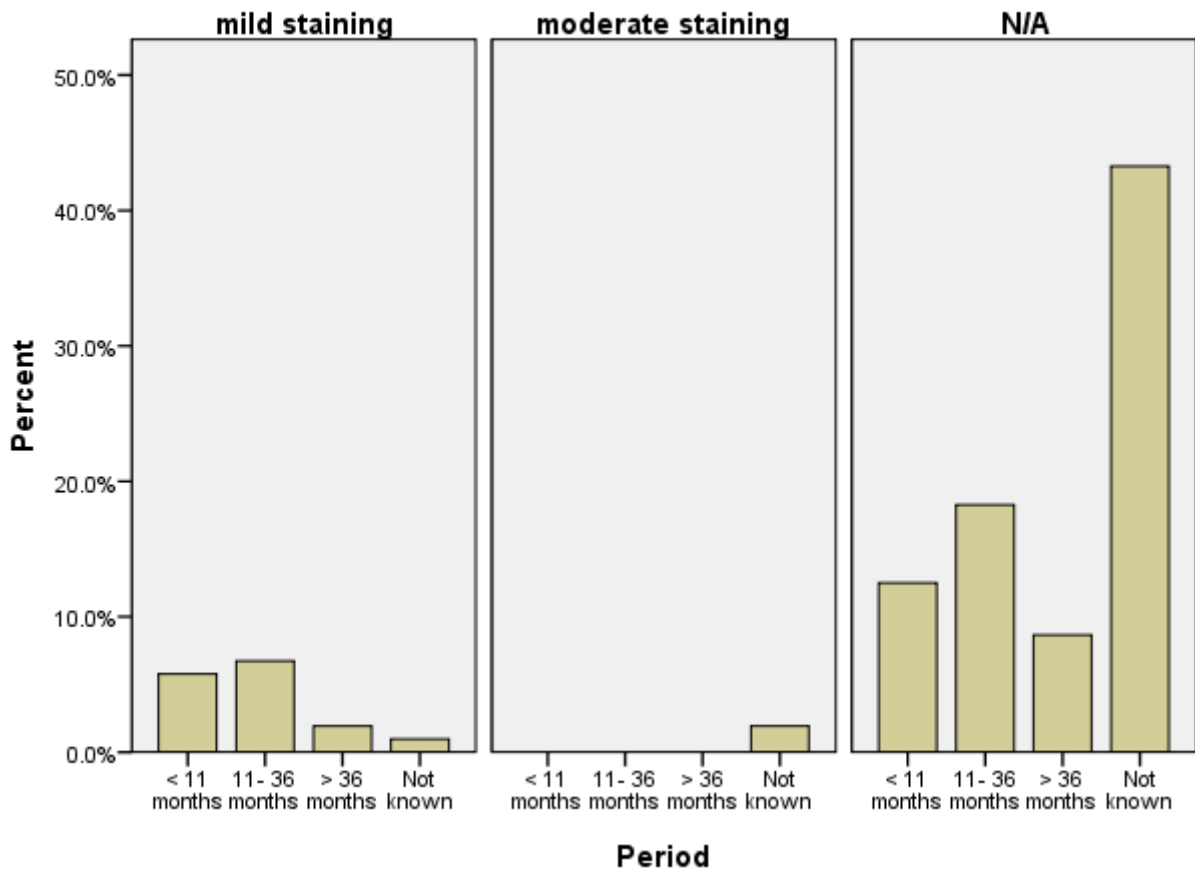
Group	Intermuscular Endothelium				
	mild staining	moderate staining	strong staining	N/A	Total
CYSTITIS	0	0	0	6	6
IRRAD	47	10	5	3	65
NONIRRAD	8	2	0	8	18
PM	8	3	1	3	15
	63	15	6	20	104

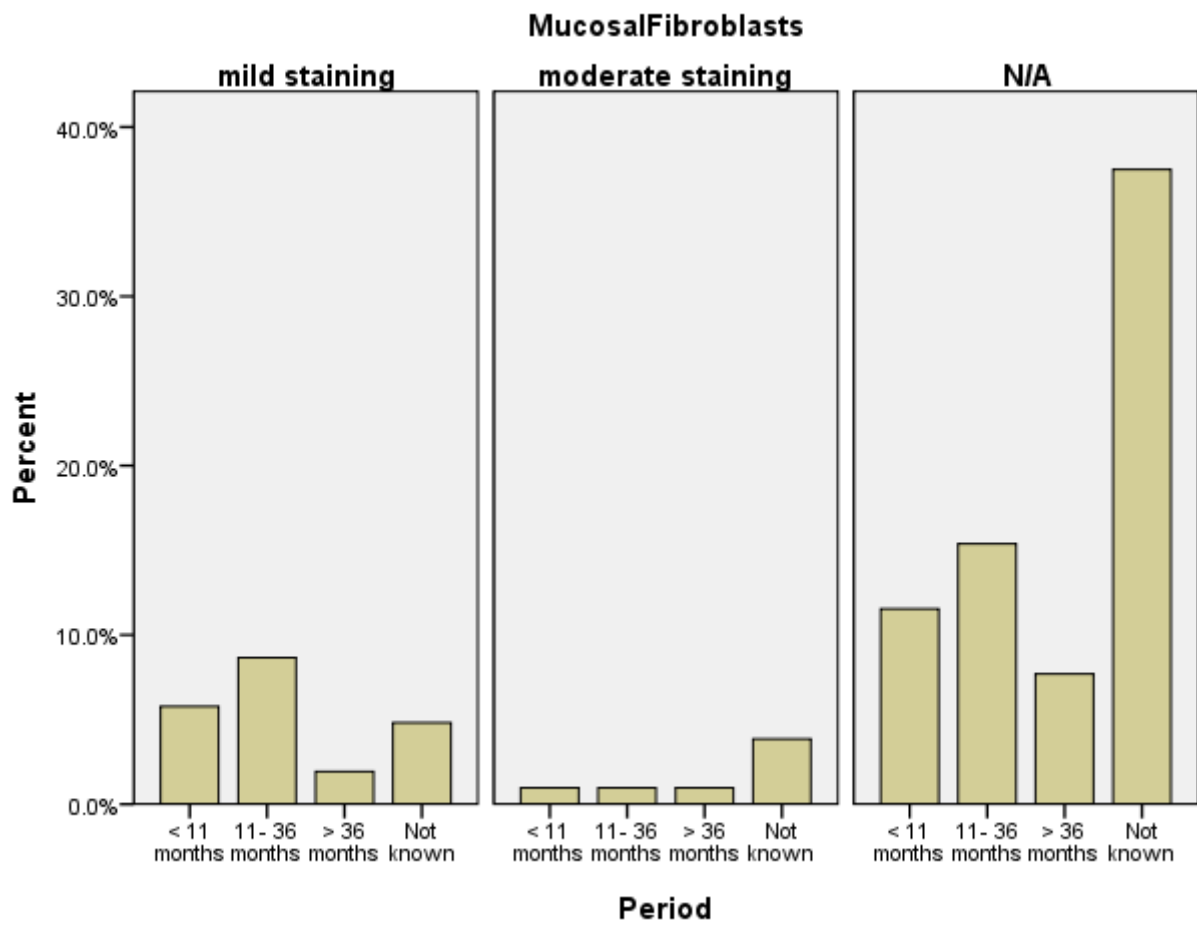
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	42.733^a	9	.000
Likelihood Ratio	39.882	9	.000
N of Valid Cases	104		

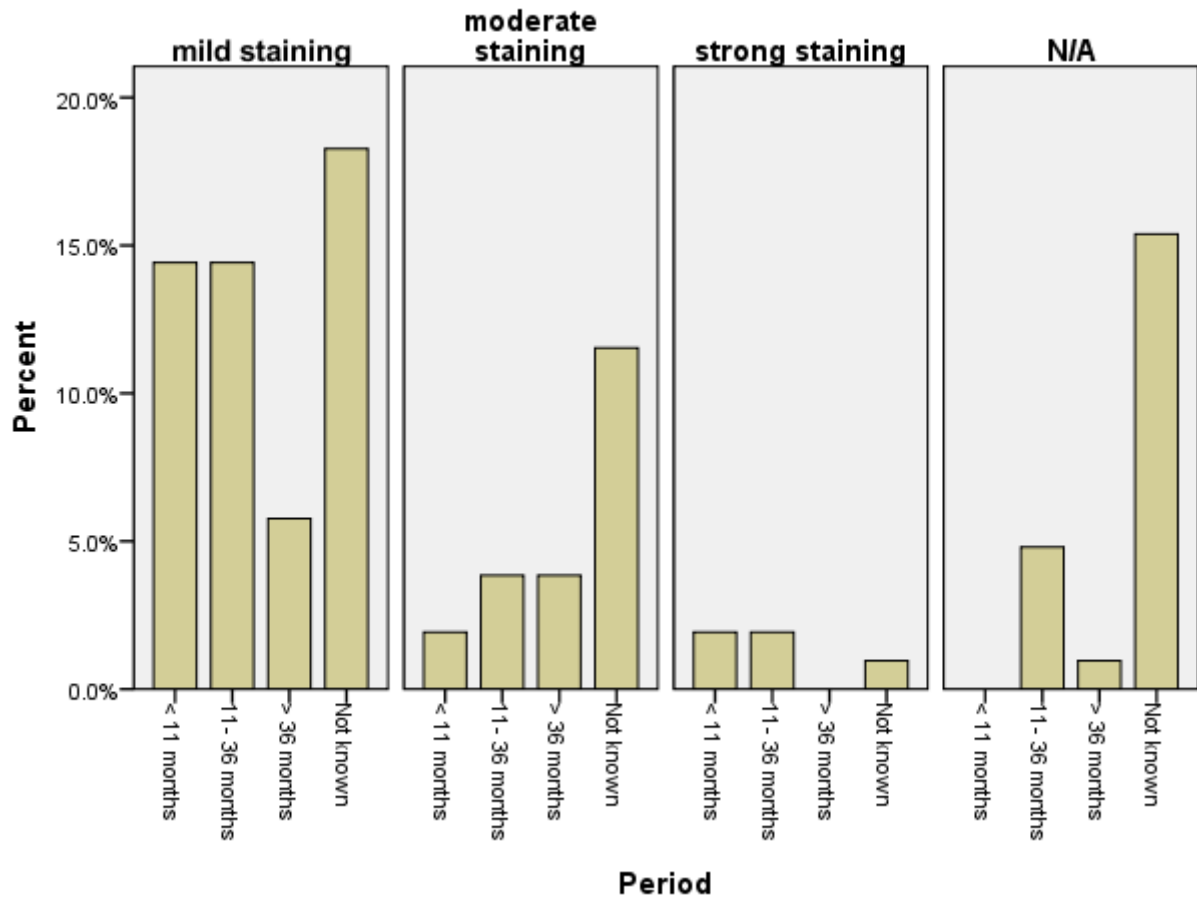


MucosalBasementMembrane

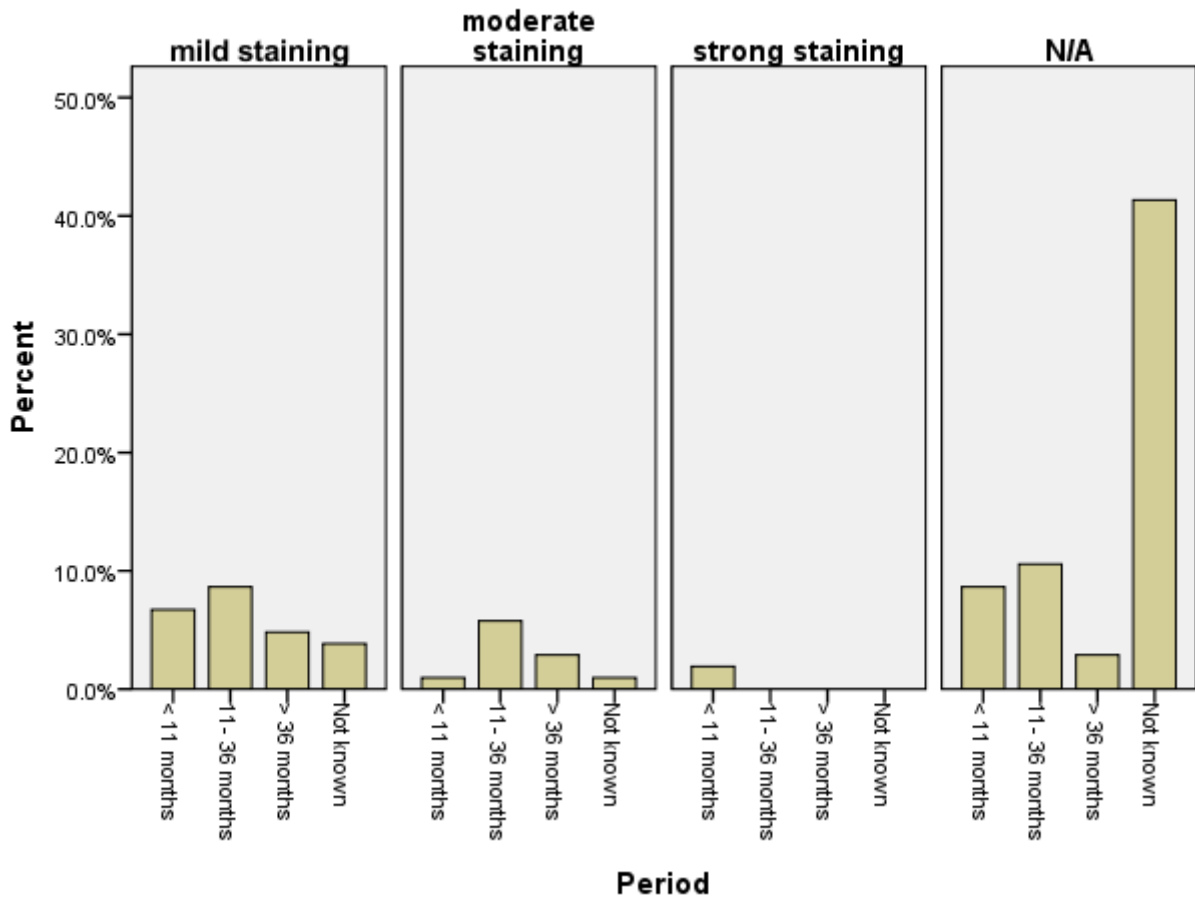


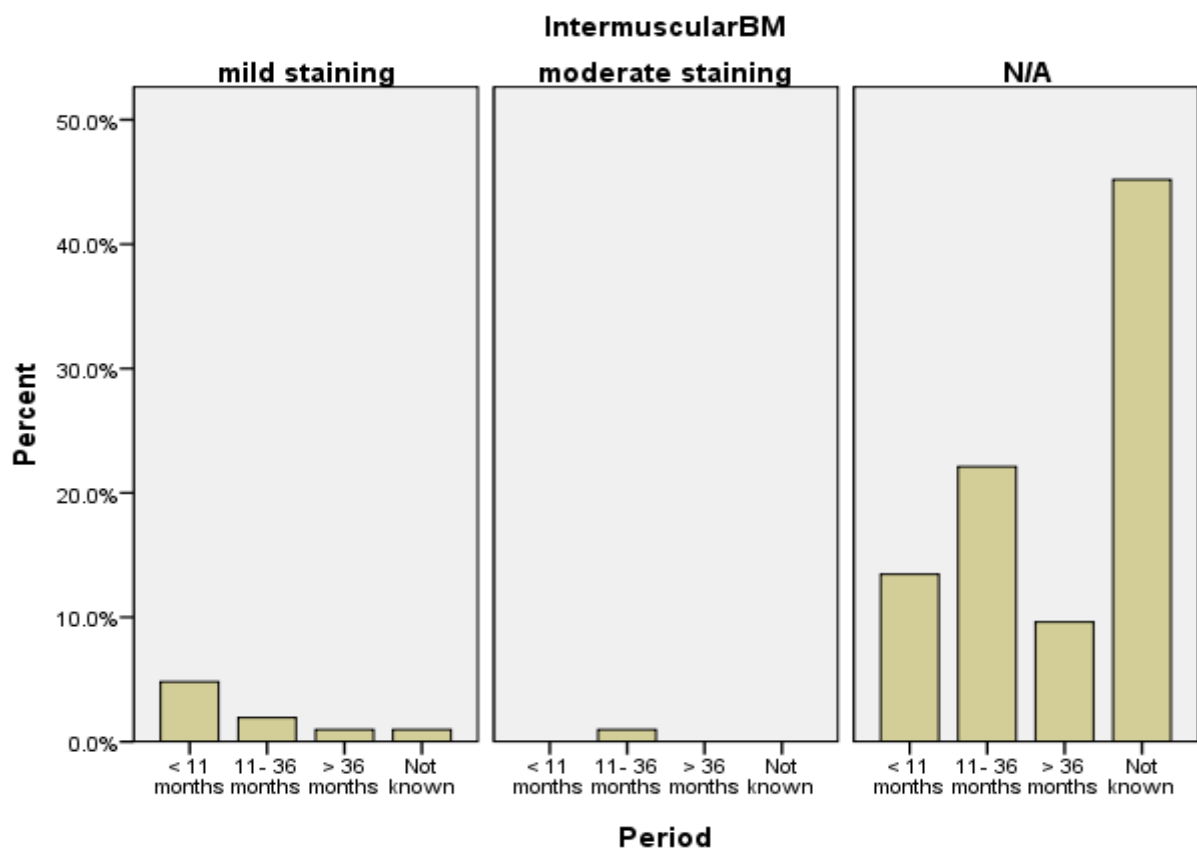


MucosalEndothelium

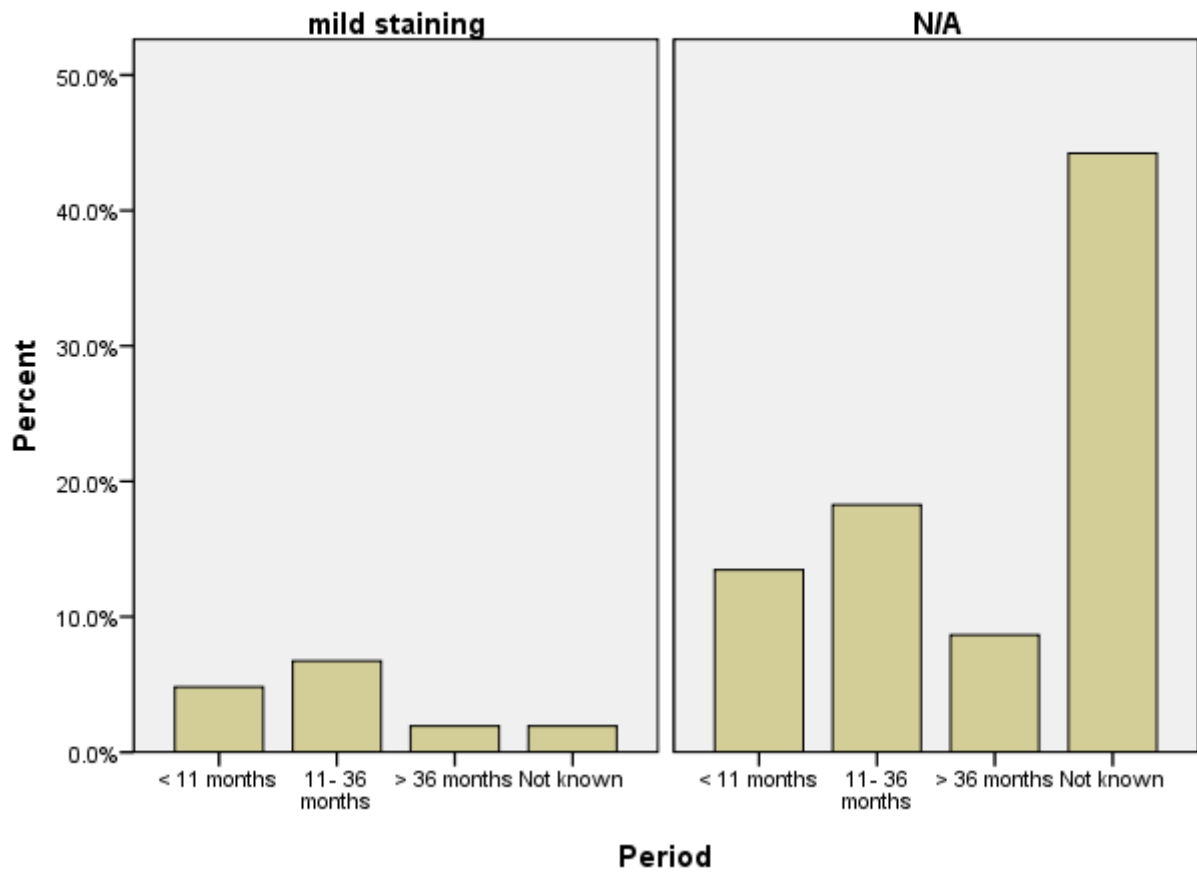


IntermuscularStroma

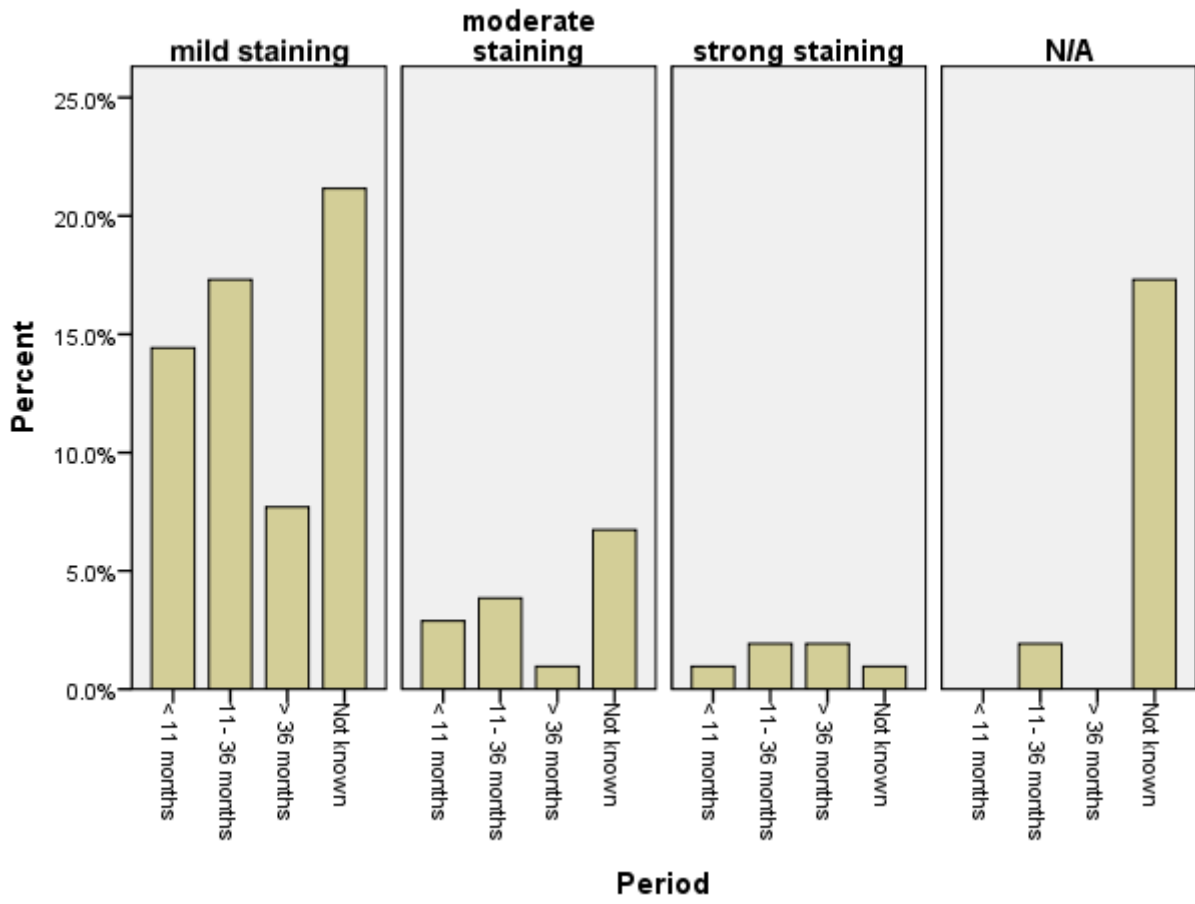




IntermuscularFibroblasts

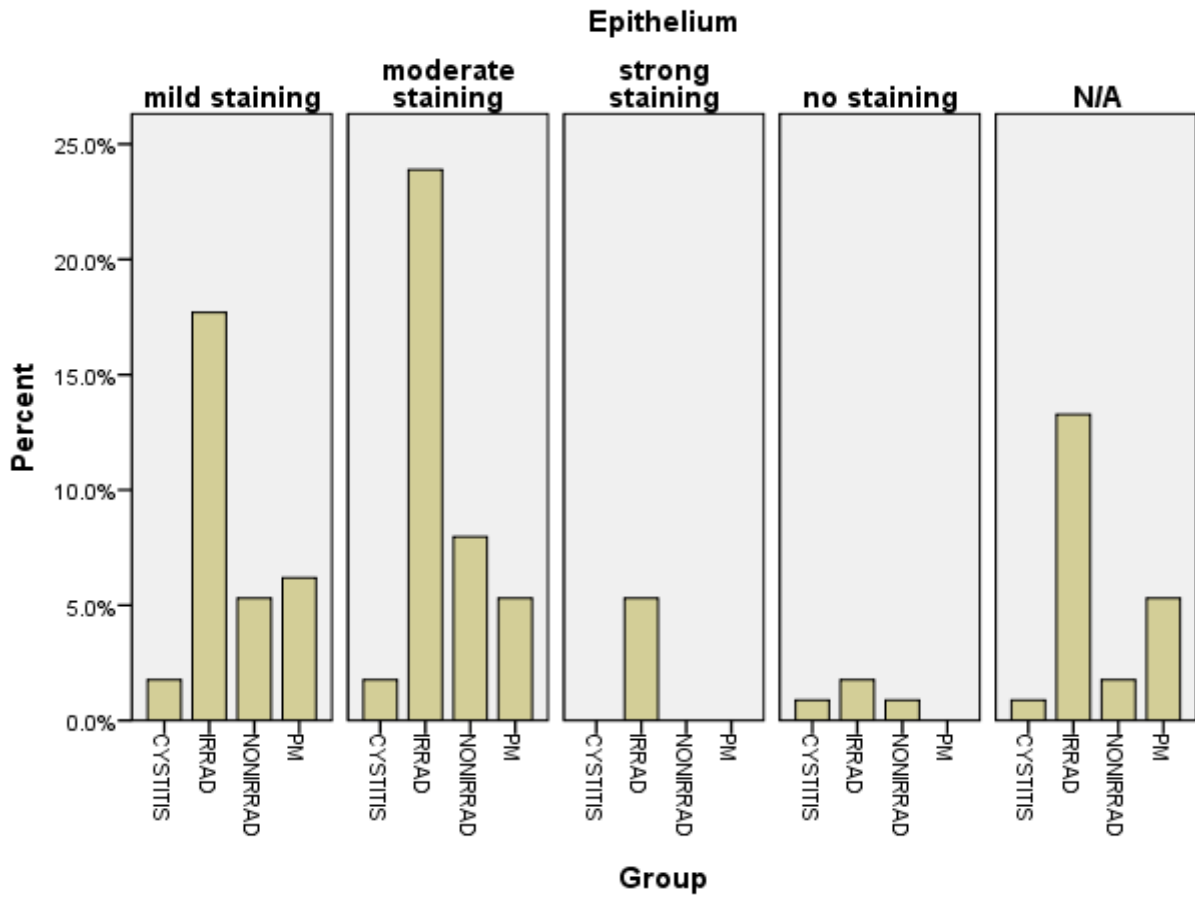


IntermuscularEndothelium



Appendix 3. Statistics for PDGF and receptor analysis (N/A = unreadable)

PDGFAA



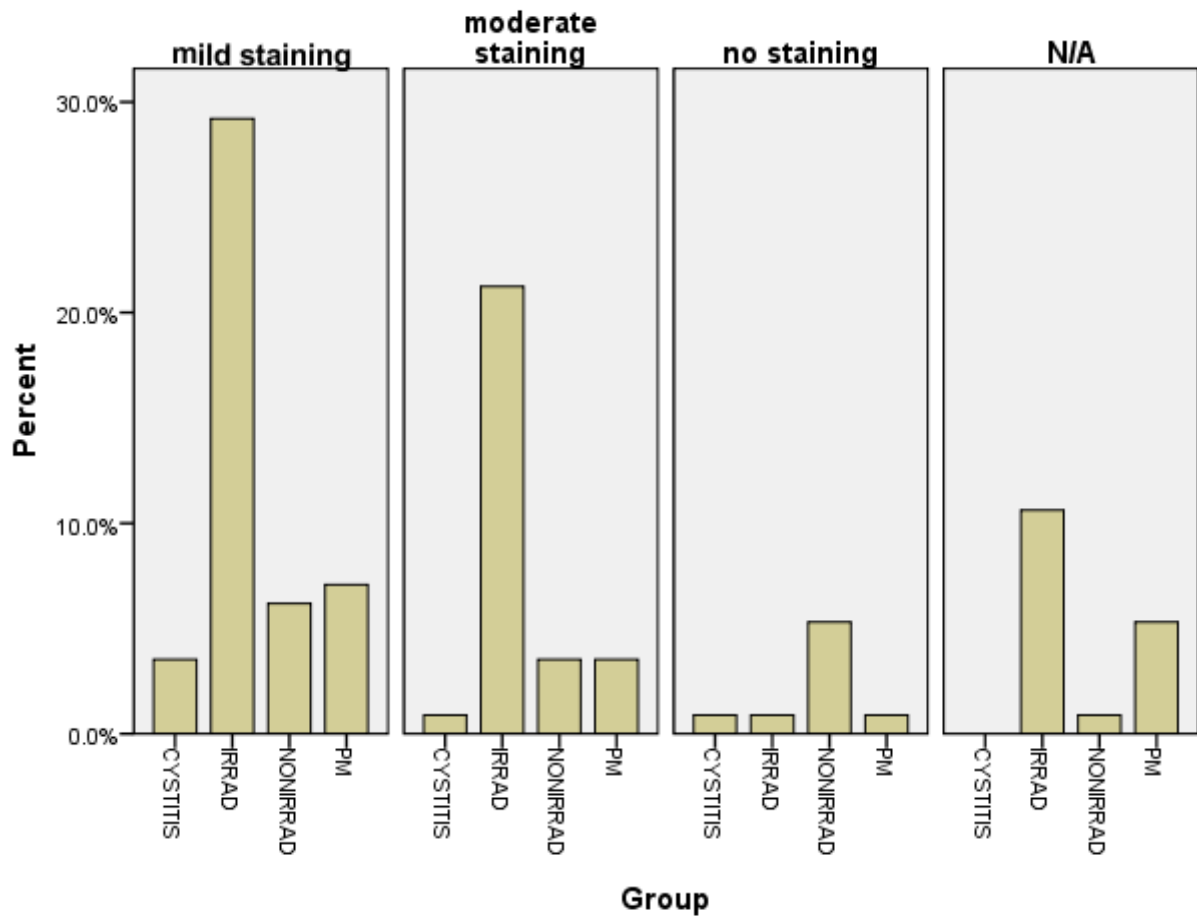
Epithelium crosstabulation

Group	Epithelium					Total
	mild staining	moderate staining	strong staining	no staining	N/A	
CYSTITIS	2	2	0	1	1	6
IRRAD	20	27	6	2	15	70
NONIRRAD	6	9	0	1	2	18
PM	7	6	0	0	6	19
Total	35	44	6	4	24	113

Chi-Square tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.729^a	12	.552
Likelihood Ratio	12.070	12	.440
N of Valid Cases	113		

SuperficialEndothelialCells



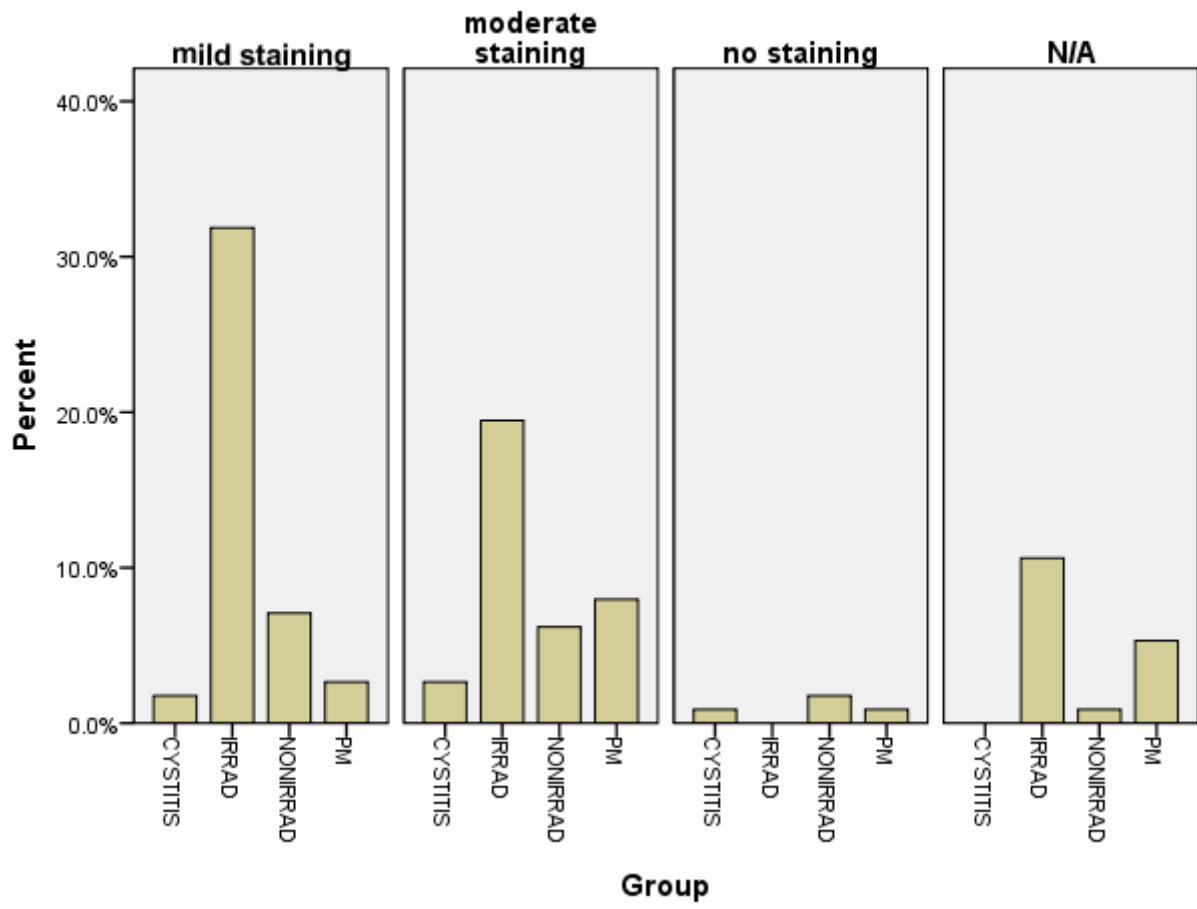
Superficial endothelial cells crosstabulation

Group	Superficial Endothelial Cells				Total
	mild staining	moderate staining	no staining	N/A	
CYSTITIS	4	1	1	0	6
IRRAD	33	24	1	12	70
NONIRRAD	7	4	6	1	18
PM	8	4	1	6	19
	52	33	9	19	113

Chi-Square tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	26.389^a	9	.002
Likelihood Ratio	22.761	9	.007
N of Valid Cases	113		

DeepEndothelialCells

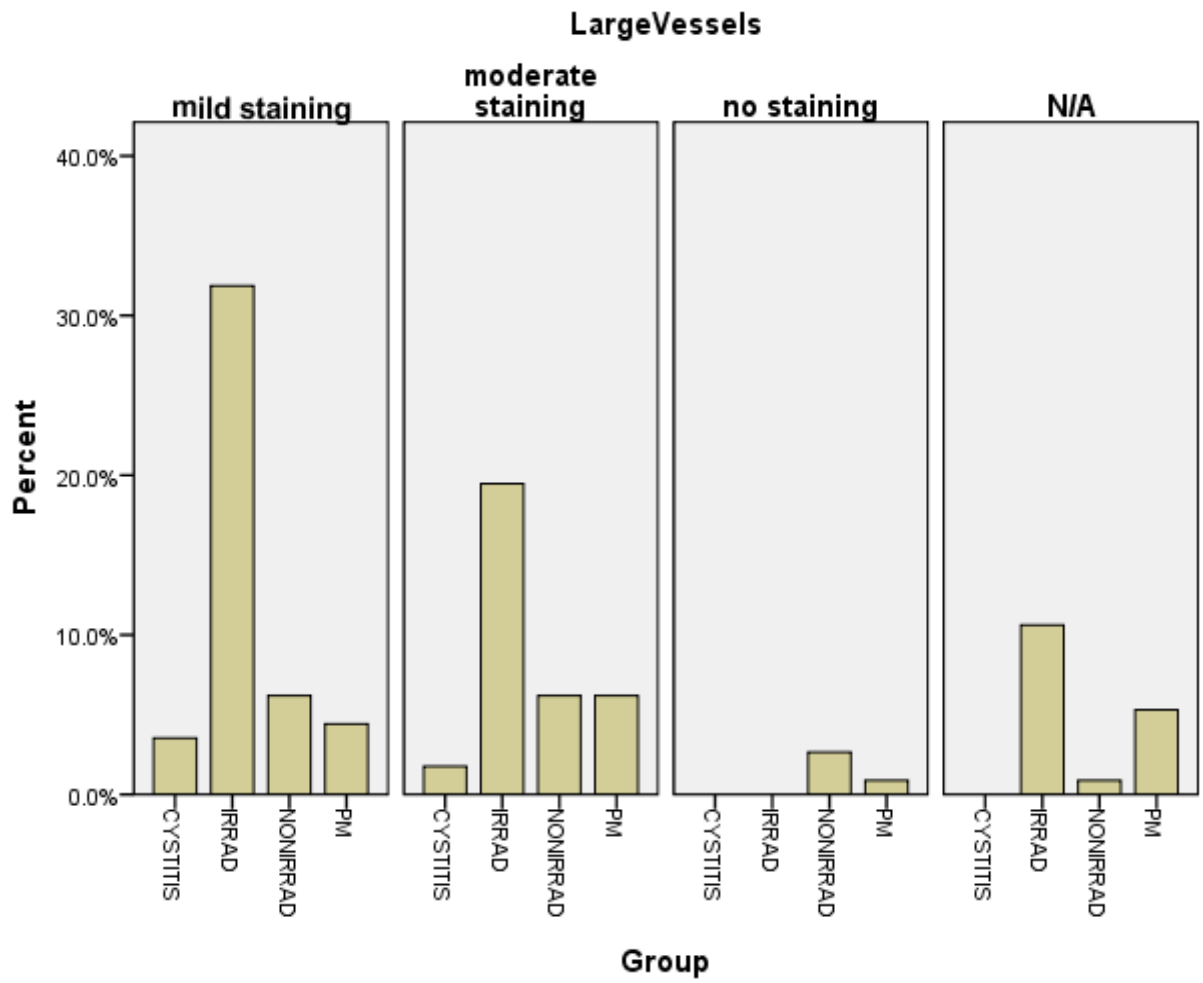


Deep endothelial cells crosstabulation

Group	Deep Endothelial Cells				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	2	3	1	0	6
IRRAD	36	22	0	12	70
NONIRRAD	8	7	2	1	18
PM	3	9	1	6	19
	49	41	4	19	113

Chi-Square tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	19.275^a	9	.023
Likelihood Ratio	21.217	9	.012
N of Valid Cases	113		



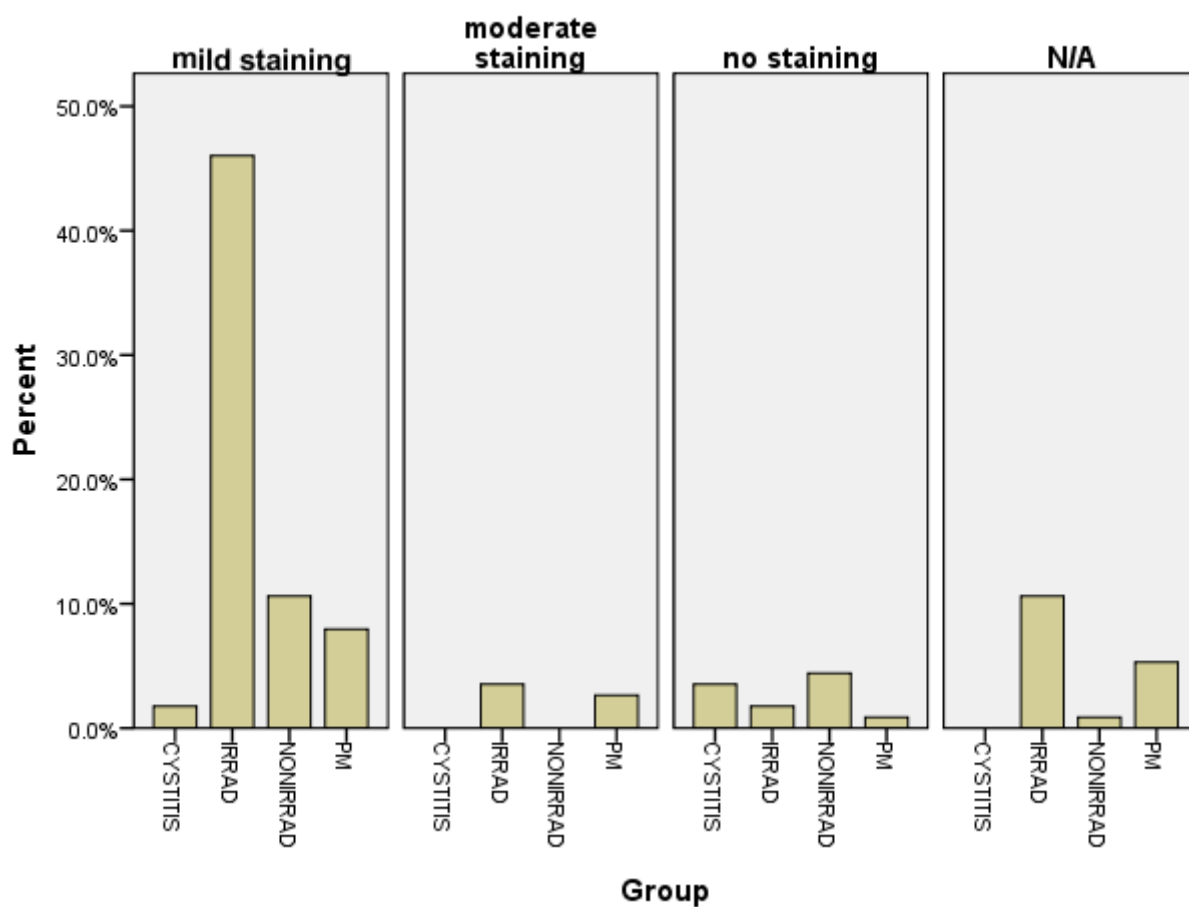
Large vessels crosstabulation

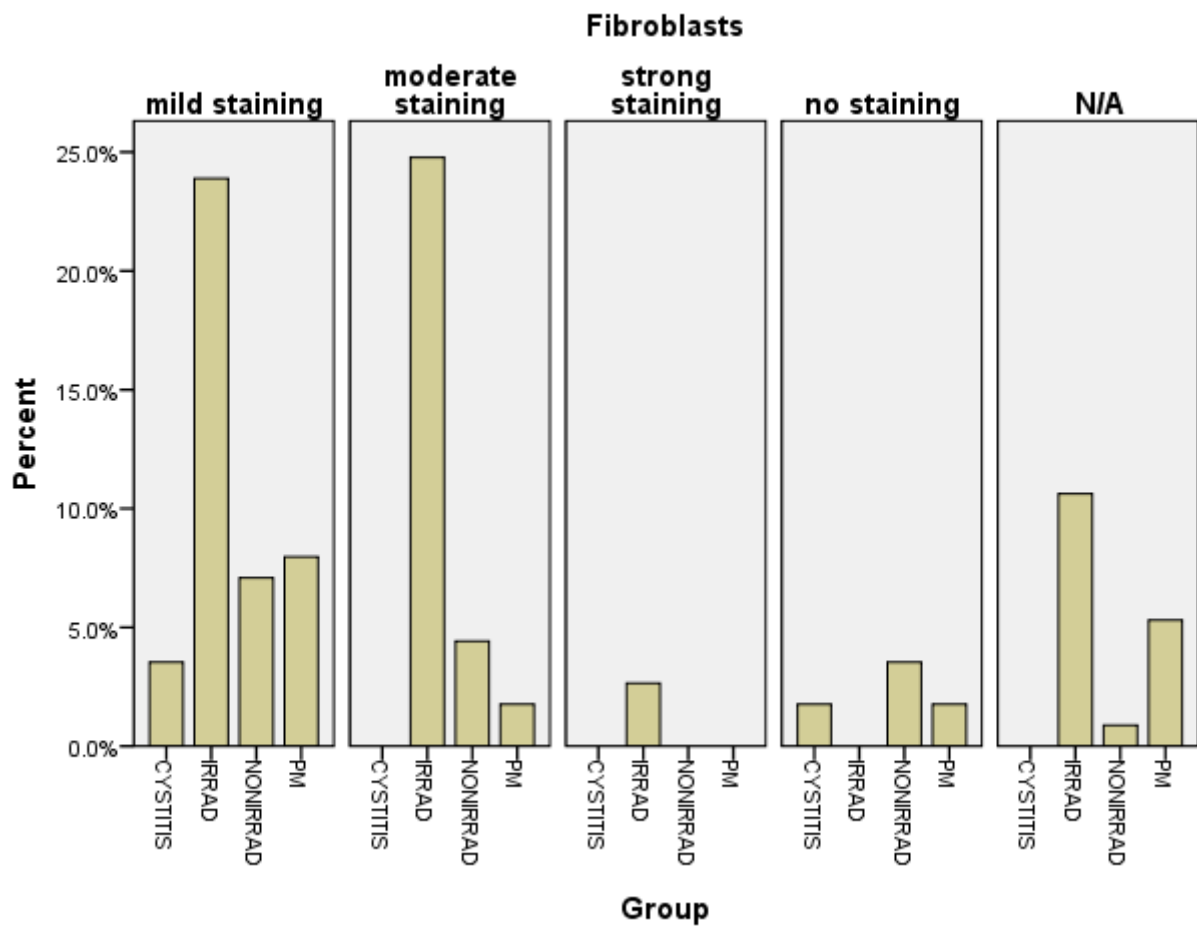
Group	Large Vessels				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	4	2	0	0	6
IRRAD	36	22	0	12	70
NONIRRAD	7	7	3	1	18
PM	5	7	1	6	19
	52	38	4	19	113

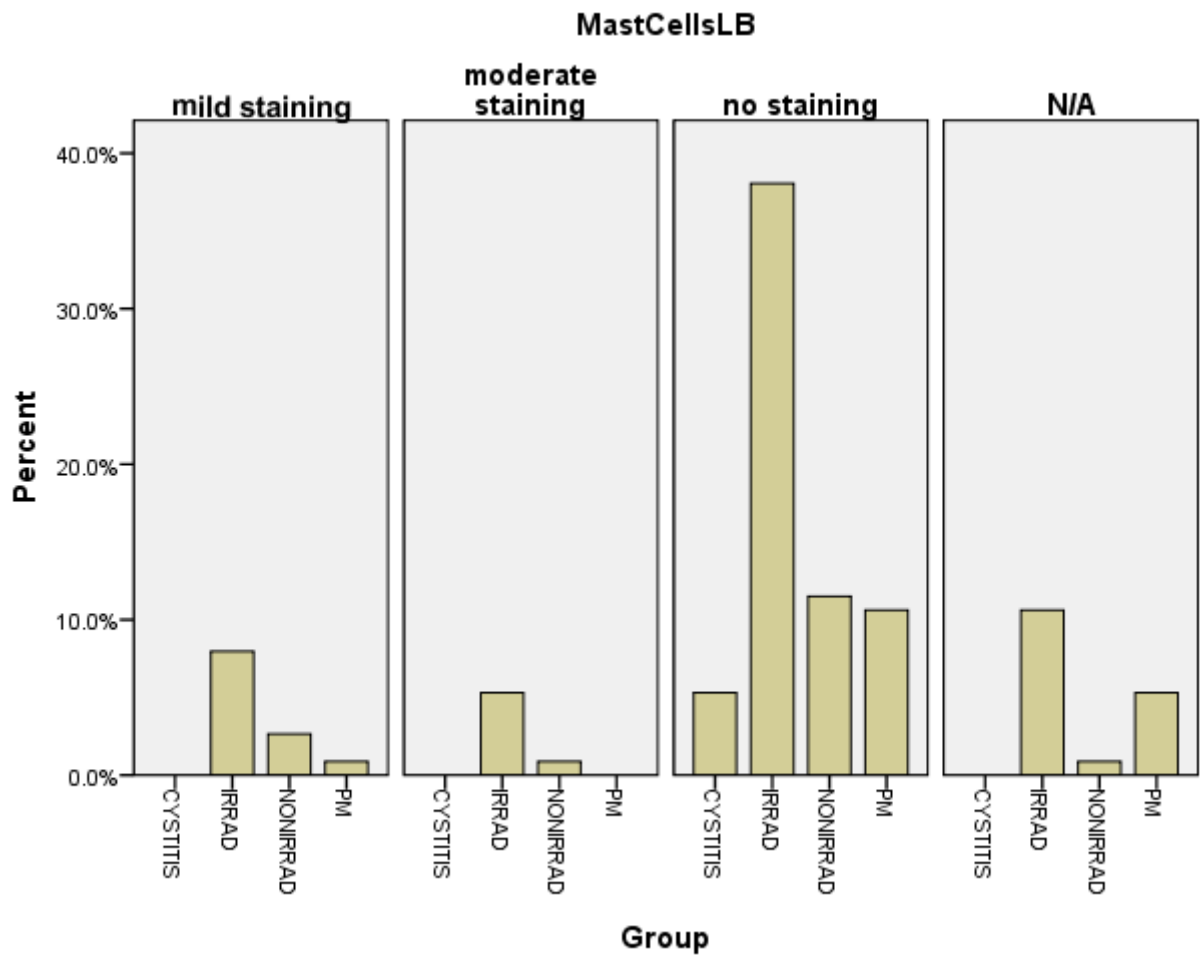
Chi-Square tests

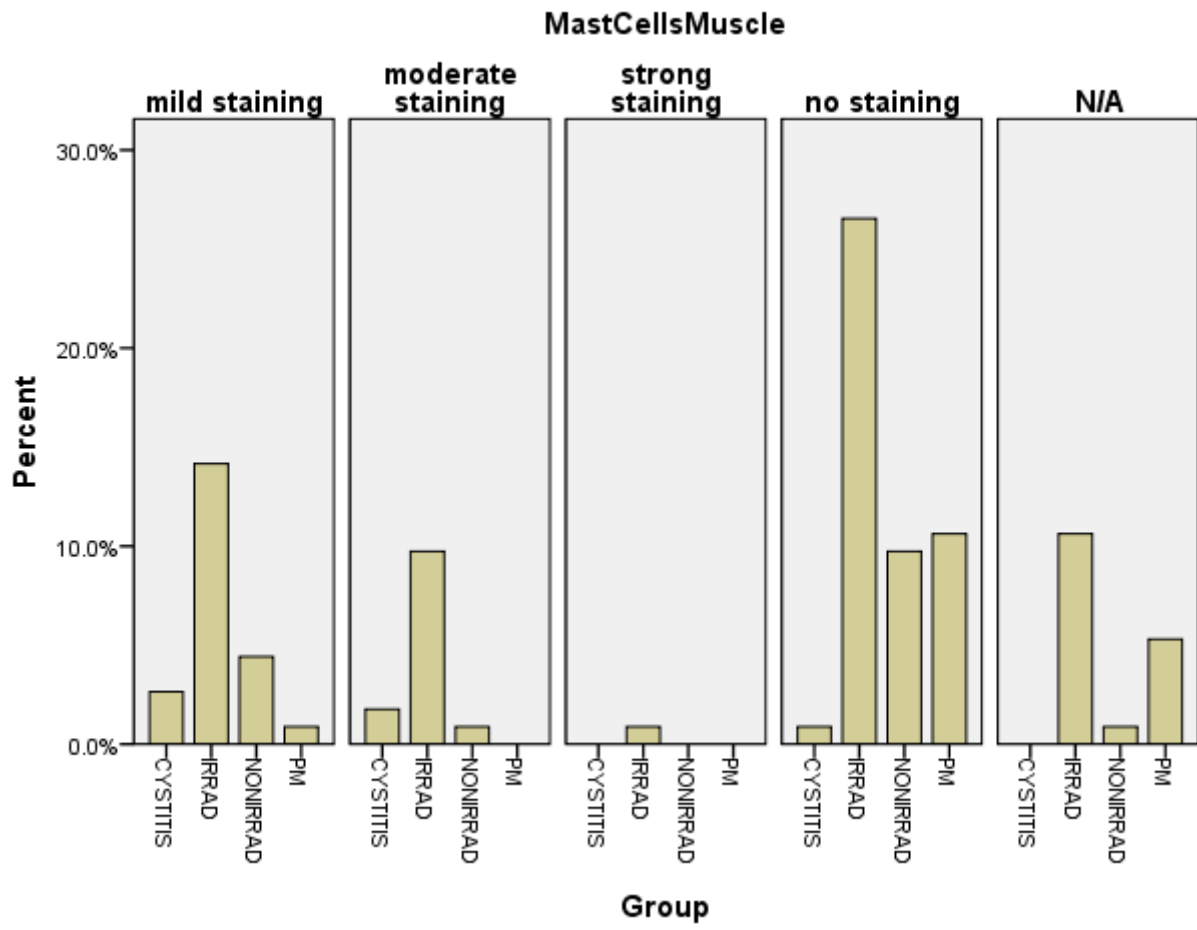
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	19.556^a	9	.021
Likelihood Ratio	19.228	9	.023
N of Valid Cases	113		

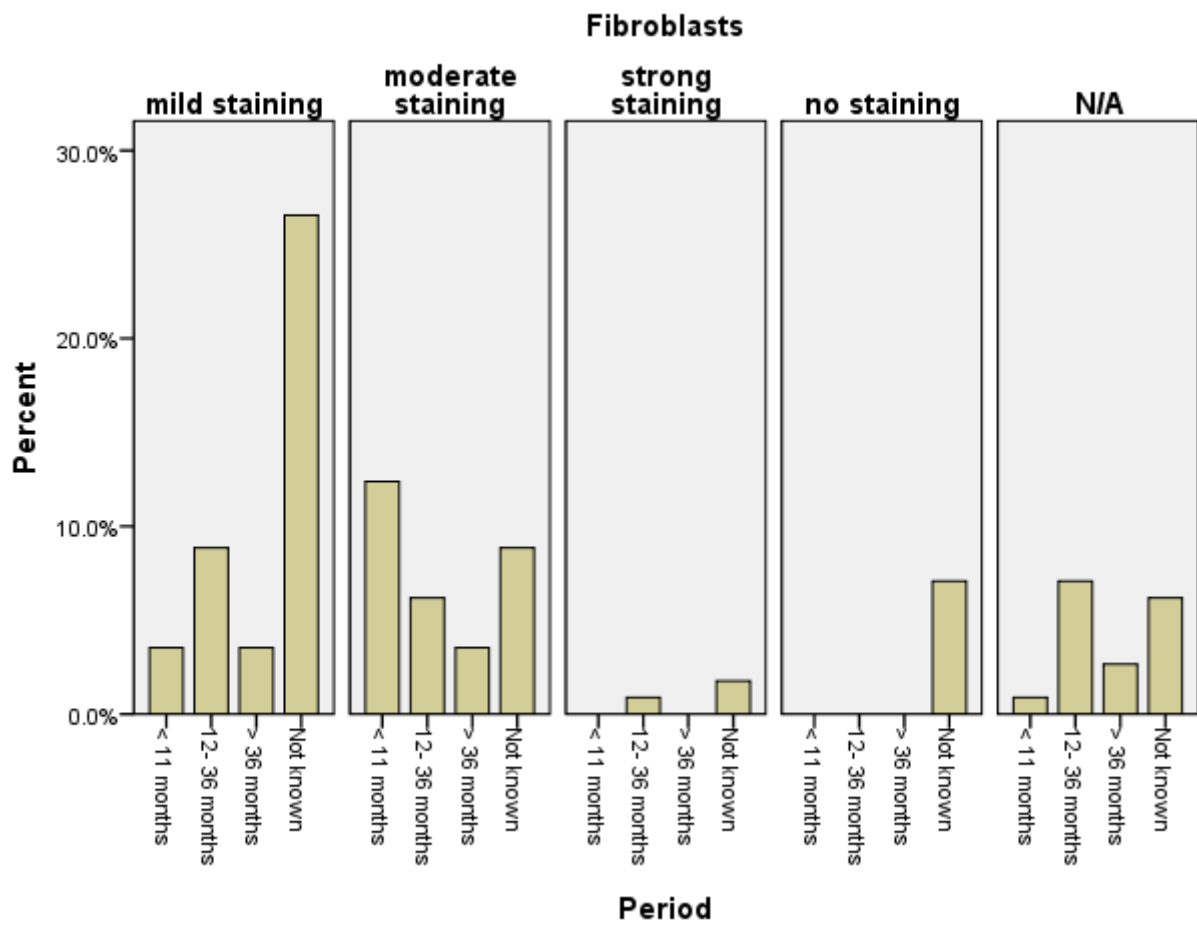
SmoothMuscle



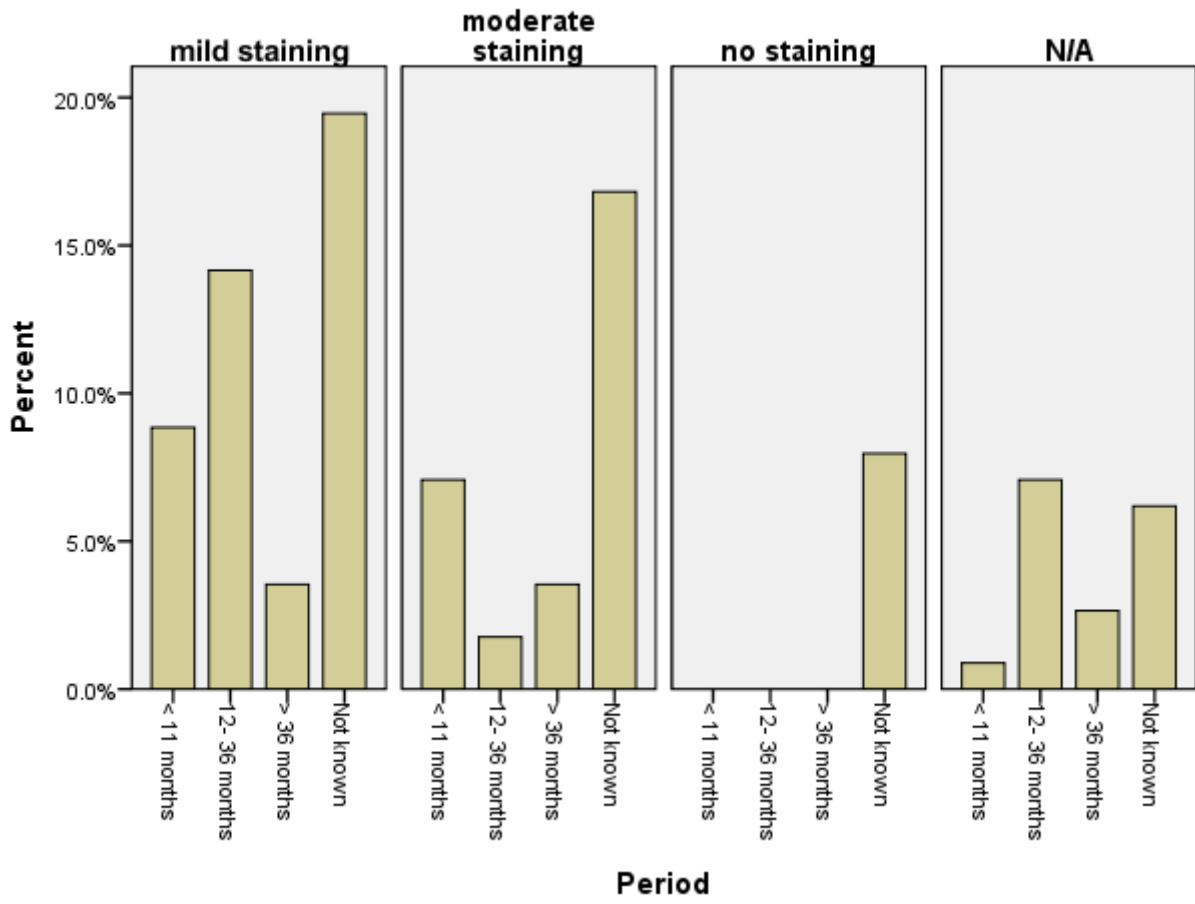




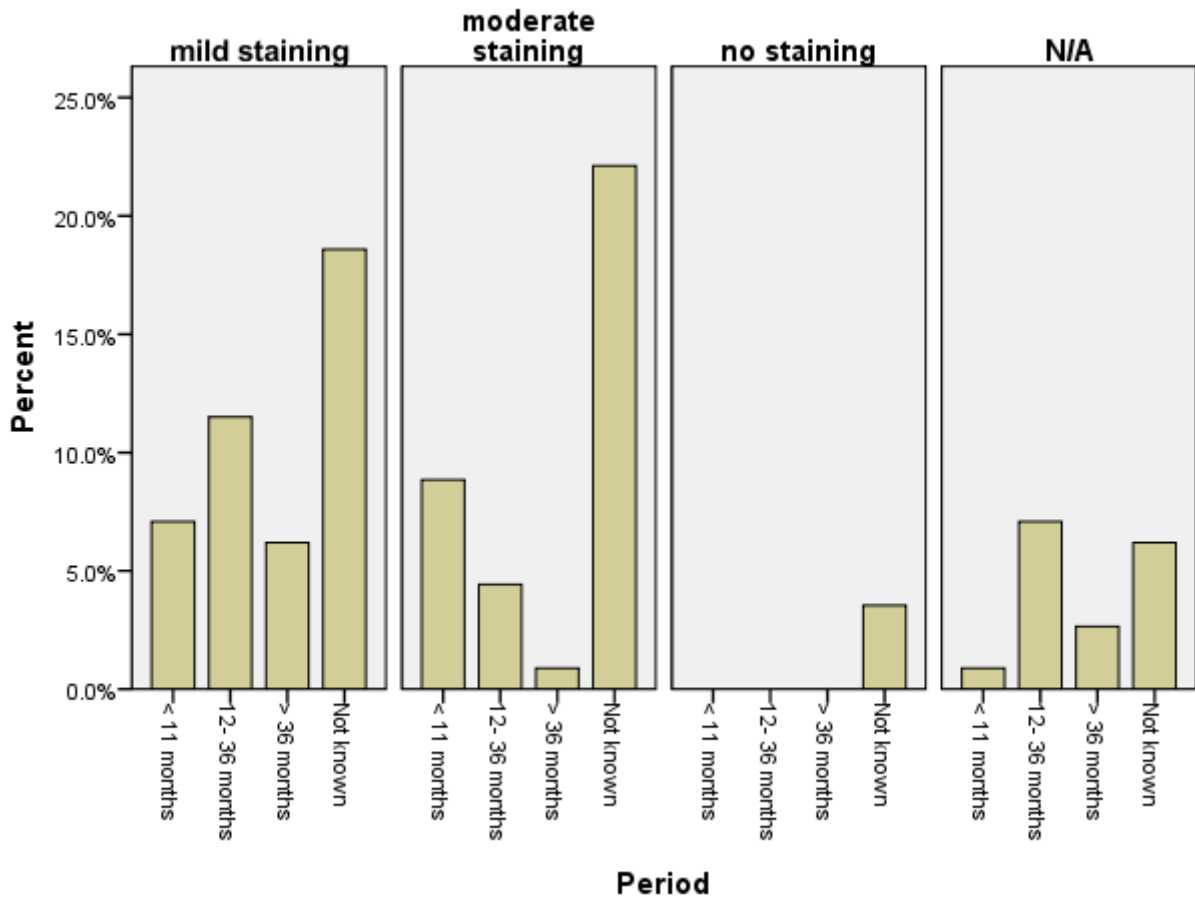


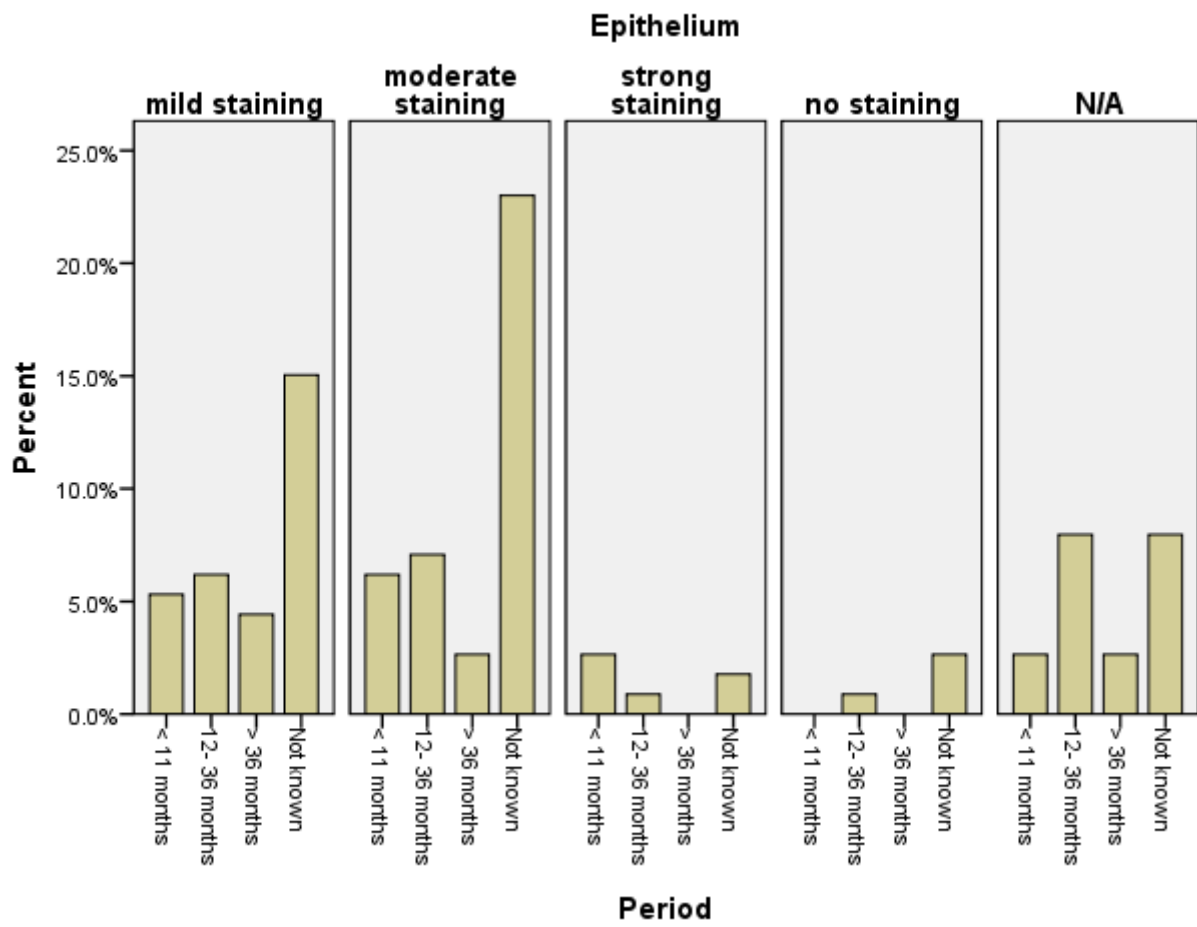


Superficial Endothelial Cells

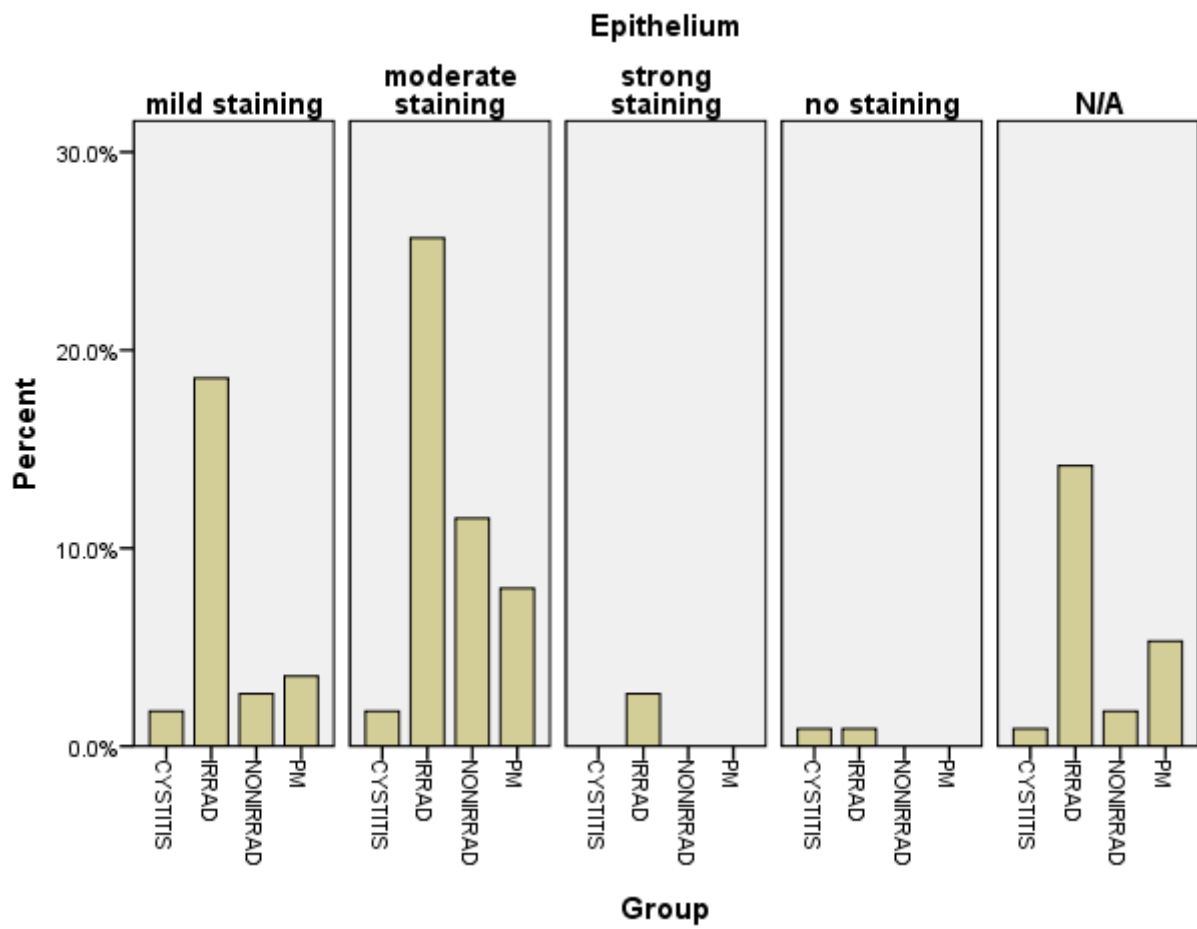


DeepEndothelialCells





PDGFAB



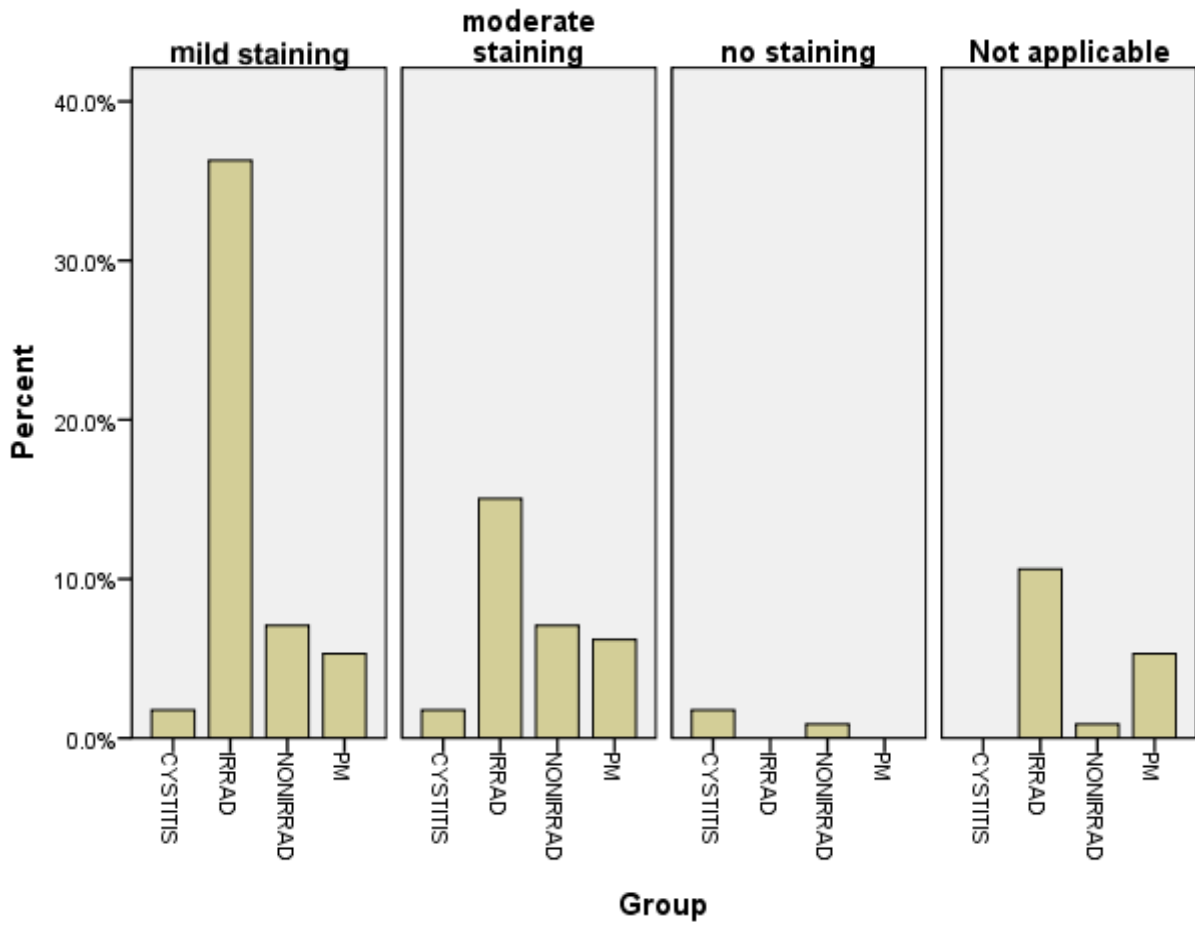
Epithelium Crosstabulation

Group	Epithelium					Total
	mild staining	moderate staining	strong staining	no staining	N/A	
CYSTITIS	2	2	0	1	1	6
IRRAD	21	29	3	1	16	70
NONIRRAD	3	13	0	0	2	18
PM	4	9	0	0	6	19
	30	53	3	2	25	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	16.359^a	12	.175
Likelihood Ratio	13.154	12	.358
N of Valid Cases	113		

SuperficialEndothelialCells



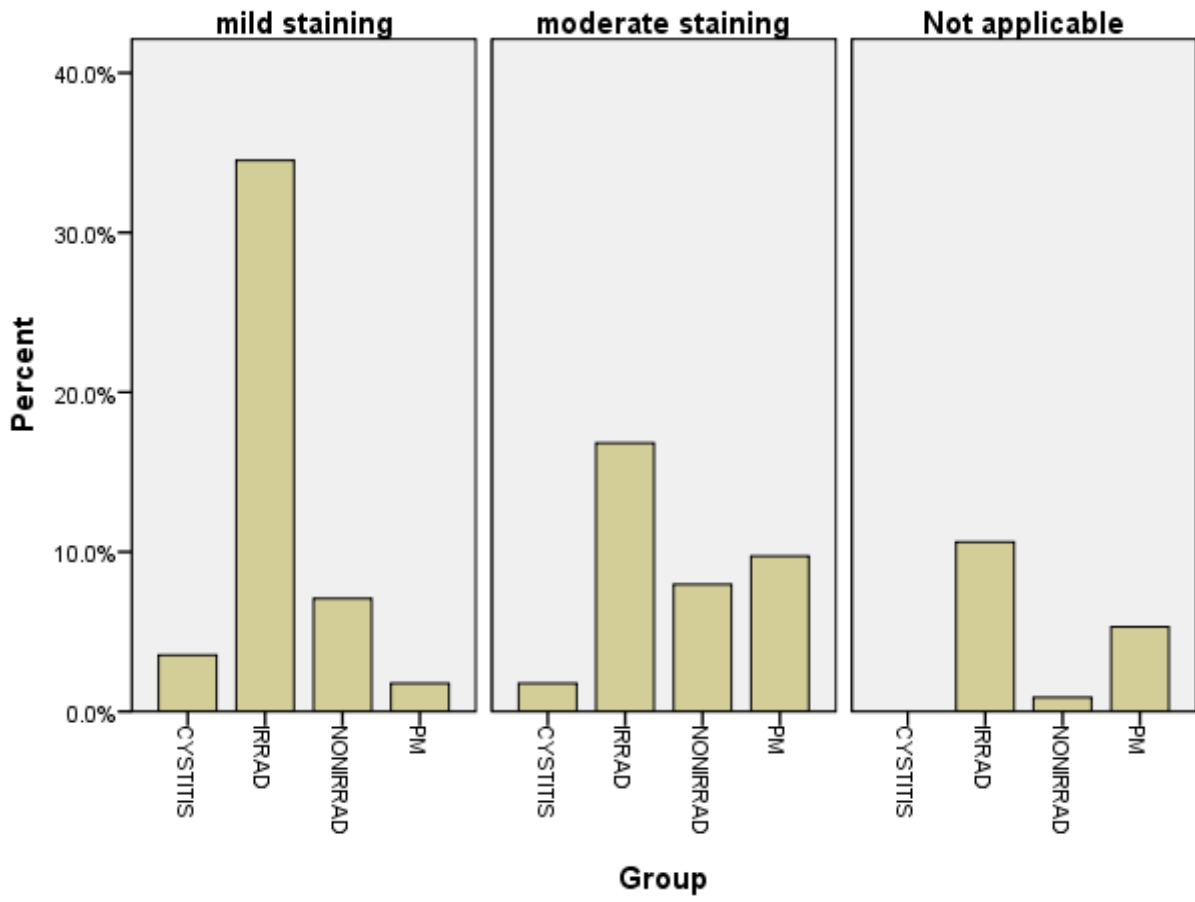
Superficial Endothelial Cells Crosstabulation

Group	Superficial Endothelial Cells				
	mild staining	moderate staining	no staining	Not applicable	Total
CYSTITIS	2	2	2	0	6
IRRAD	41	17	0	12	70
NONIRRAD	8	8	1	1	18
PM	6	7	0	6	19
	57	34	3	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	34.097^a	9	.000
Likelihood Ratio	22.561	9	.007
N of Valid Cases	113		

DeepEndothelialCells



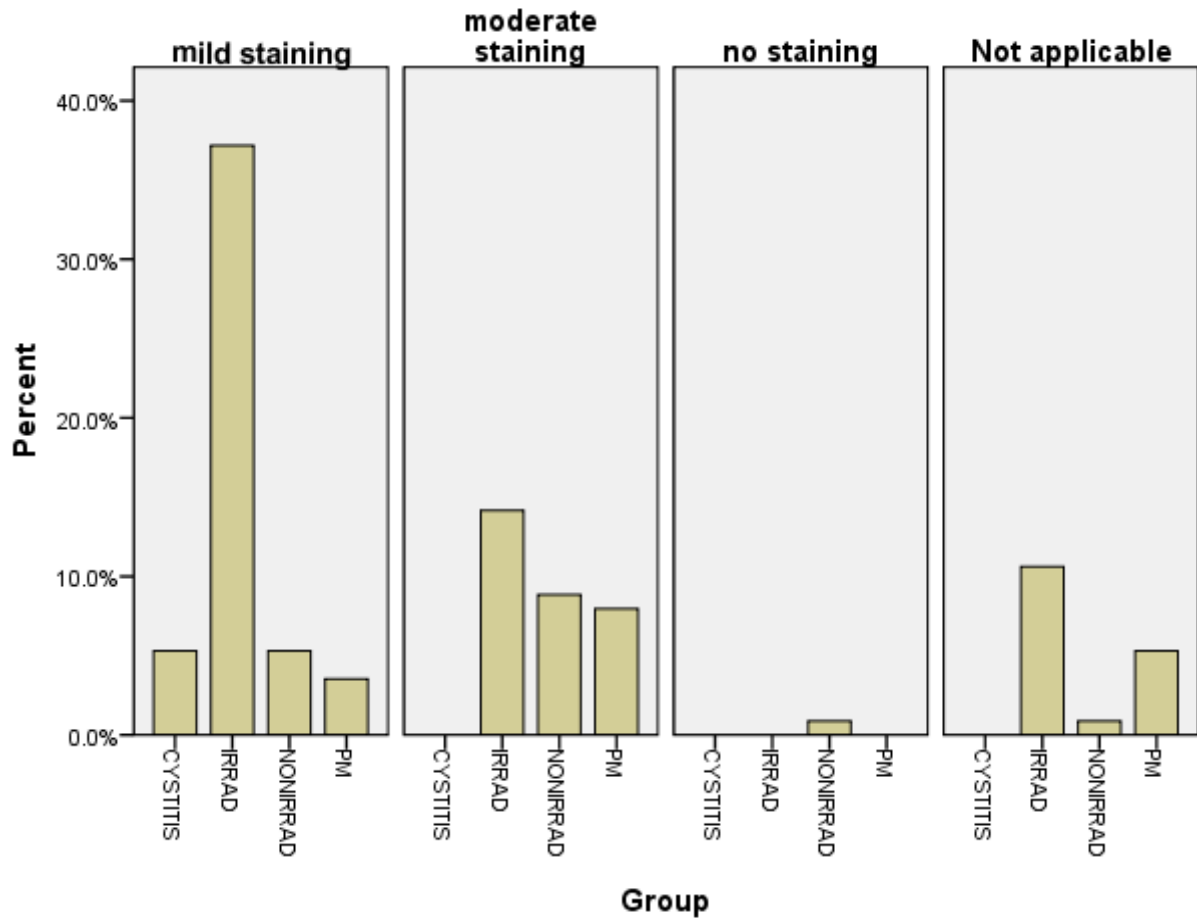
Deep Endothelial Cells Crosstabulation

Group	Deep Endothelial Cells			
	mild staining	moderate staining	Not applicable	Total
CYSTITIS	4	2	0	6
IRRAD	39	19	12	70
NONIRRAD	8	9	1	18
PM	2	11	6	19
	53	41	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	16.881^a	6	.010
Likelihood Ratio	19.900	6	.003
N of Valid Cases	113		

LargeVessels

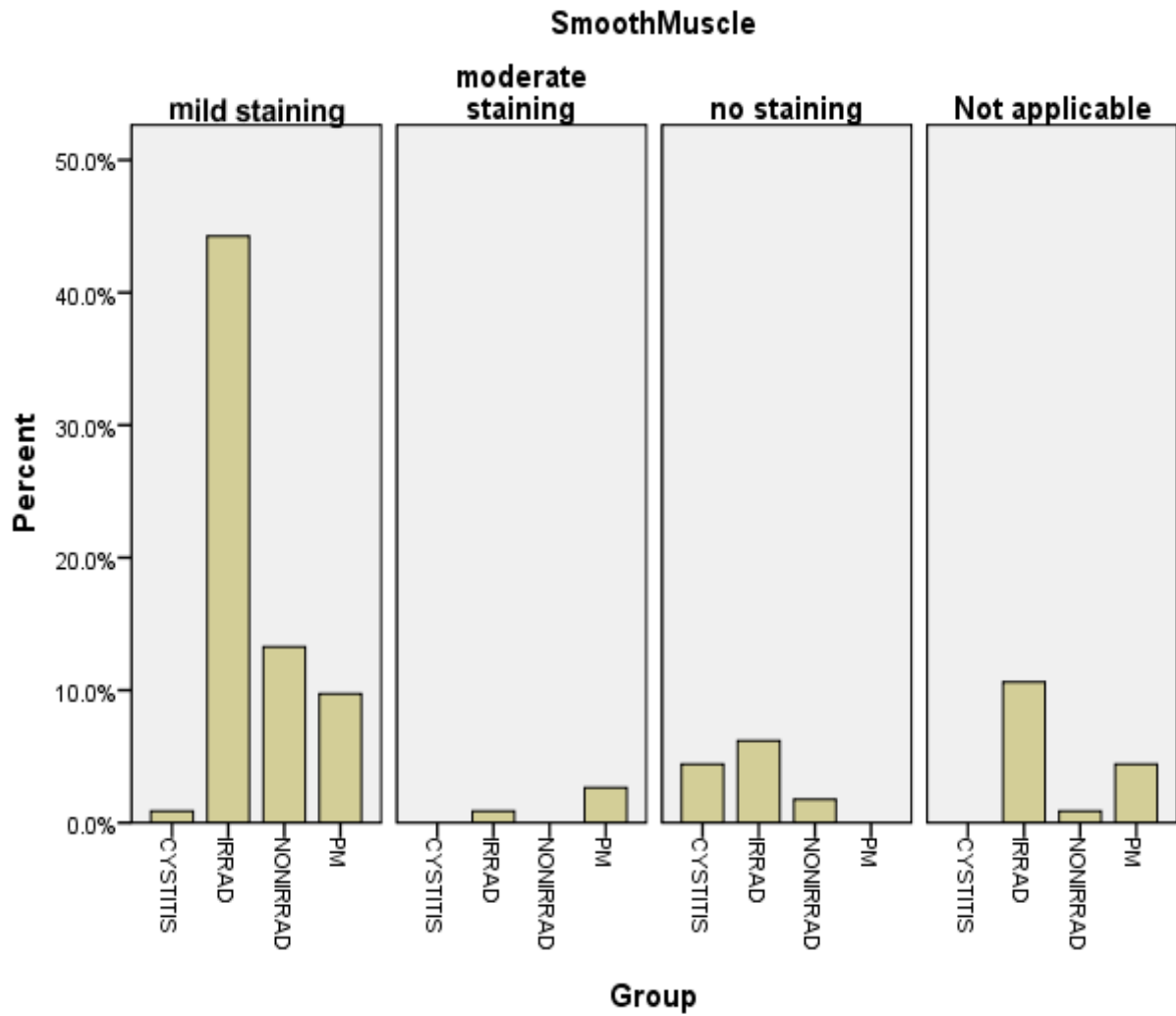


Large Vessels Crosstabulation

Group	Large Vessels				Total
	mild staining	moderate staining	no staining	Not applicable	
CYSTITIS	6	0	0	0	6
IRRAD	42	16	0	12	70
NONIRRAD	6	10	1	1	18
PM	4	9	0	6	19
	58	35	1	19	113

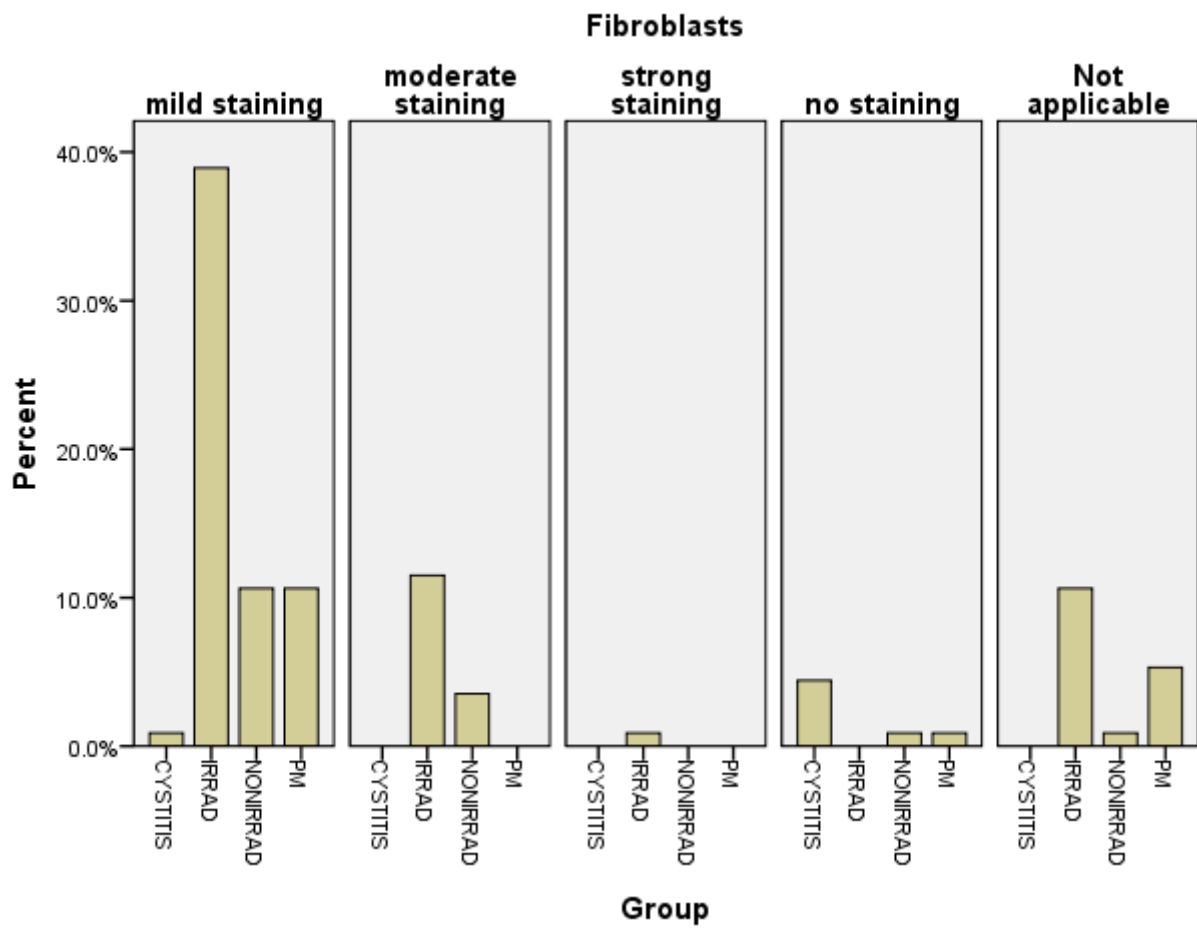
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	26.943^a	9	.001
Likelihood Ratio	27.903	9	.001
N of Valid Cases	113		



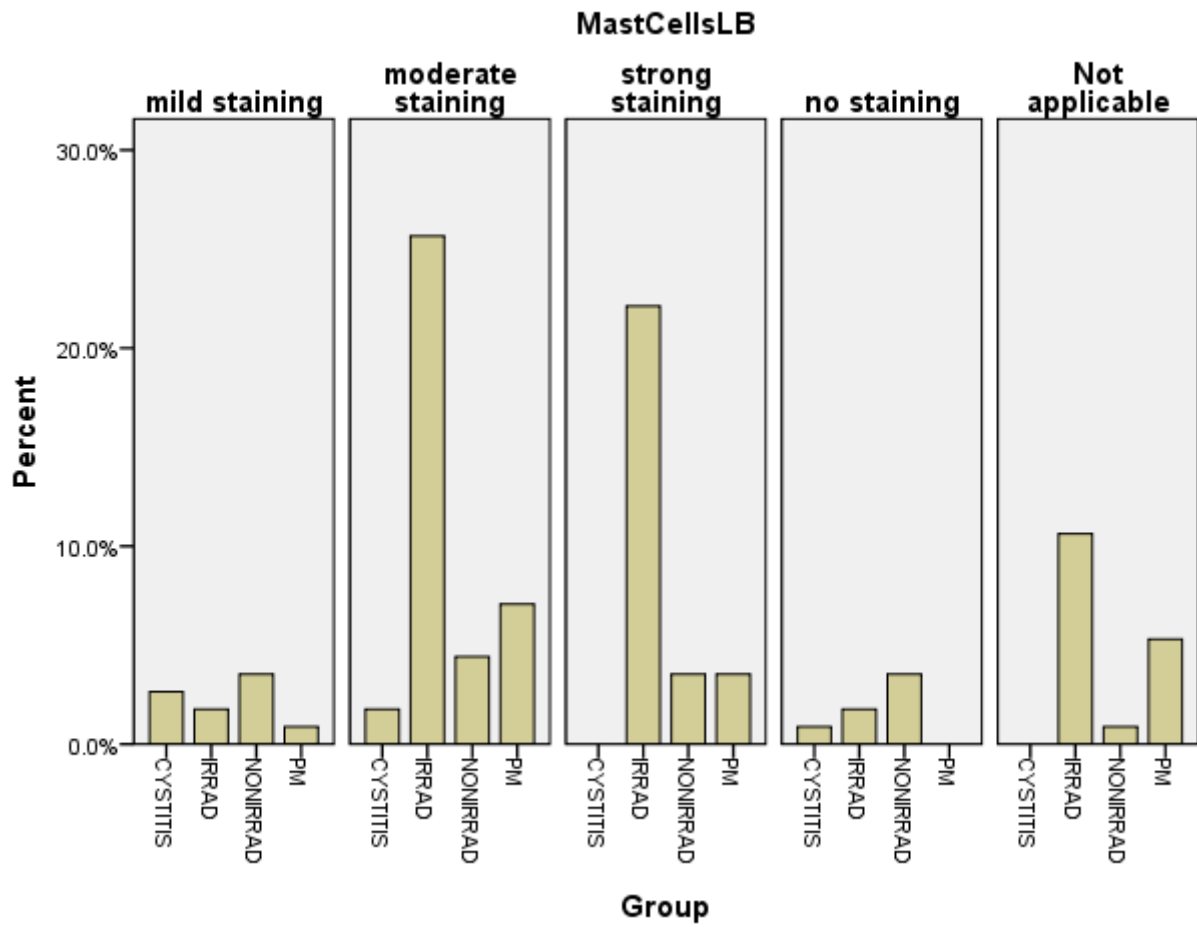
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	50.549 ^a	15	.000
Likelihood Ratio	42.274	15	.000
N of Valid Cases	118		



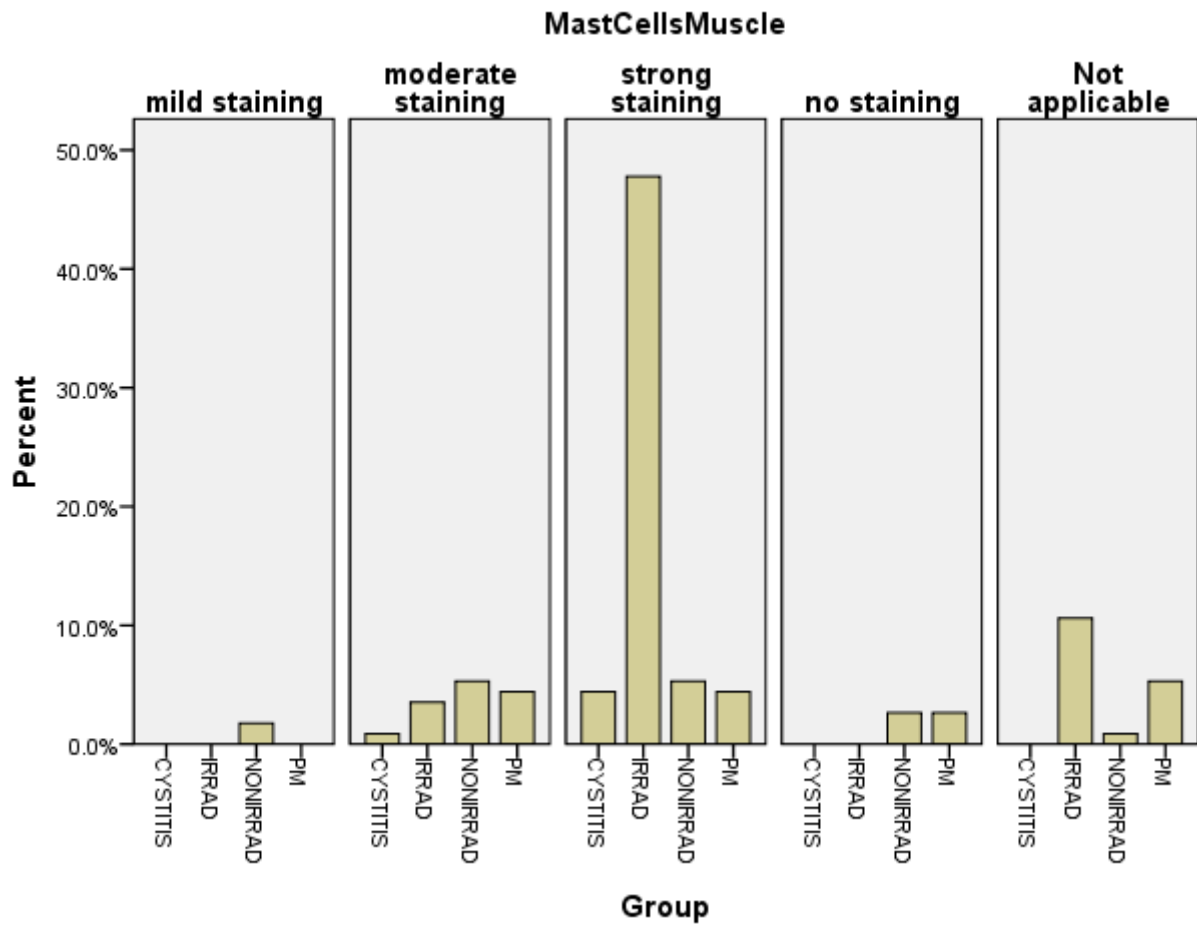
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	84.777 ^a	20	.000
Likelihood Ratio	57.815	20	.000
N of Valid Cases	118		



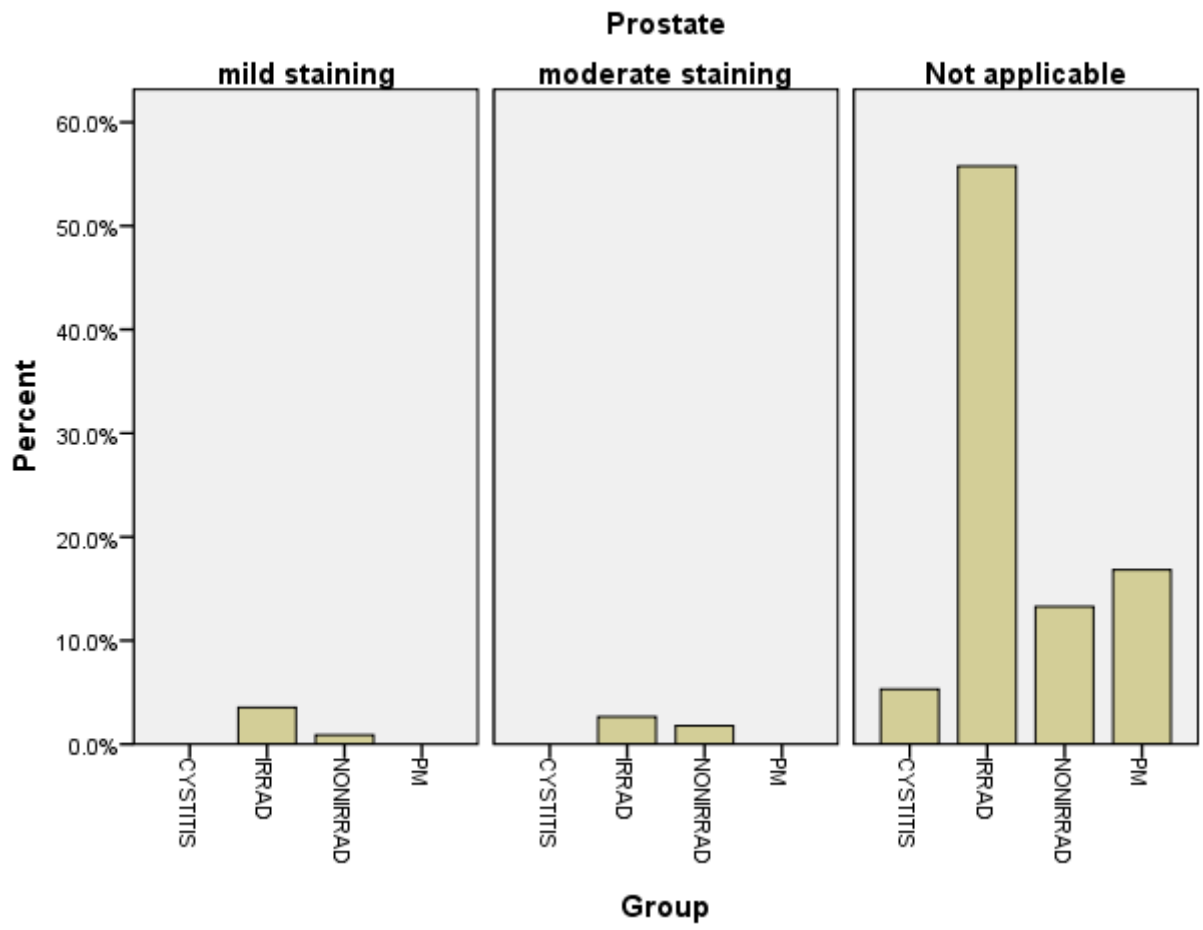
Chi-Square Tests

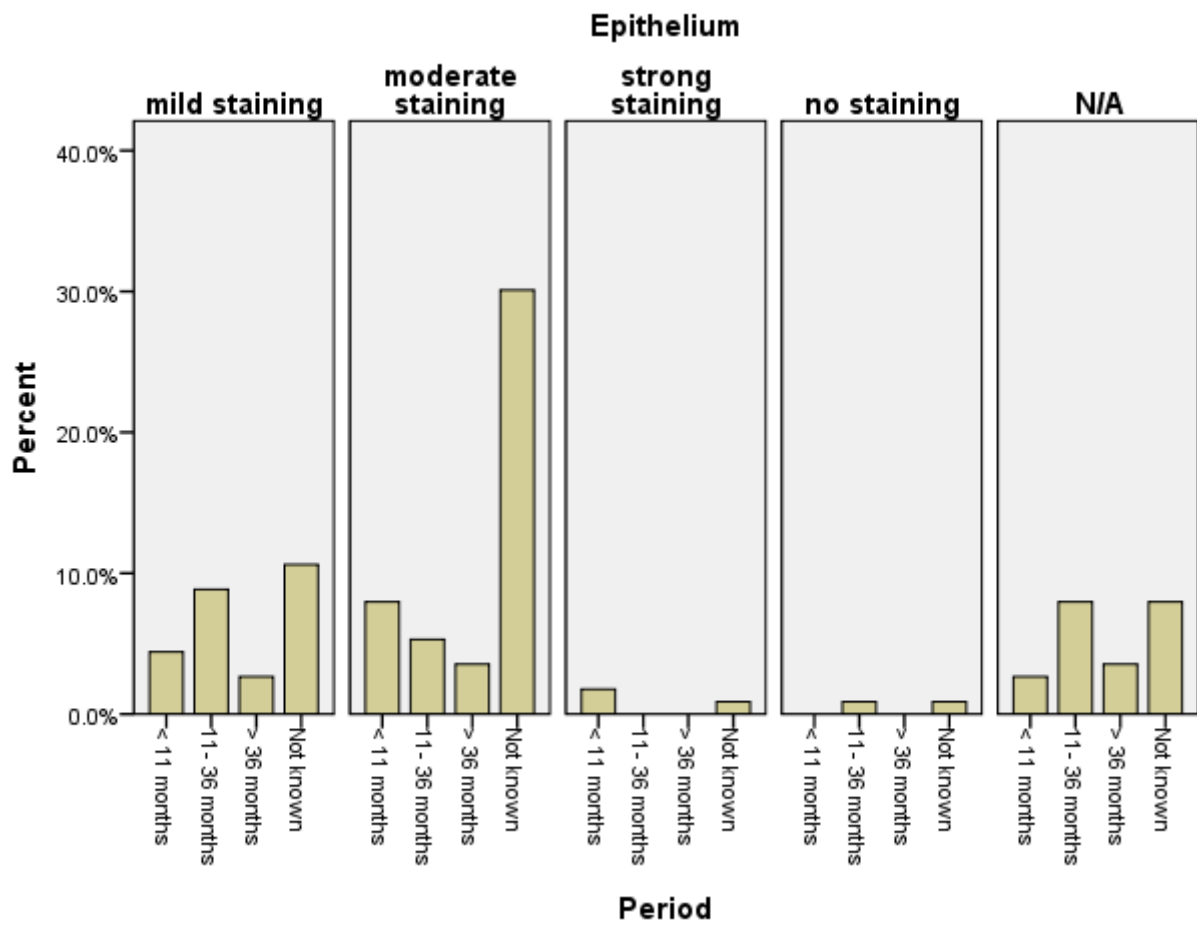
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	45.515 ^a	20	.001
Likelihood Ratio	43.618	20	.002
N of Valid Cases	118		



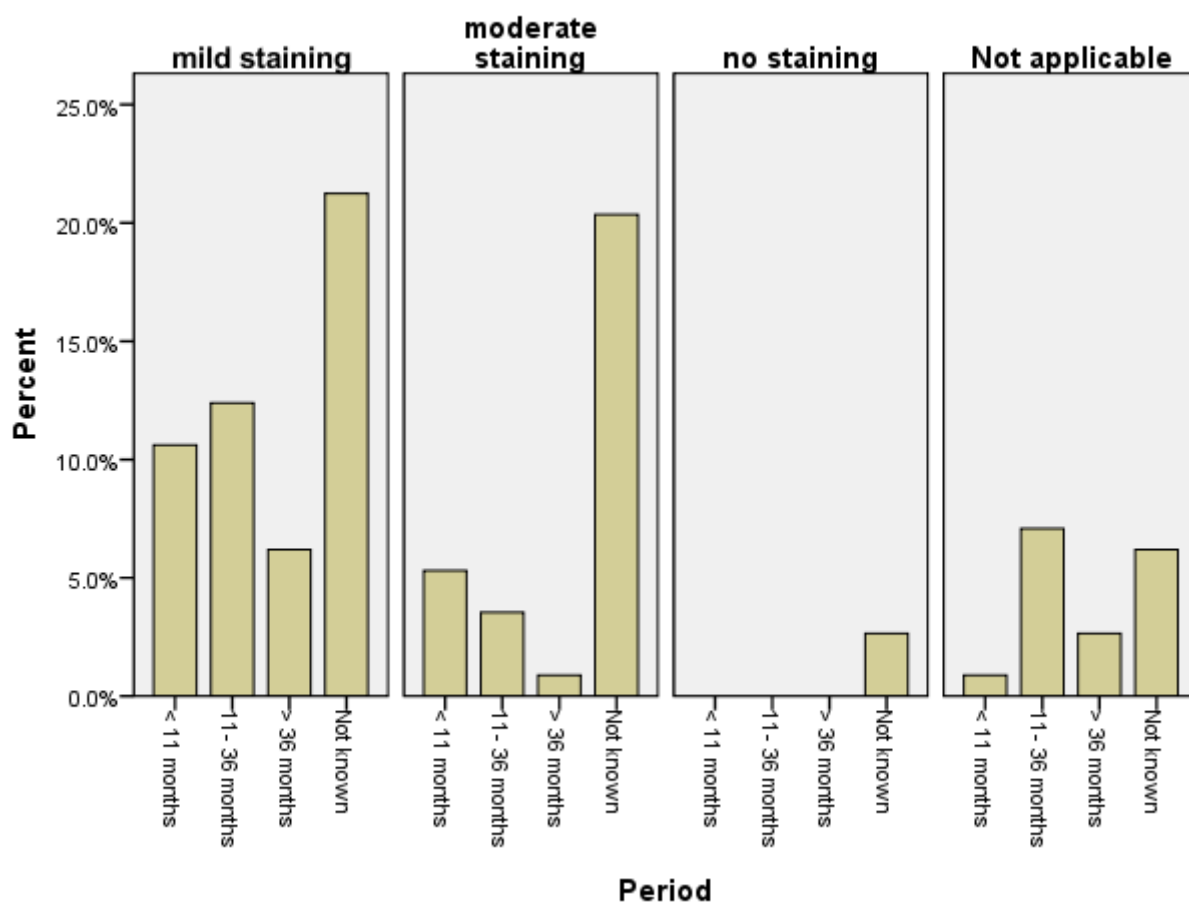
Chi-Square Tests

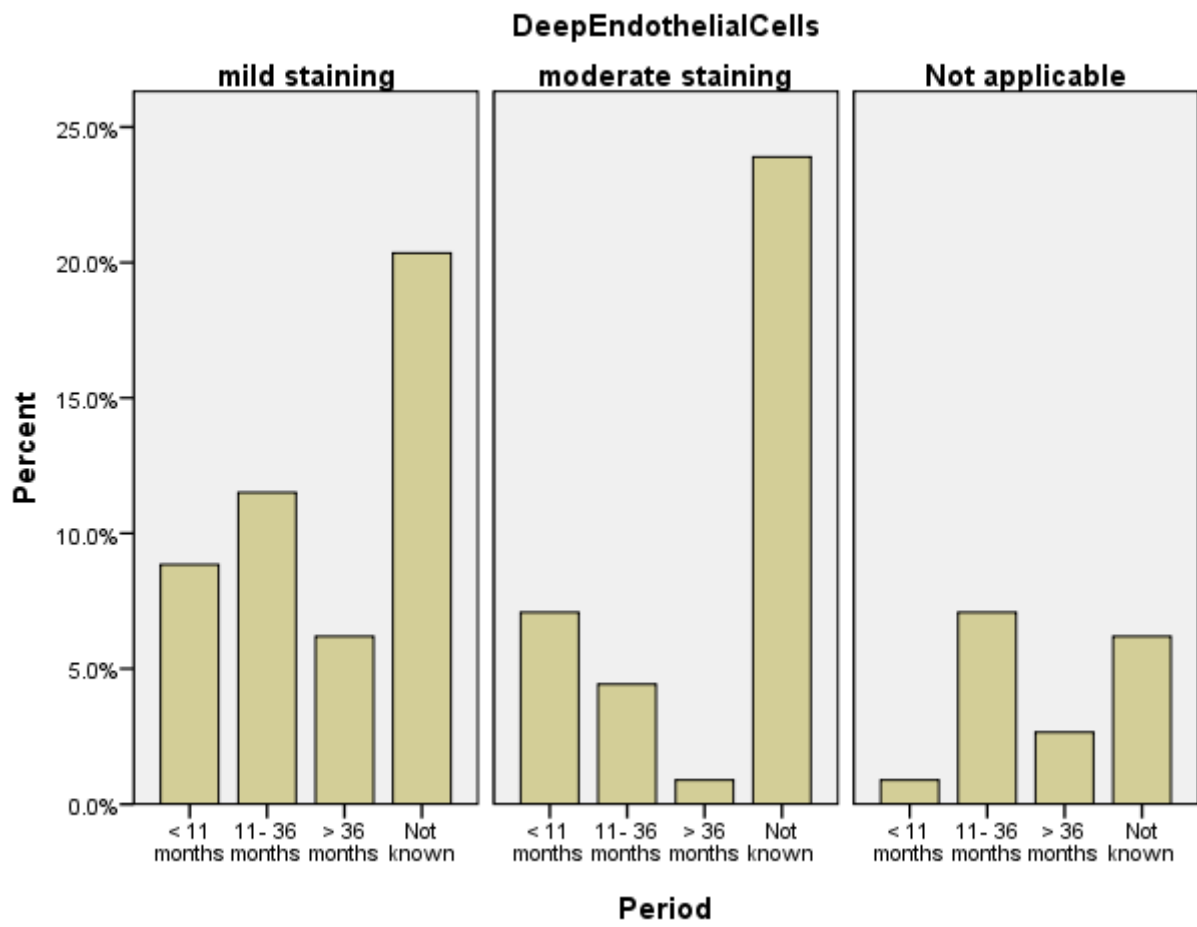
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	53.856 ^a	20	.000
Likelihood Ratio	56.761	20	.000
N of Valid Cases	118		

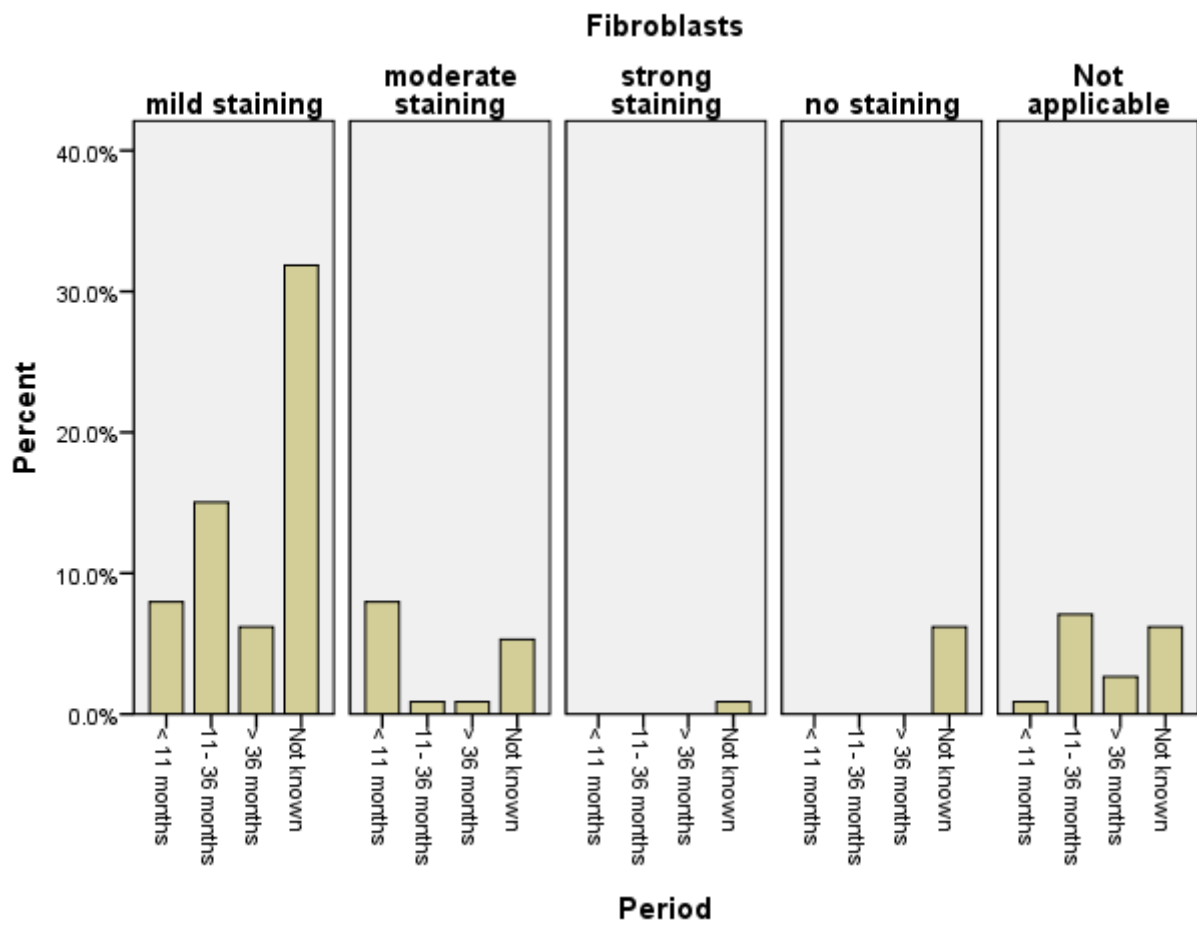


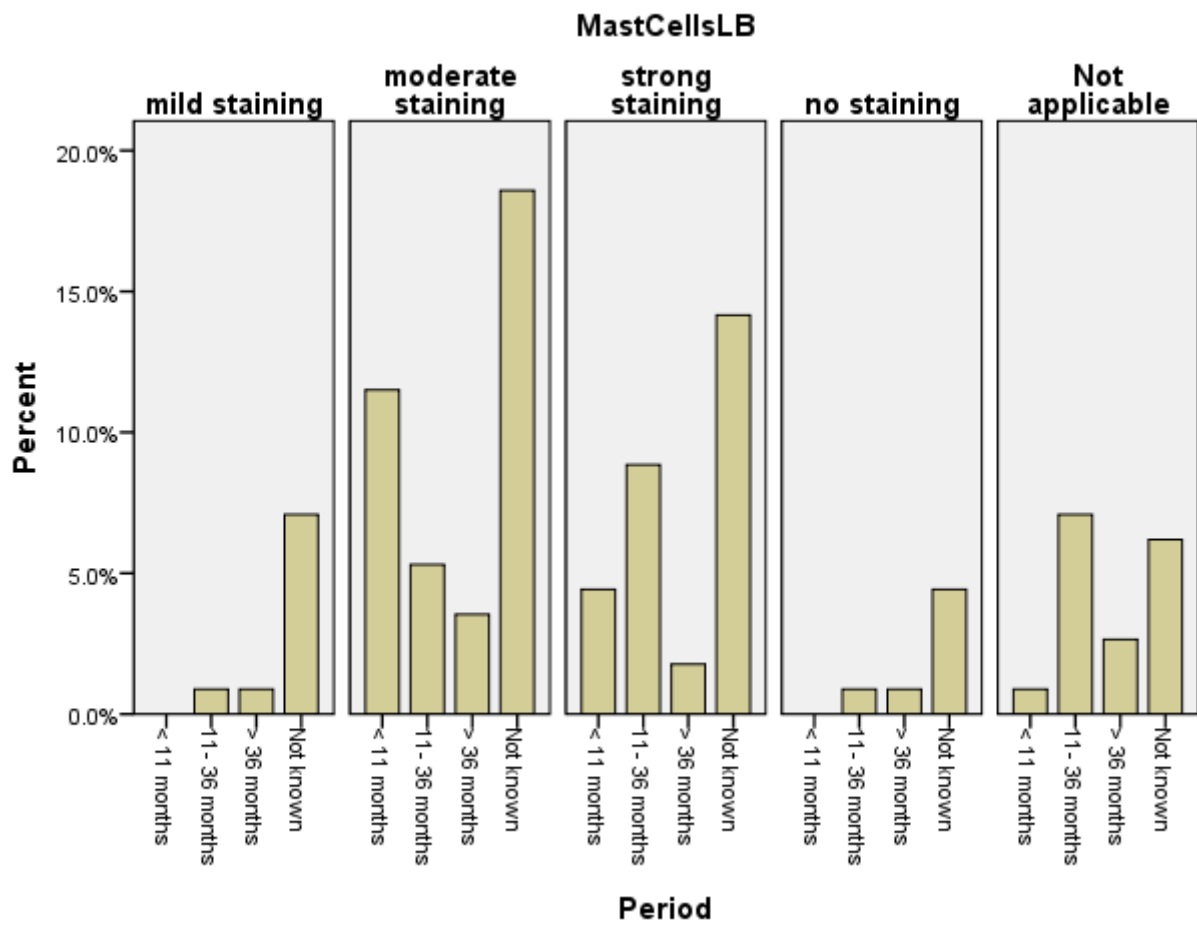


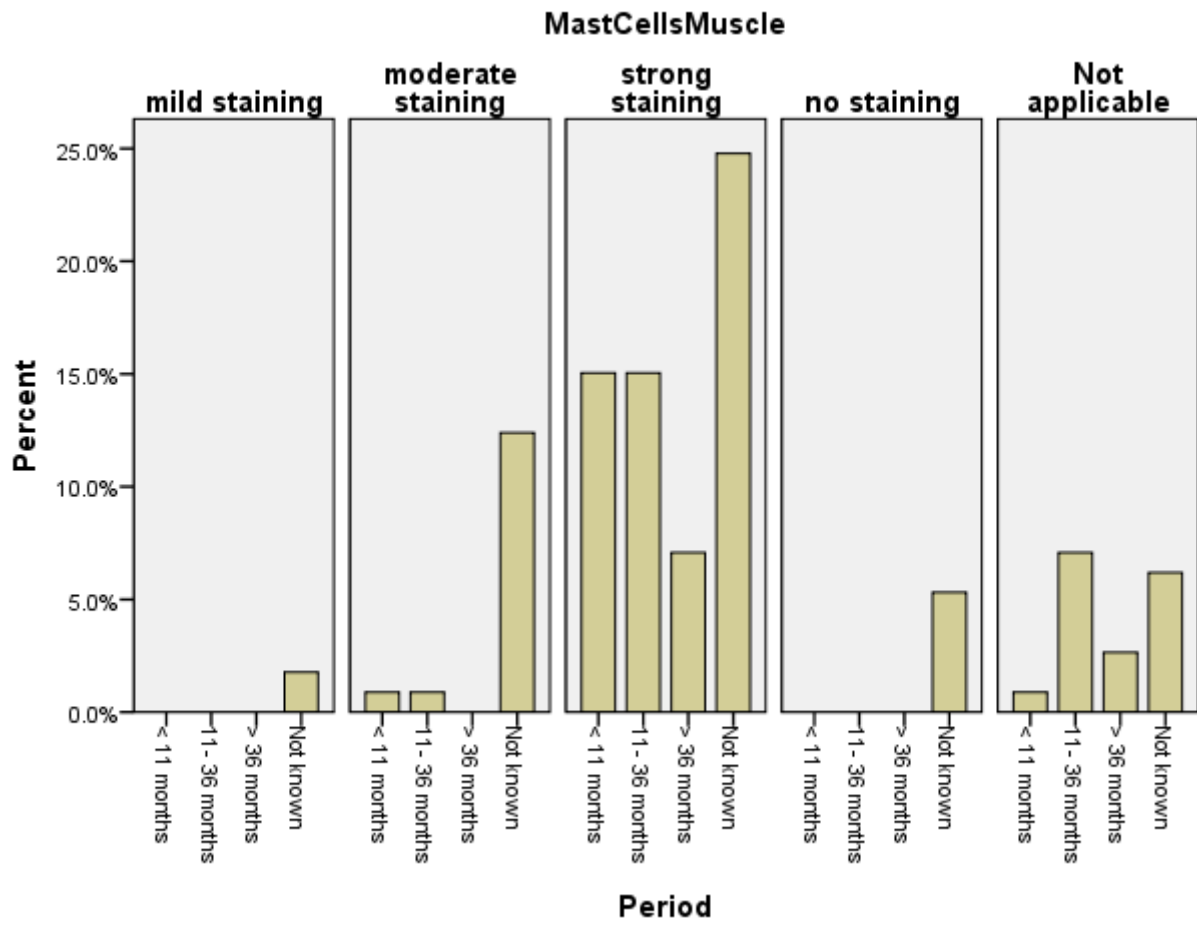
Superficial Endothelial Cells



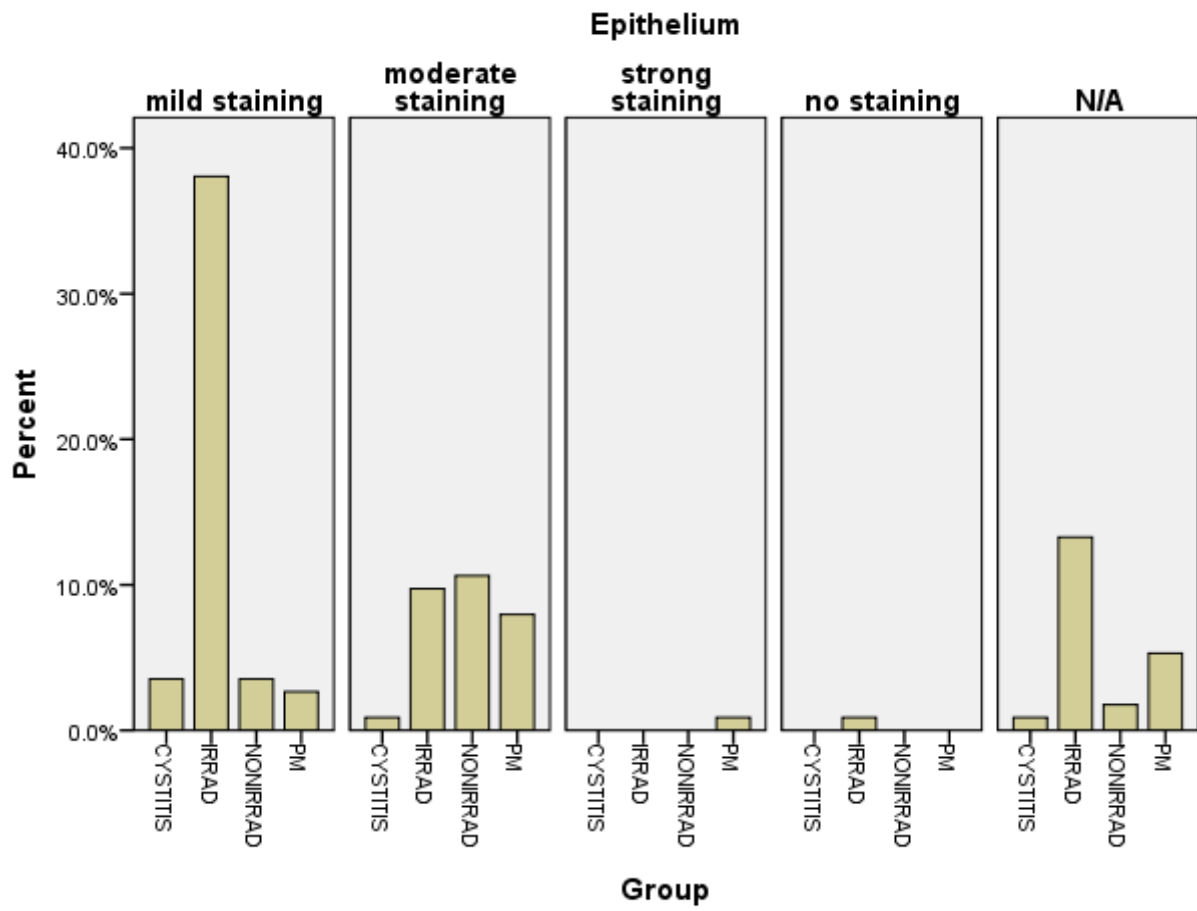








PDGFR-alpha



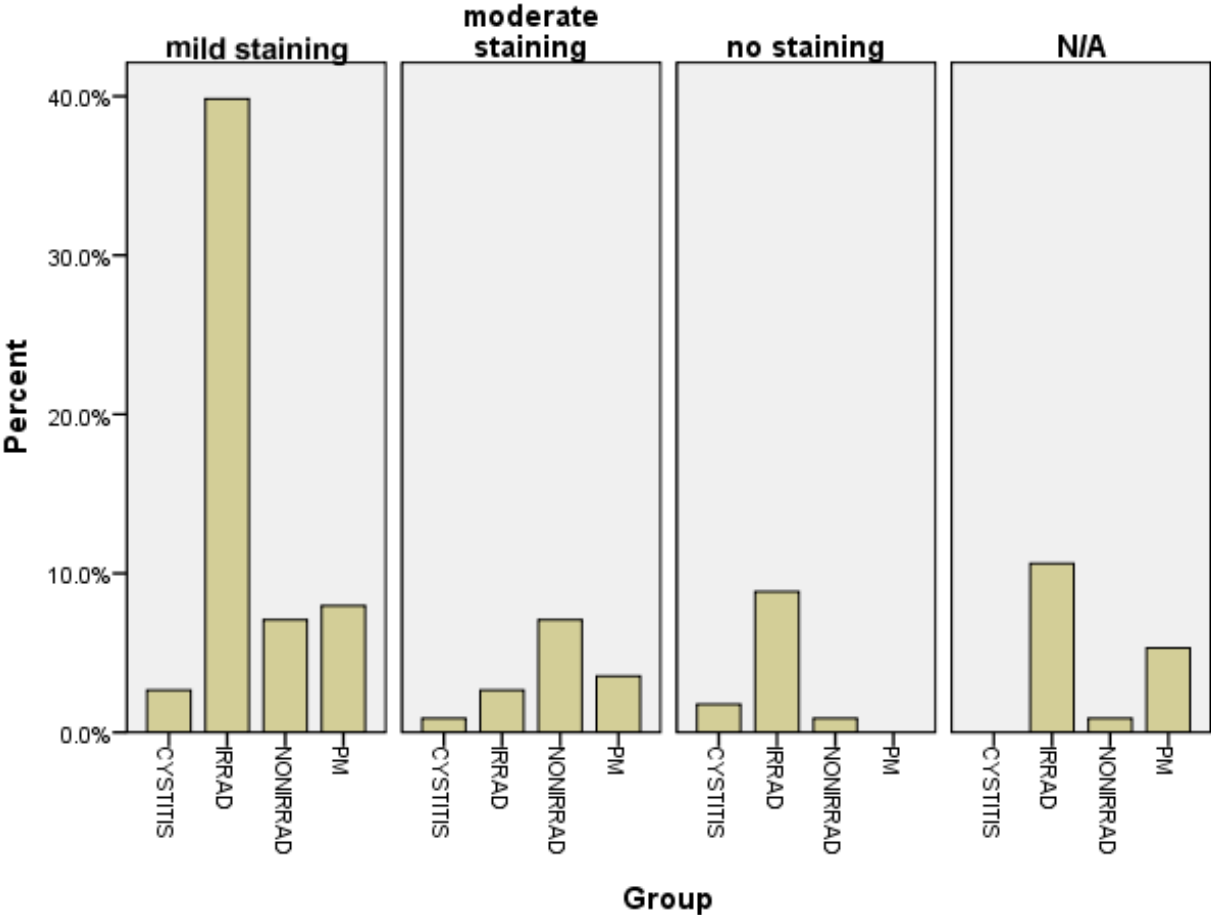
Epithelium Crosstabulation

Group	Epithelium					
	mild staining	moderate staining	strong staining	no staining	N/A	Total
CYSTITIS	4	1	0	0	1	6
IRRAD	43	11	0	1	15	70
NONIRRAD	4	12	0	0	2	18
PM	3	9	1	0	6	19
	54	33	1	1	24	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	32.635^a	12	.001
Likelihood Ratio	31.723	12	.002
N of Valid Cases	113		

SuperficialEndothelialCells



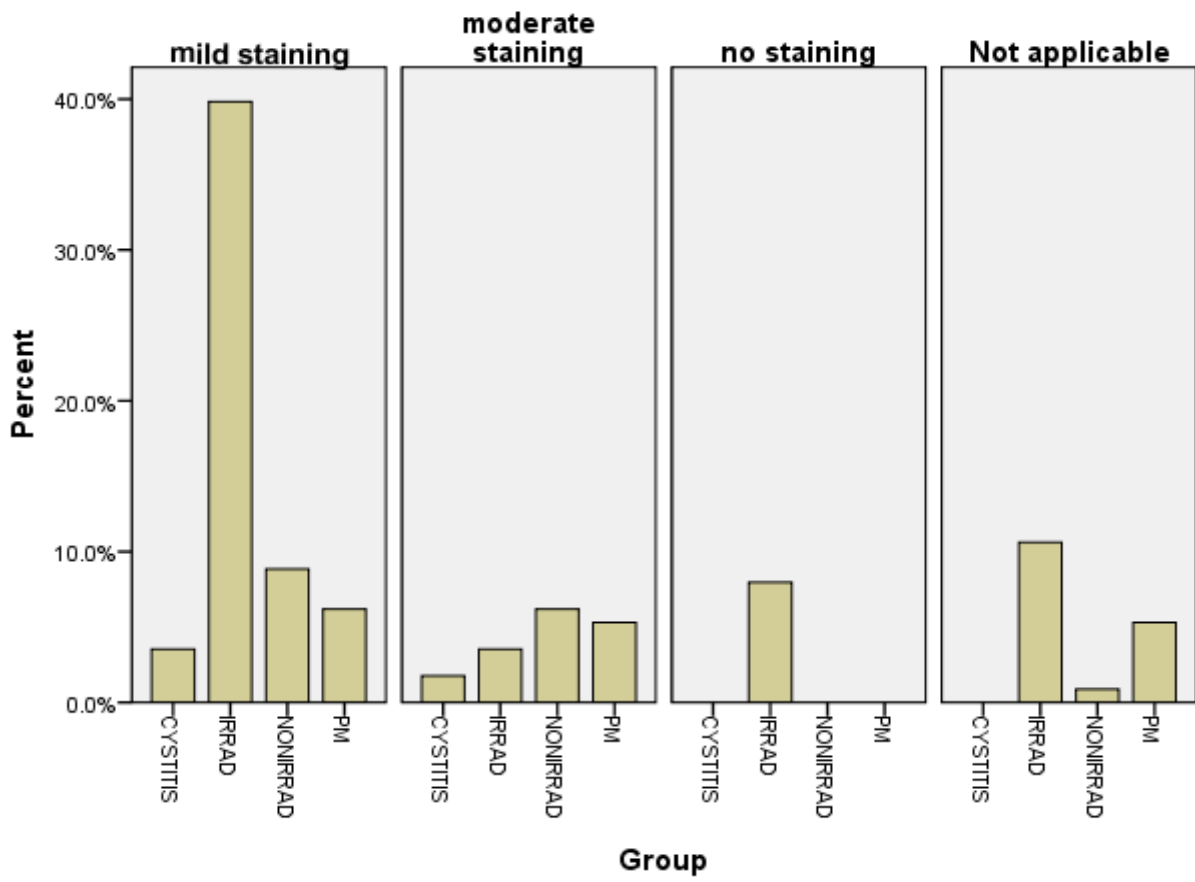
Superficial Endothelial Cells Crosstabulation

Group	Superficial Endothelial Cells				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	3	1	2	0	6
IRRAD	45	3	10	12	70
NONIRRAD	8	8	1	1	18
PM	9	4	0	6	19
	65	16	13	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	29.164^a	9	.001
Likelihood Ratio	29.115	9	.001
N of Valid Cases	113		

DeepEndothelialCells

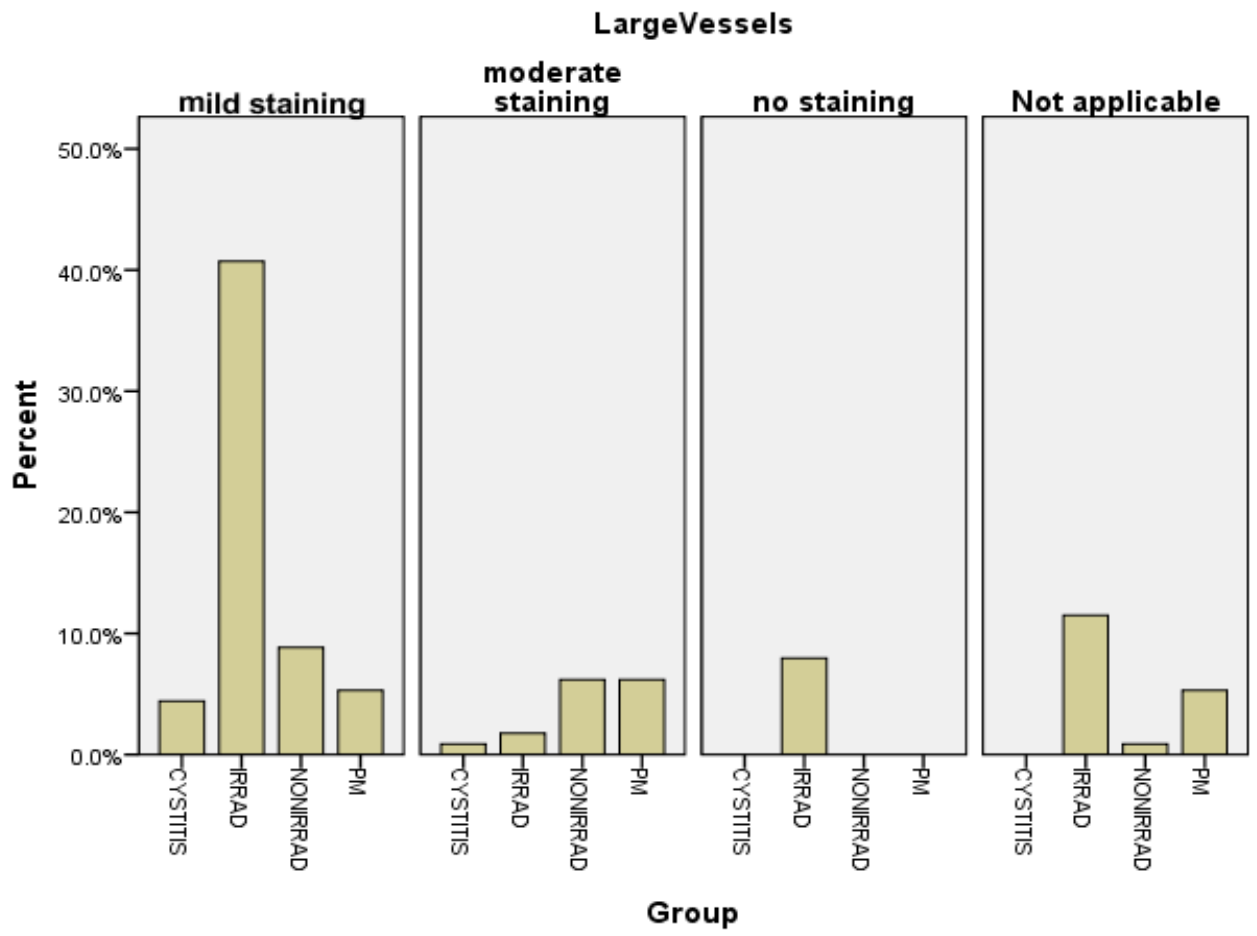


Deep Endothelial Cells Crosstabulation

Group	Deep Endothelial Cells				
	mild staining	moderate staining	no staining	Not applicable	Total
CYSTITIS	4	2	0	0	6
IRRAD	45	4	9	12	70
NONIRRAD	10	7	0	1	18
PM	7	6	0	6	19
	66	19	9	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	26.167^a	9	.002
Likelihood Ratio	30.075	9	.000
N of Valid Cases	113		

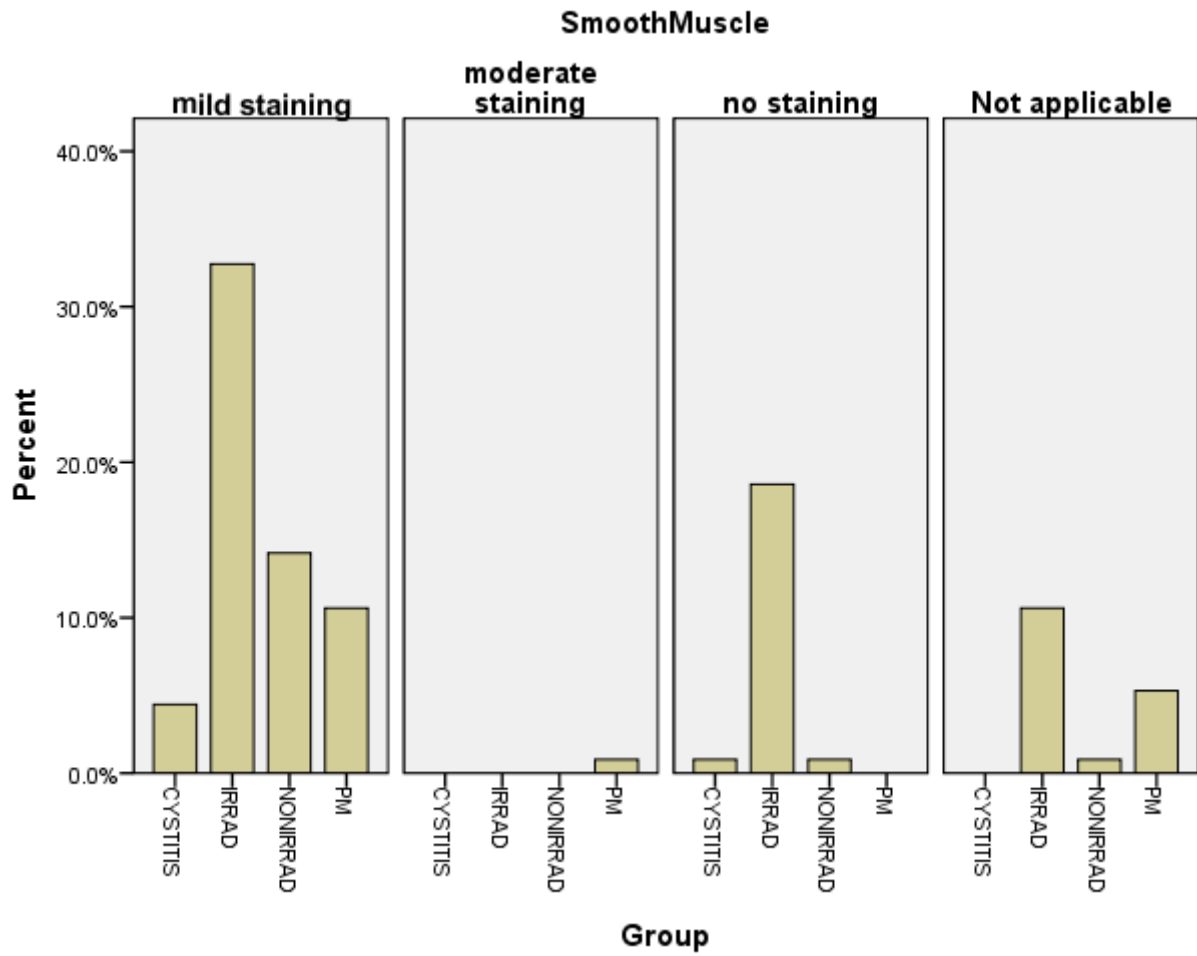


Large Vessels Crosstabulation

Group	Large Vessels				
	mild staining	moderate staining	no staining	Not applicable	Total
CYSTITIS	5	1	0	0	6
IRRAD	46	2	9	13	70
NONIRRAD	10	7	0	1	18
PM	6	7	0	6	19
	67	17	9	20	113

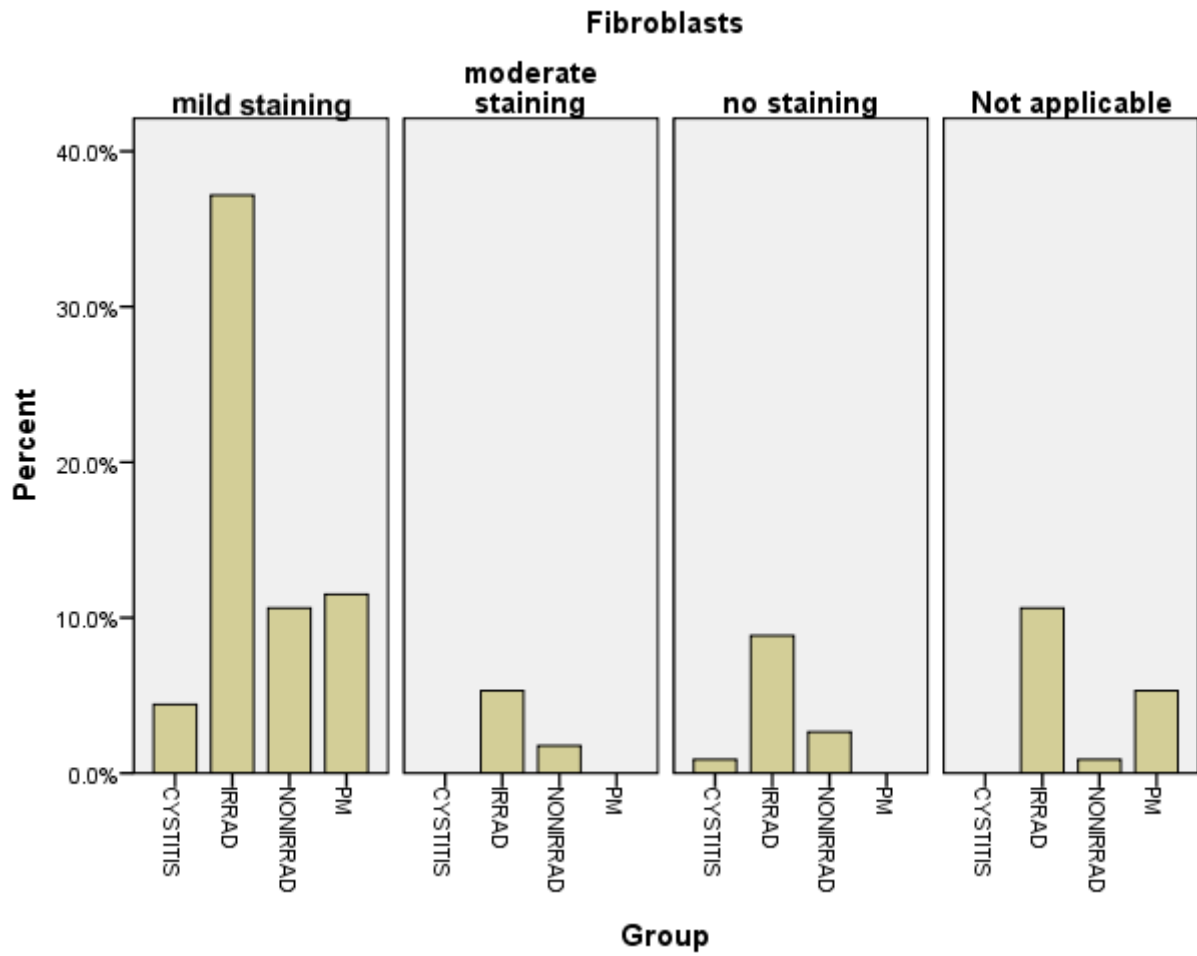
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	34.277^a	12	.001
Likelihood Ratio	38.790	12	.000
N of Valid Cases	113		



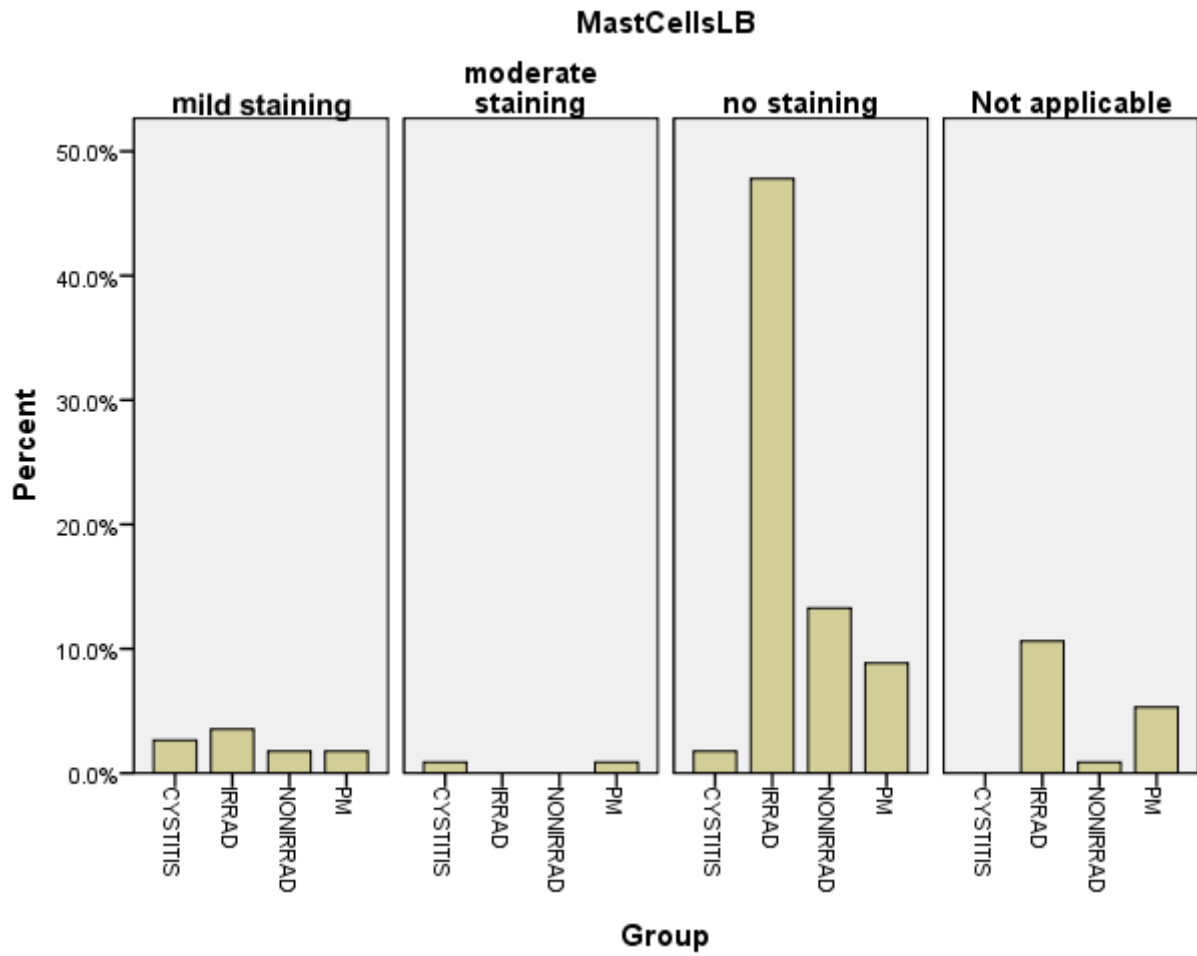
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	43.671 ^a	20	.002
Likelihood Ratio	44.459	20	.001
N of Valid Cases	118		



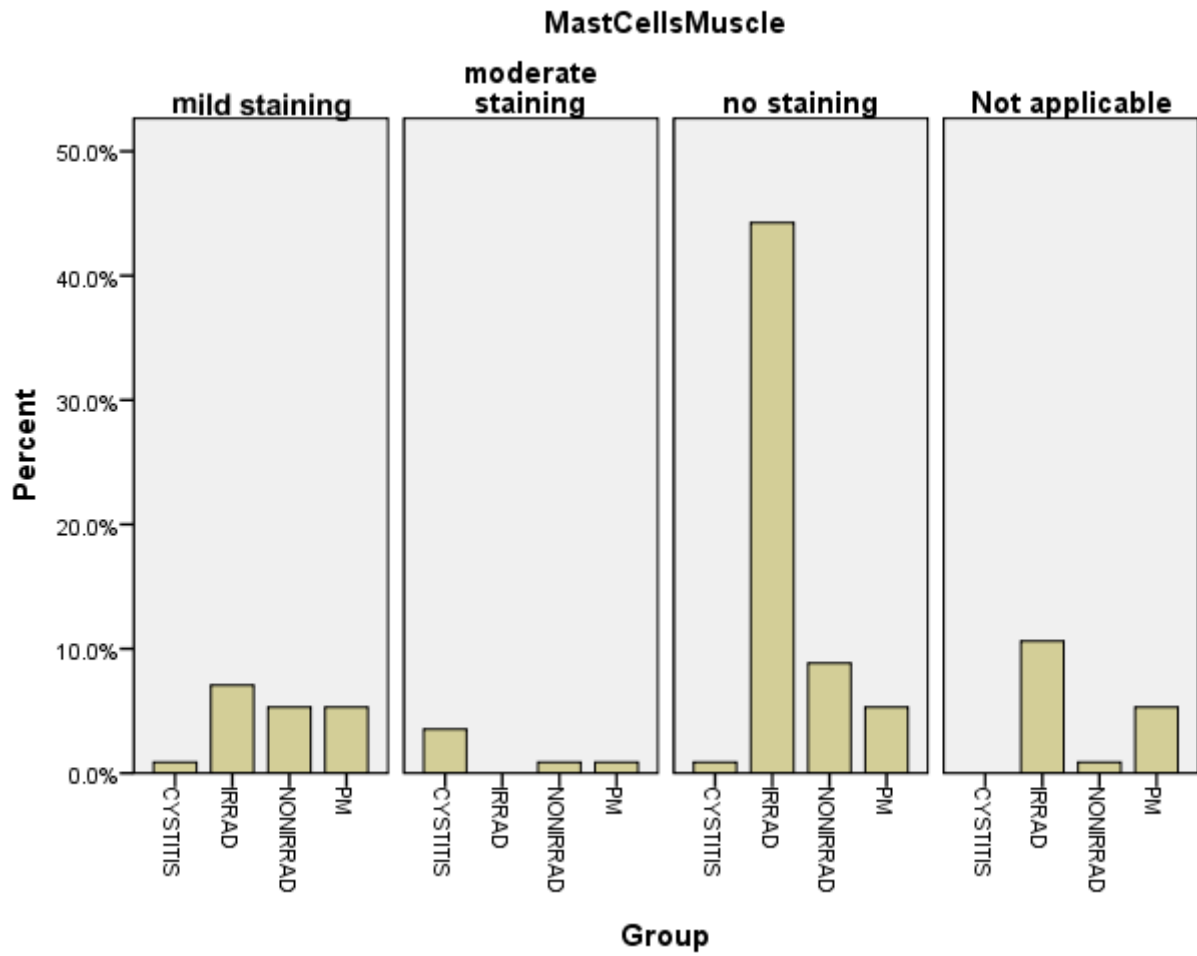
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	50.495 ^a	20	.000
Likelihood Ratio	48.636	20	.000
N of Valid Cases	118		



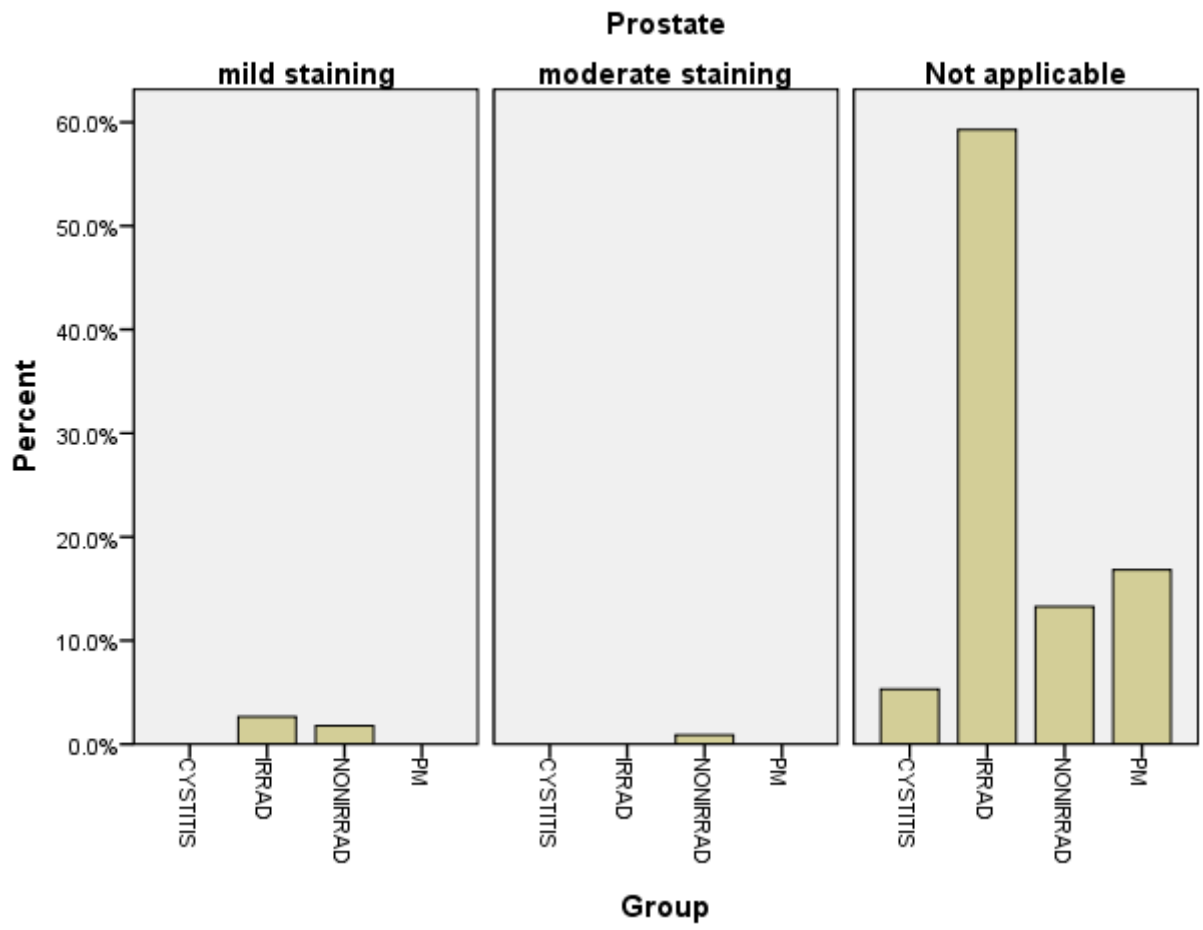
Chi-Square Tests

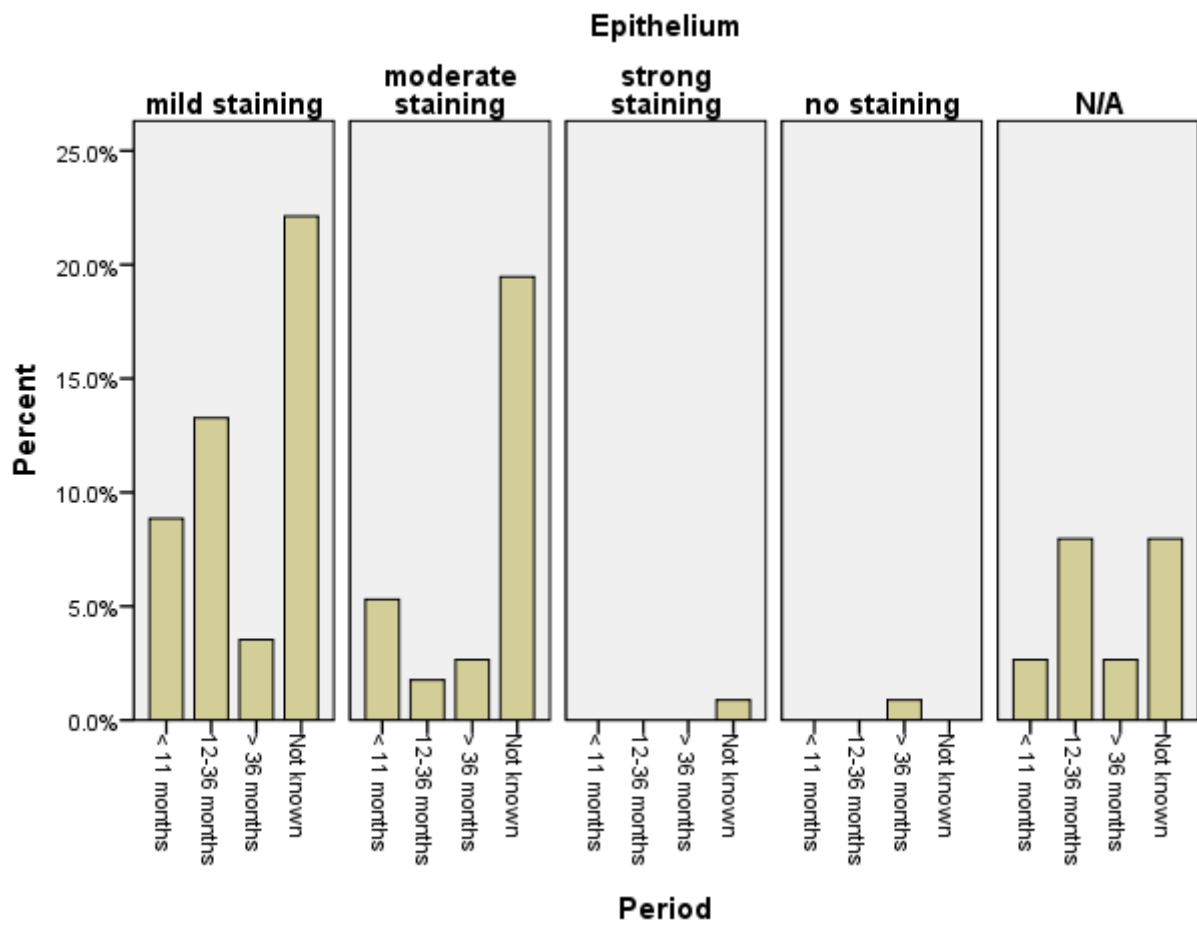
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	41.400 ^a	20	.003
Likelihood Ratio	36.201	20	.015
N of Valid Cases	118		



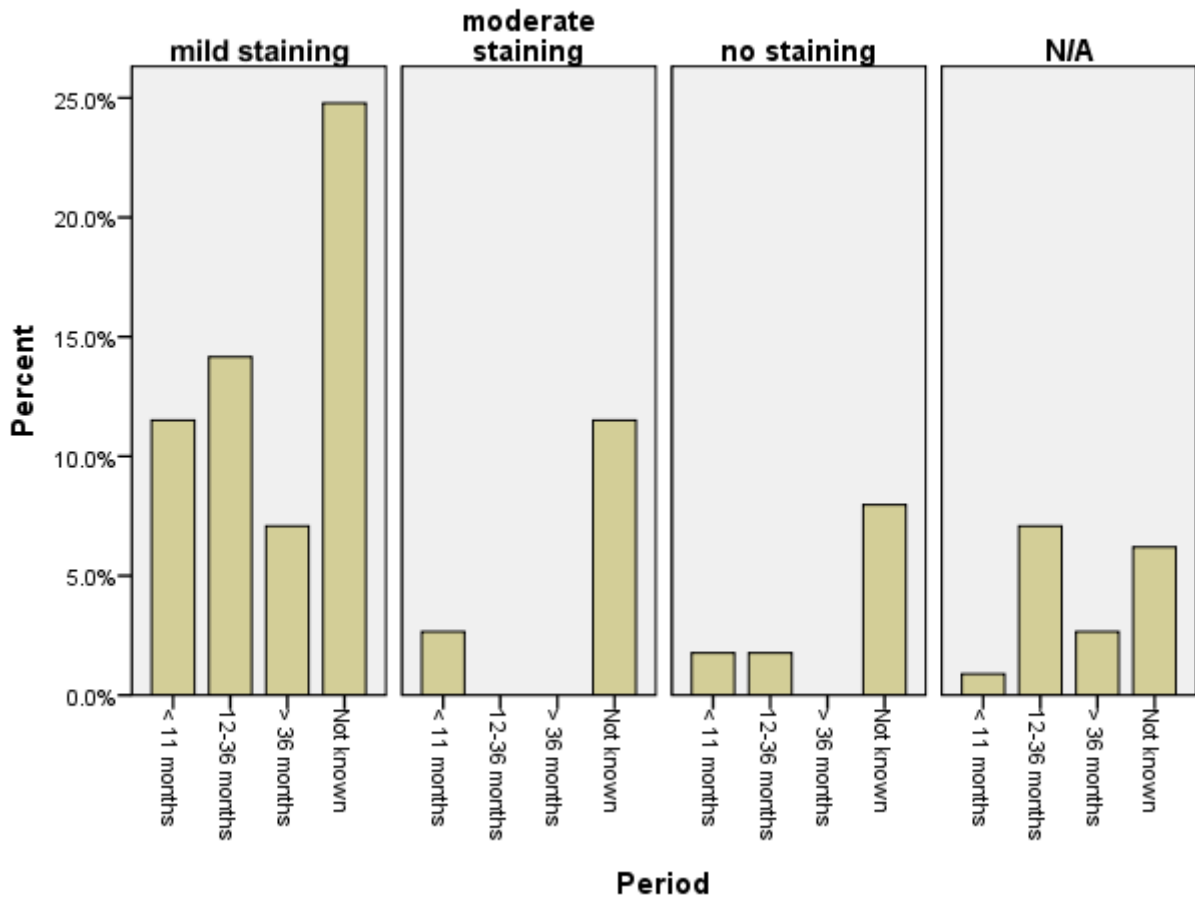
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	73.418 ^a	20	.000
Likelihood Ratio	53.660	20	.000
N of Valid Cases	118		

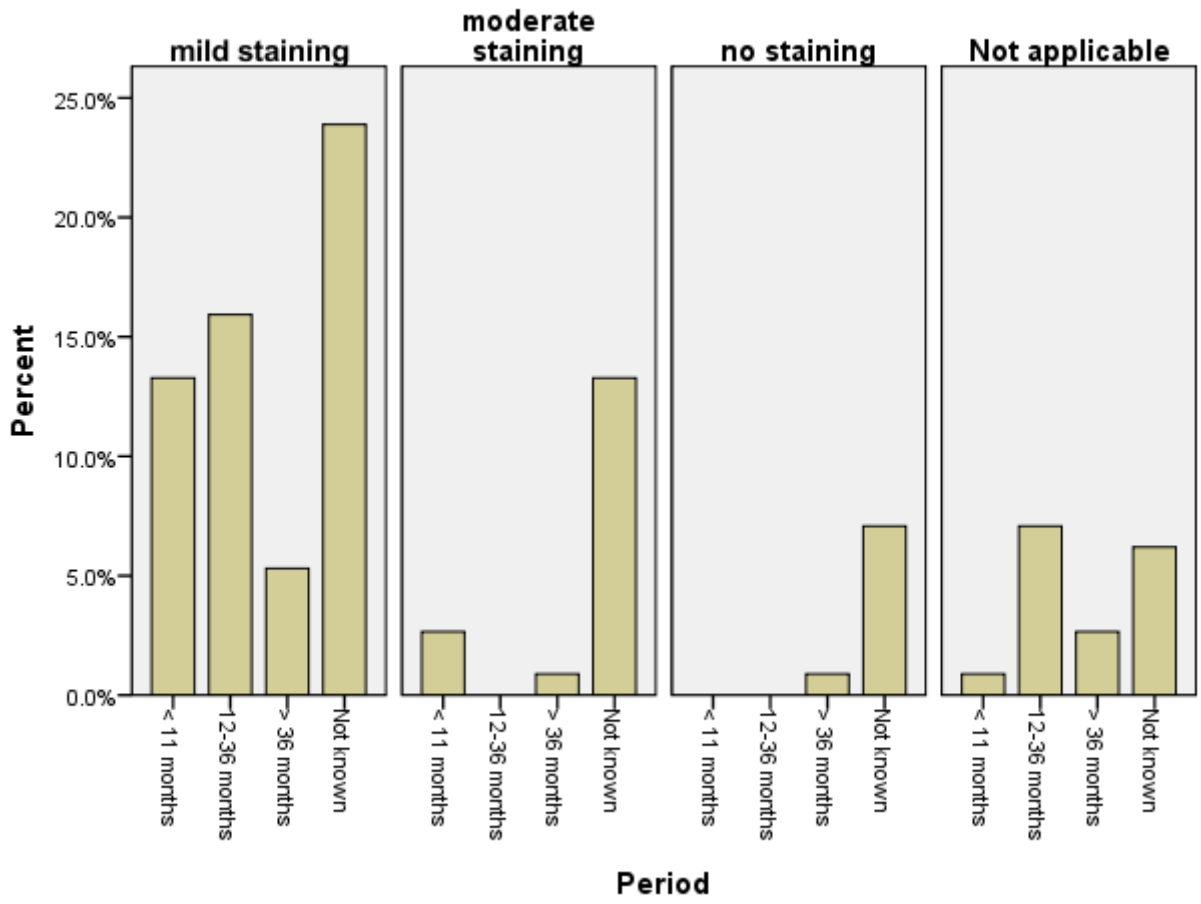




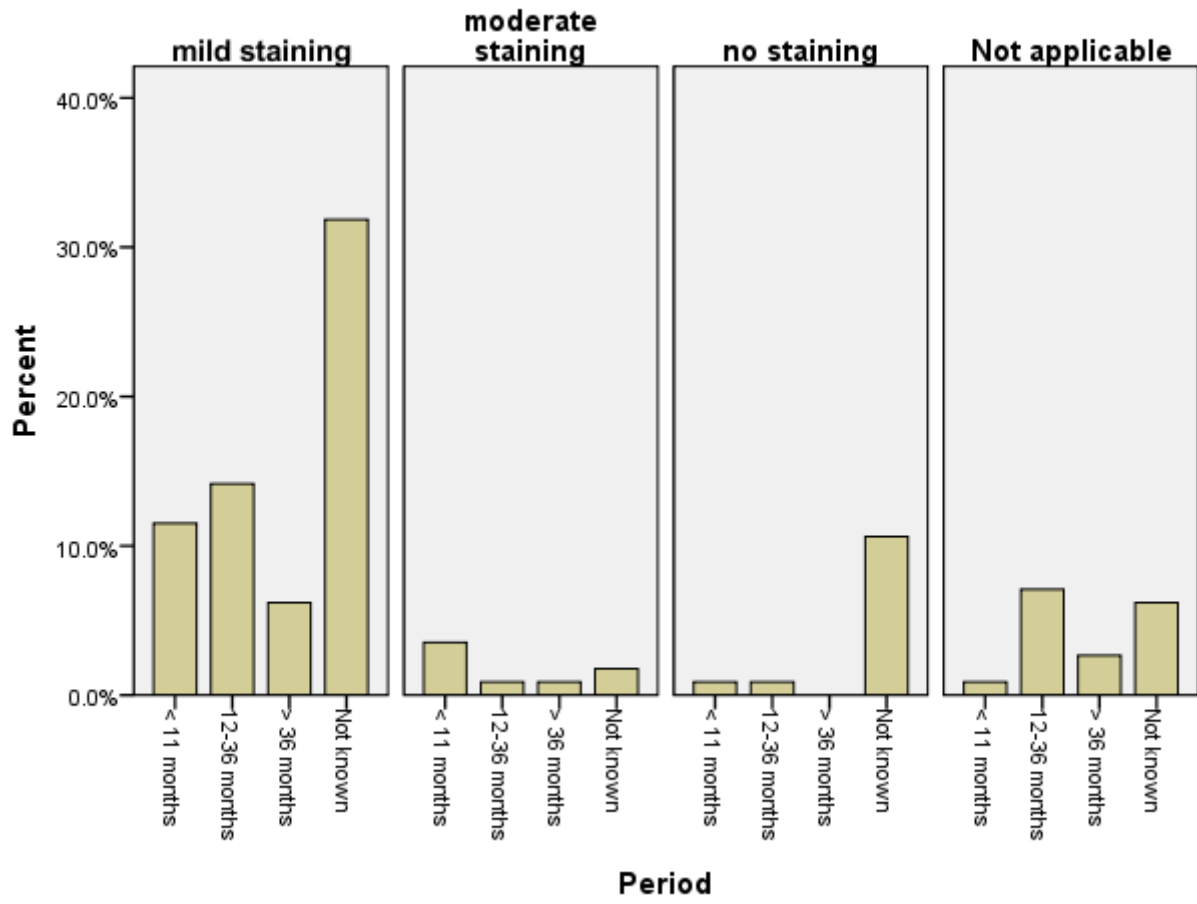
Superficial Endothelial Cells



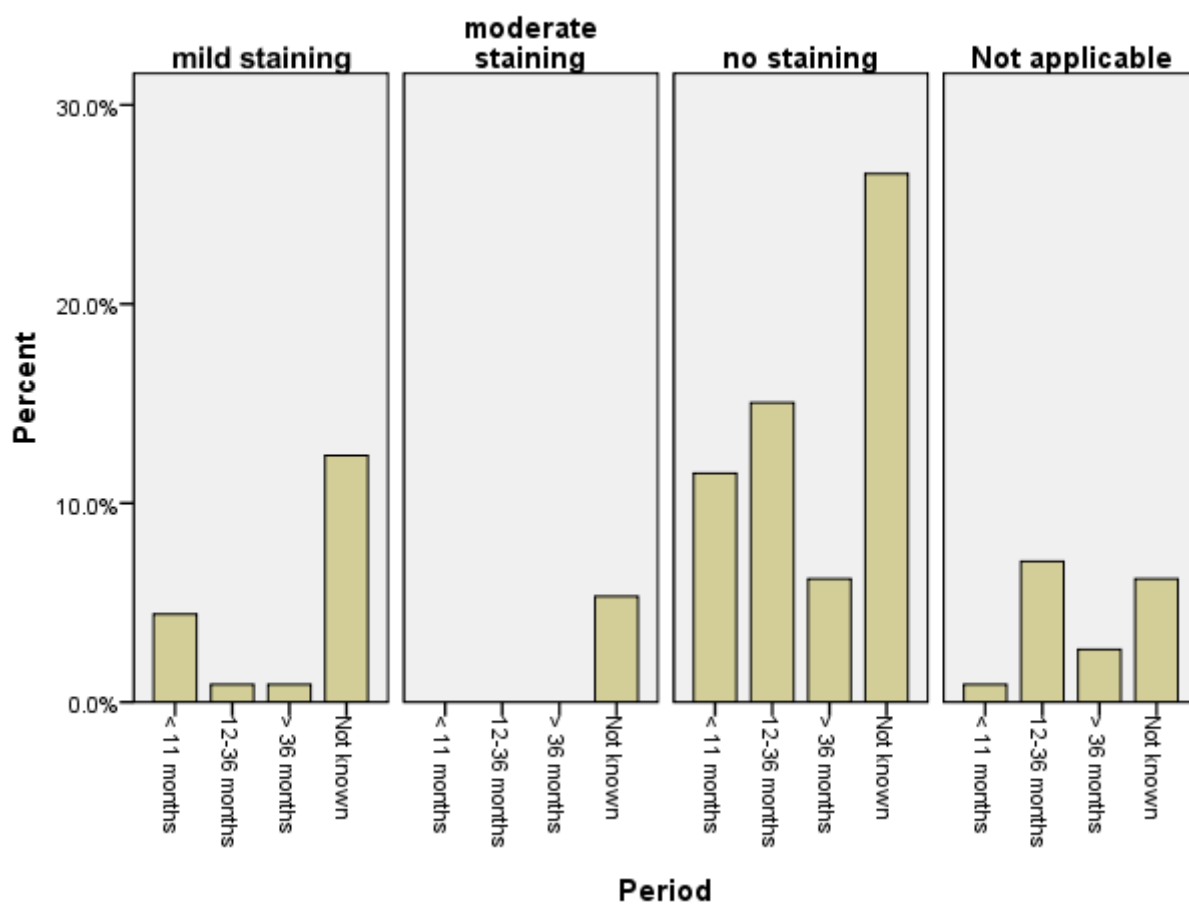
DeepEndothelialCells

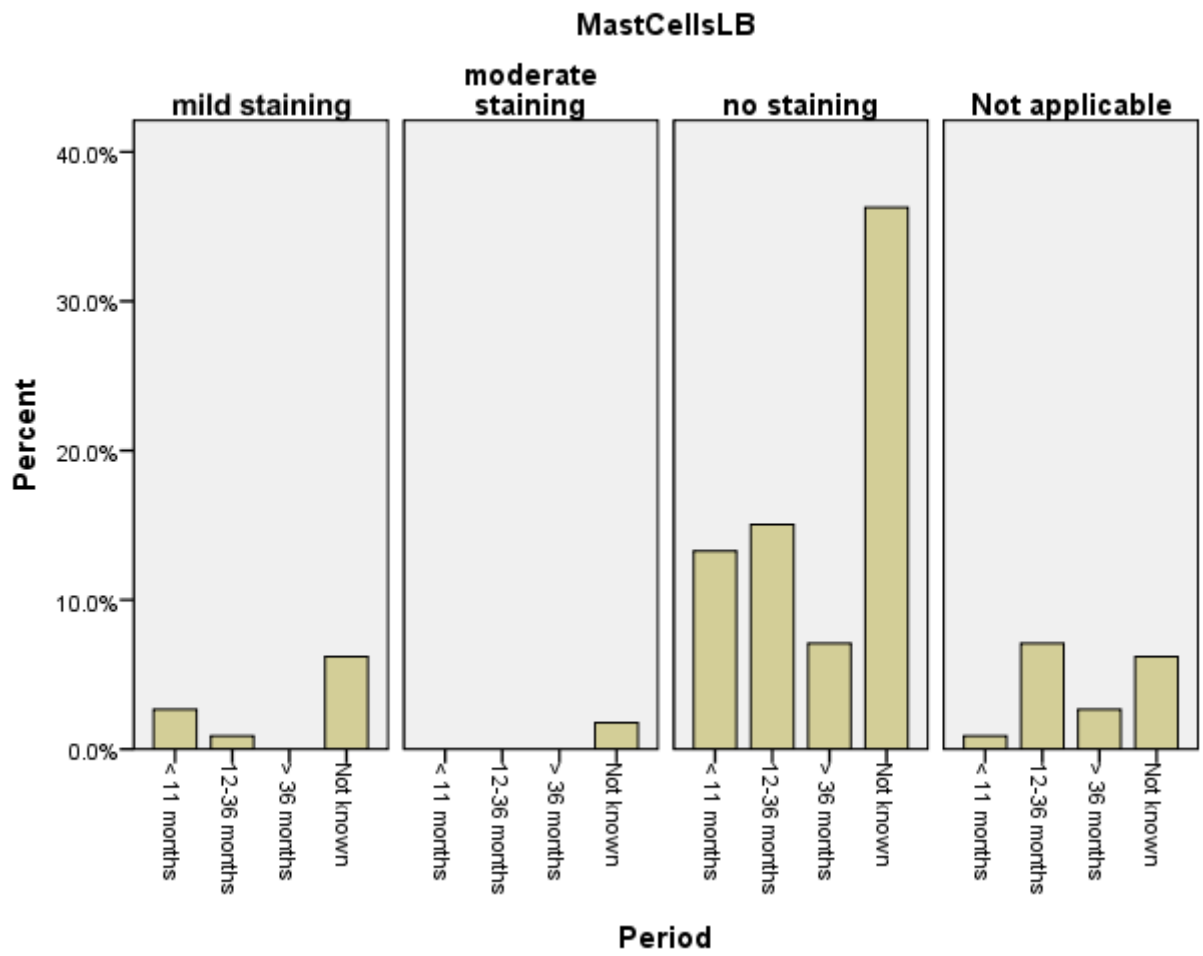


Fibroblasts

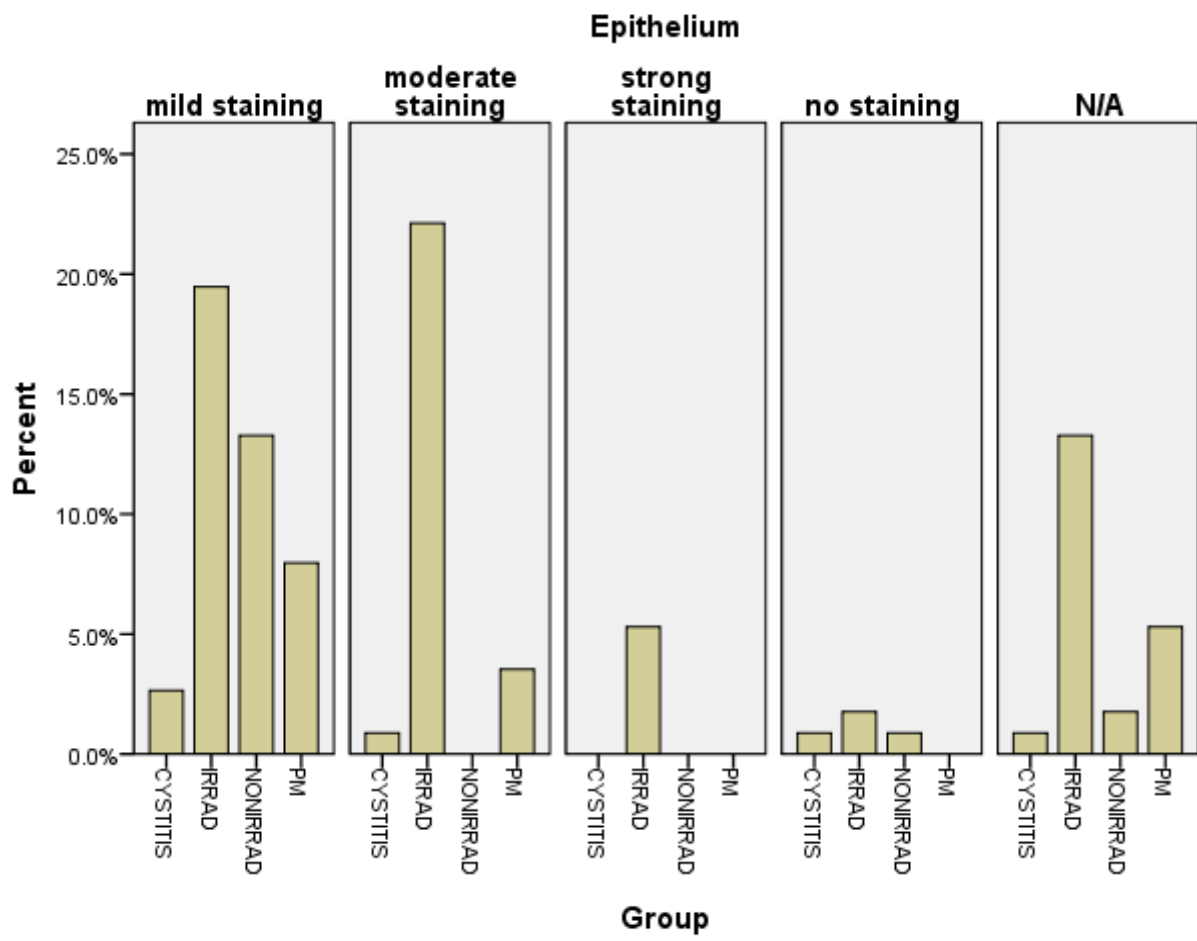


MastCellsMuscle





PDGFBB



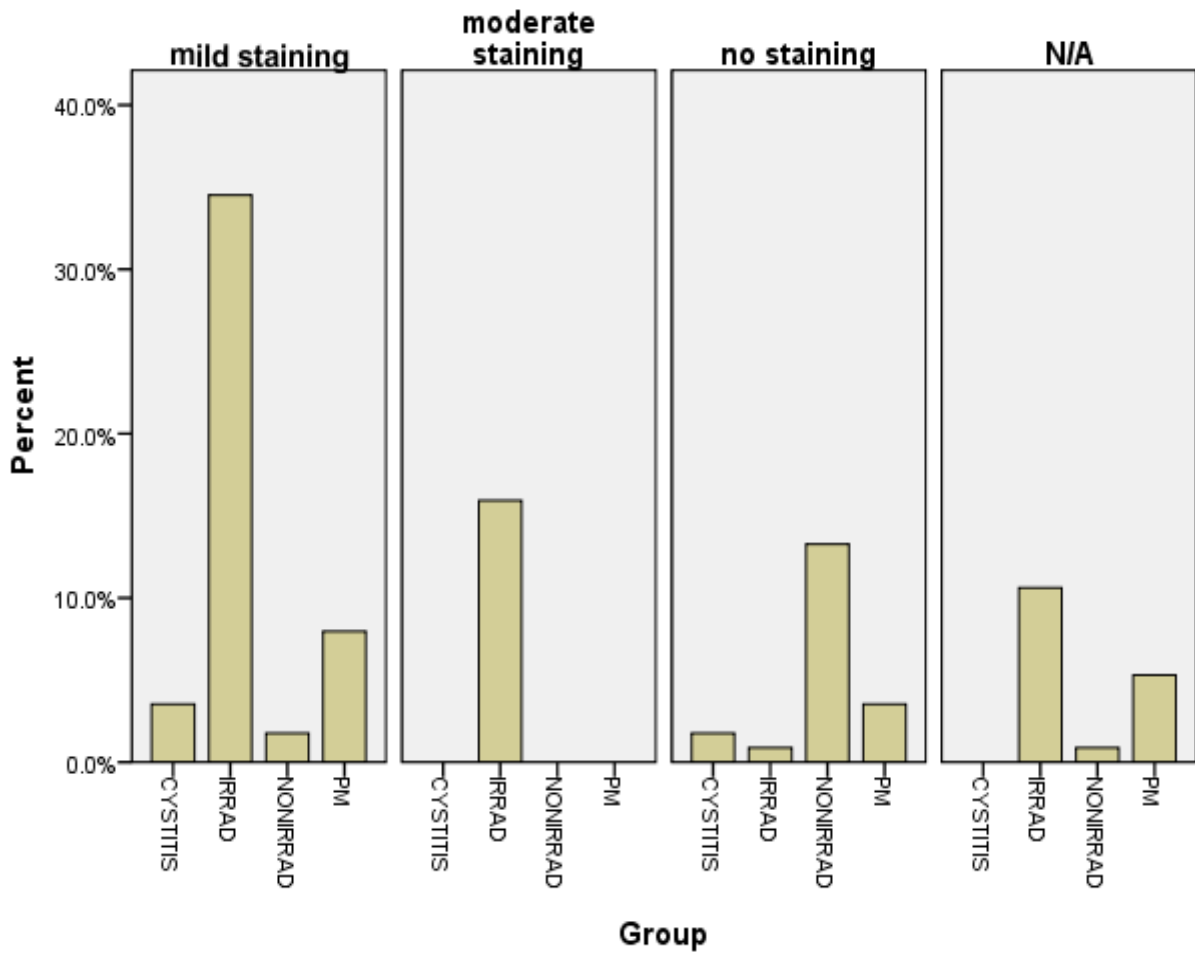
Epithelium crosstabulation

Group	Epithelium					Total
	mild staining	moderate staining	strong staining	no staining	N/A	
CYSTITIS	3	1	0	1	1	6
IRRAD	22	25	6	2	15	70
NONIRRAD	15	0	0	1	2	18
PM	9	4	0	0	6	19
	49	30	6	4	24	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	25.957^a	12	.011
Likelihood Ratio	30.761	12	.002
N of Valid Cases	113		

SuperficialEndothelialCells



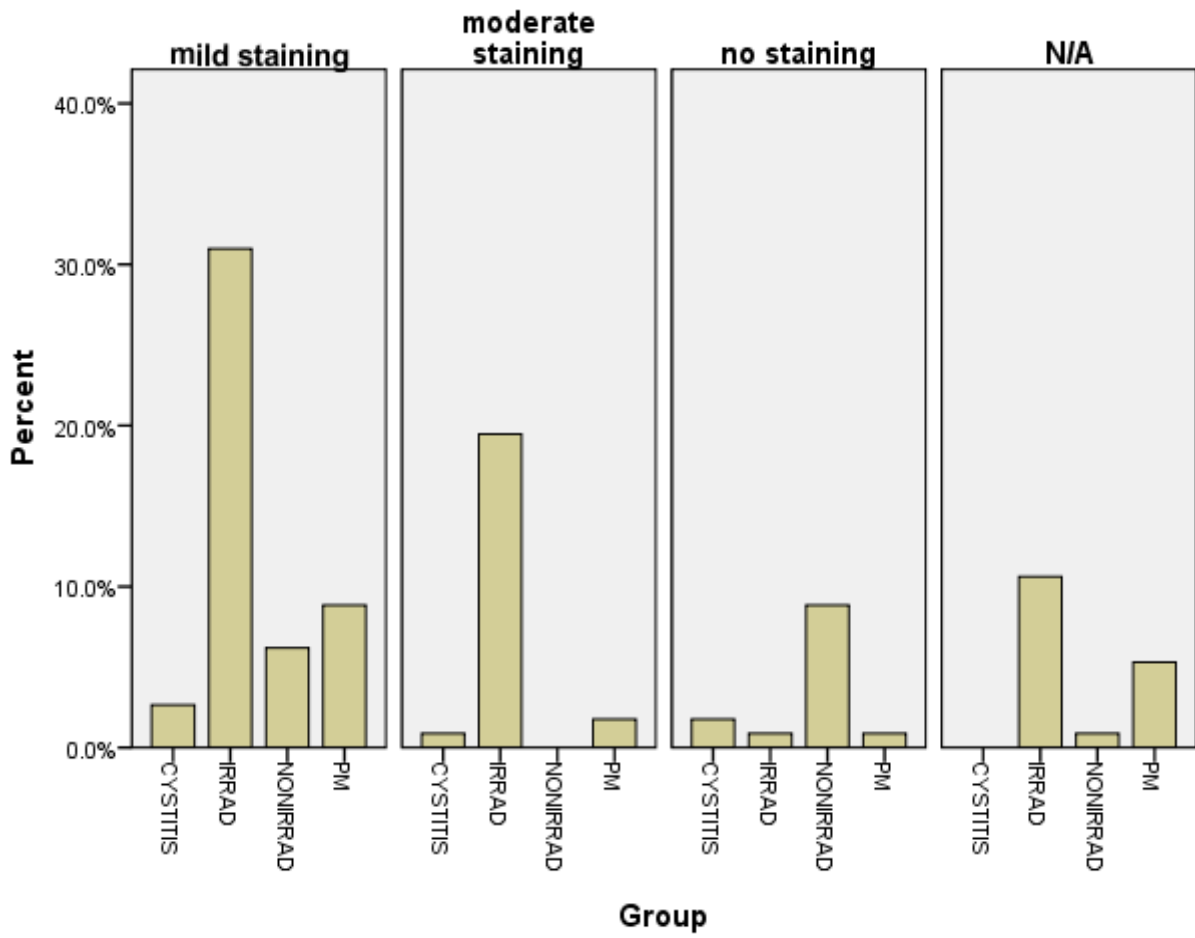
Superficial Endothelial Cells Crosstabulation

Group	Superficial Endothelial Cells				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	4	0	2	0	6
IRRAD	39	18	1	12	70
NONIRRAD	2	0	15	1	18
PM	9	0	4	6	19
	54	18	22	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	72.354^a	9	.000
Likelihood Ratio	72.866	9	.000
N of Valid Cases	113		

DeepEndothelialCells

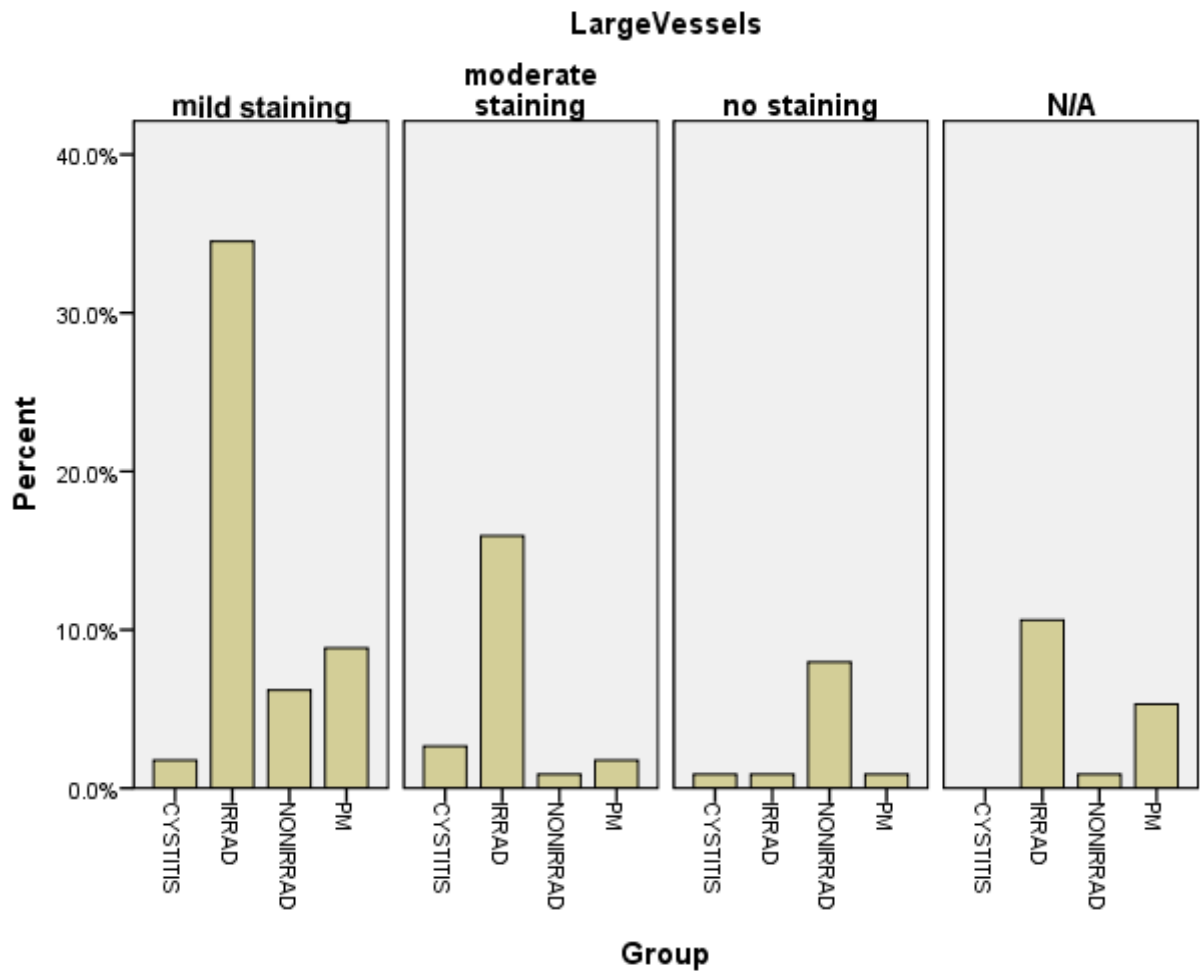


Deep Endothelial Cells Crosstabulation

Group	Deep Endothelial Cells				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	3	1	2	0	6
IRRAD	35	22	1	12	70
NONIRRAD	7	0	10	1	18
PM	10	2	1	6	19
	55	25	14	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	49.996^a	9	.000
Likelihood Ratio	46.127	9	.000
N of Valid Cases	113		

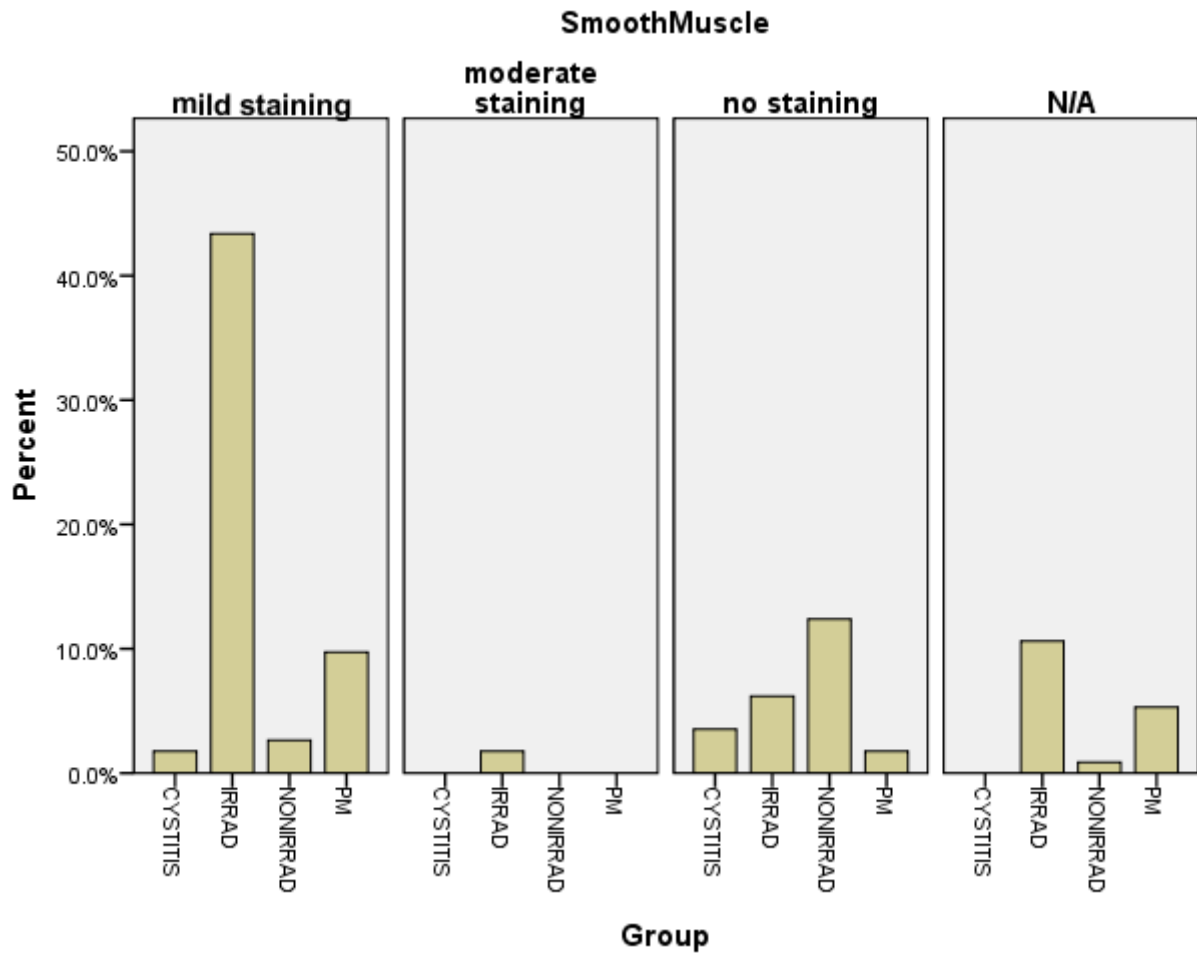


Large Vessels Crosstabulation

Group	Large Vessels				Total
	mild staining	moderate staining	no staining	N/A	
CYSTITIS	2	3	1	0	6
IRRAD	39	18	1	12	70
NONIRRAD	7	1	9	1	18
PM	10	2	1	6	19
	58	24	12	19	113

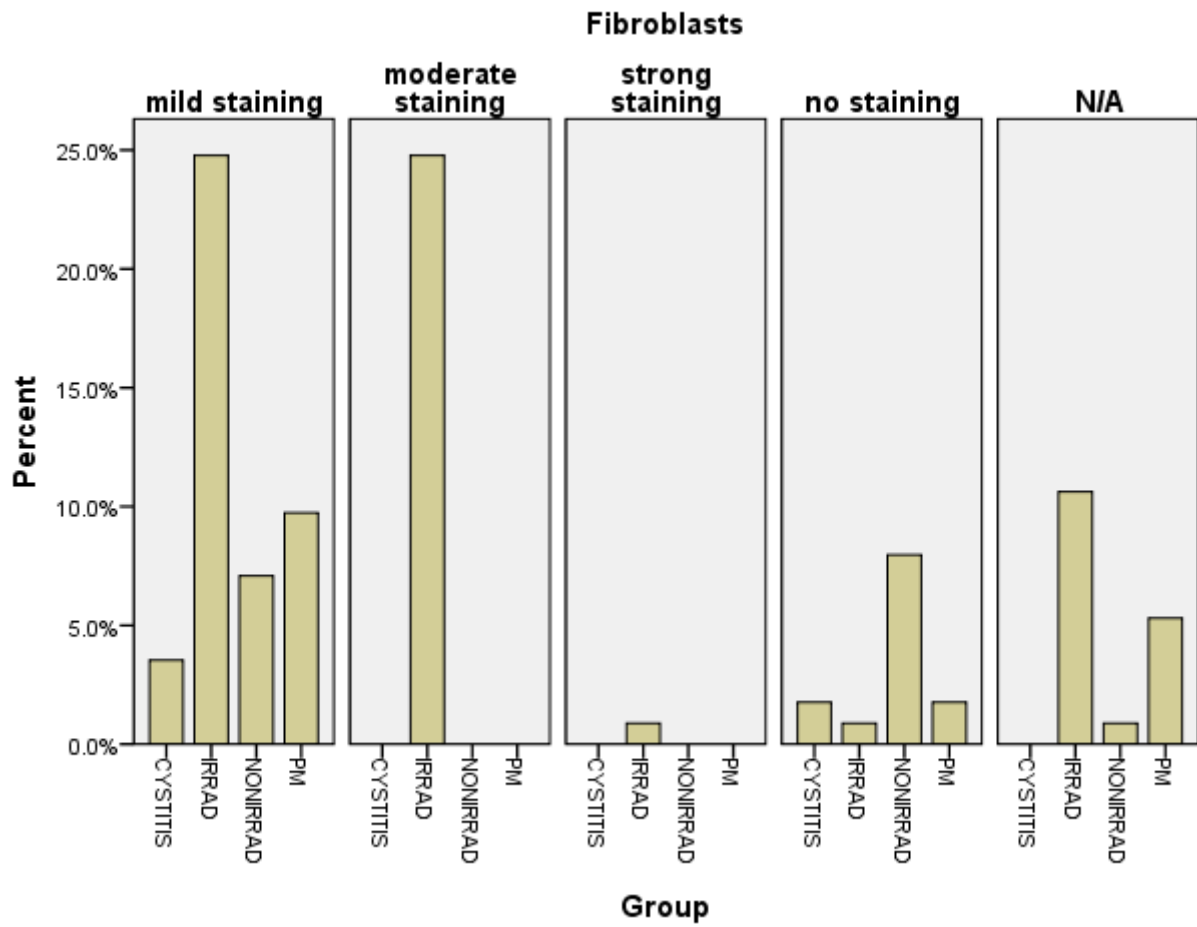
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	44.706^a	9	.000
Likelihood Ratio	37.004	9	.000
N of Valid Cases	113		



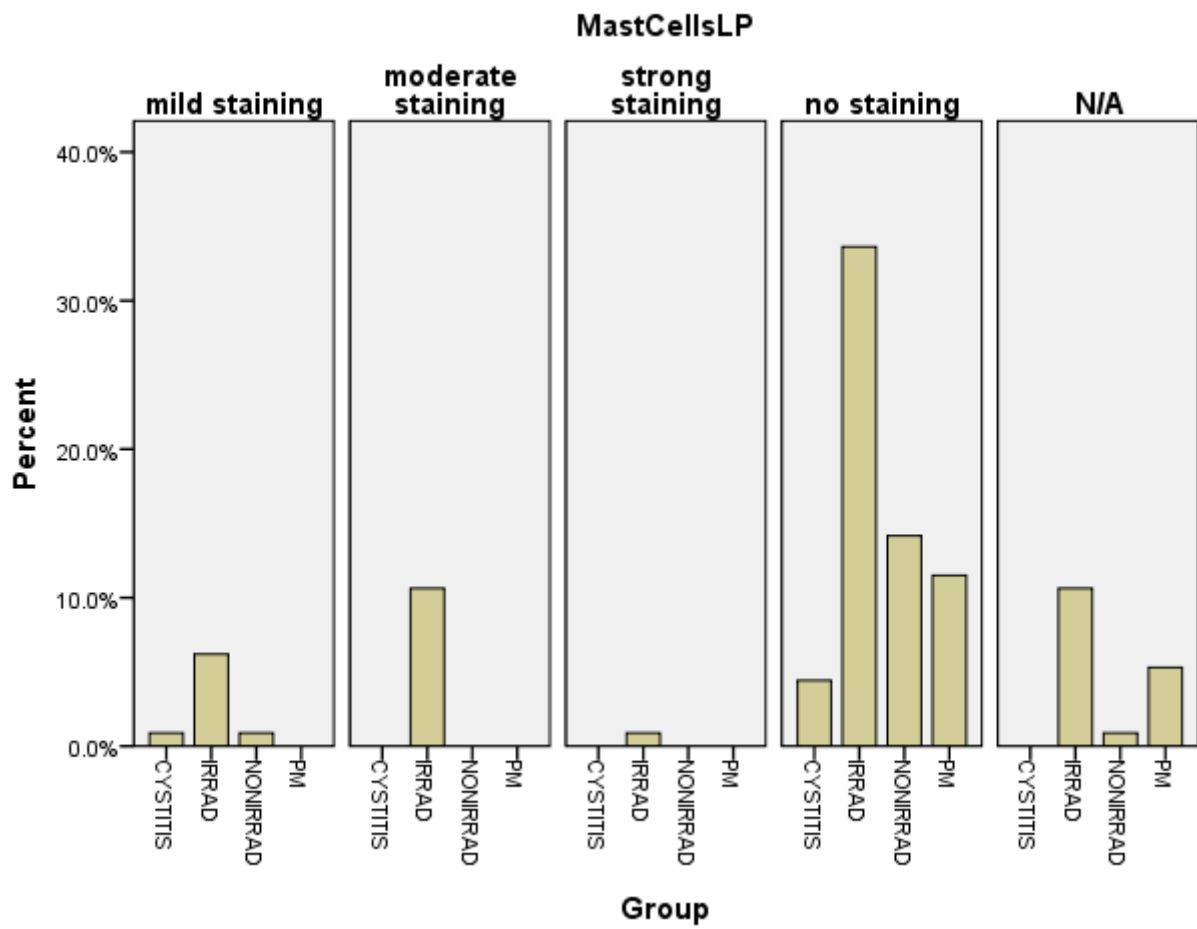
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	55.481 ^a	15	.000
Likelihood Ratio	54.028	15	.000
N of Valid Cases	118		



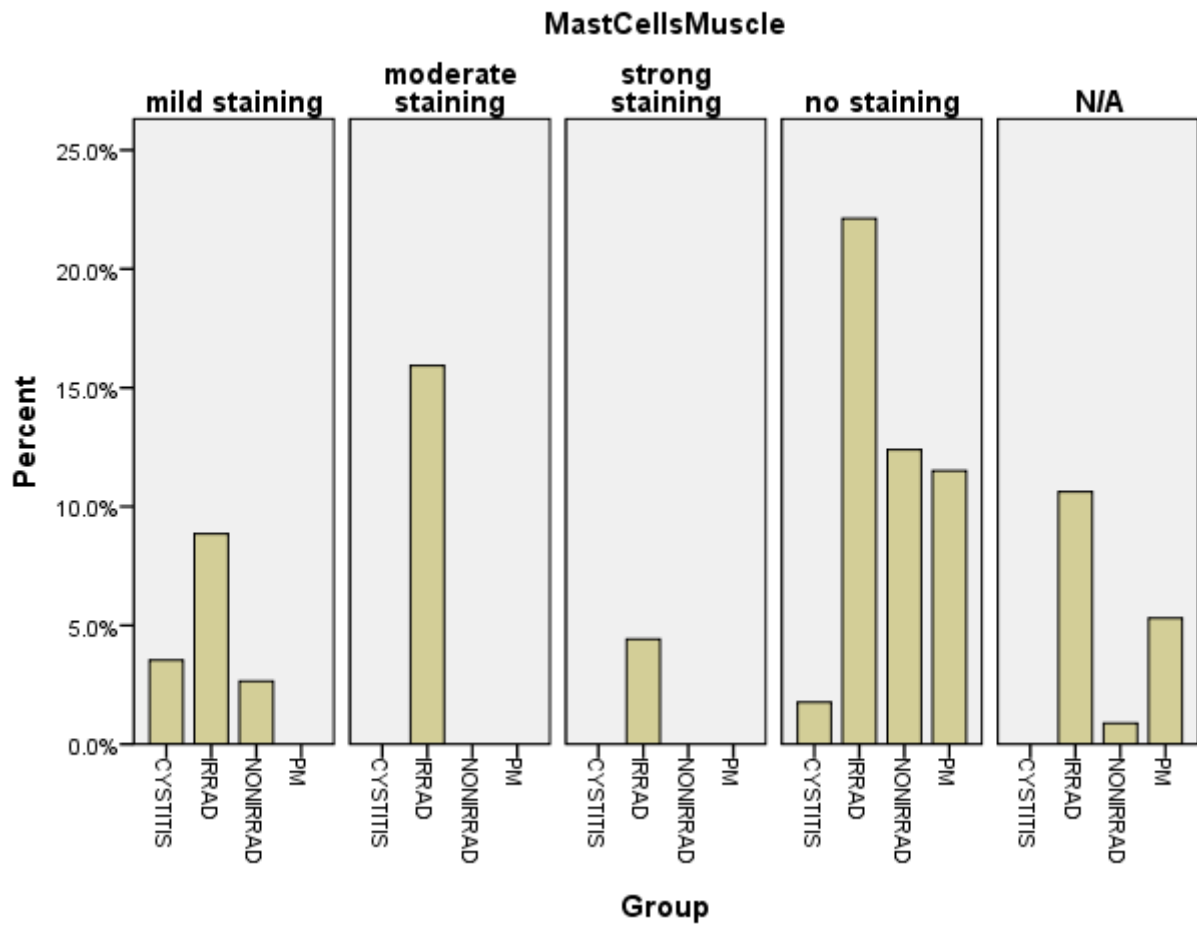
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	68.514 ^a	20	.000
Likelihood Ratio	77.089	20	.000
N of Valid Cases	118		



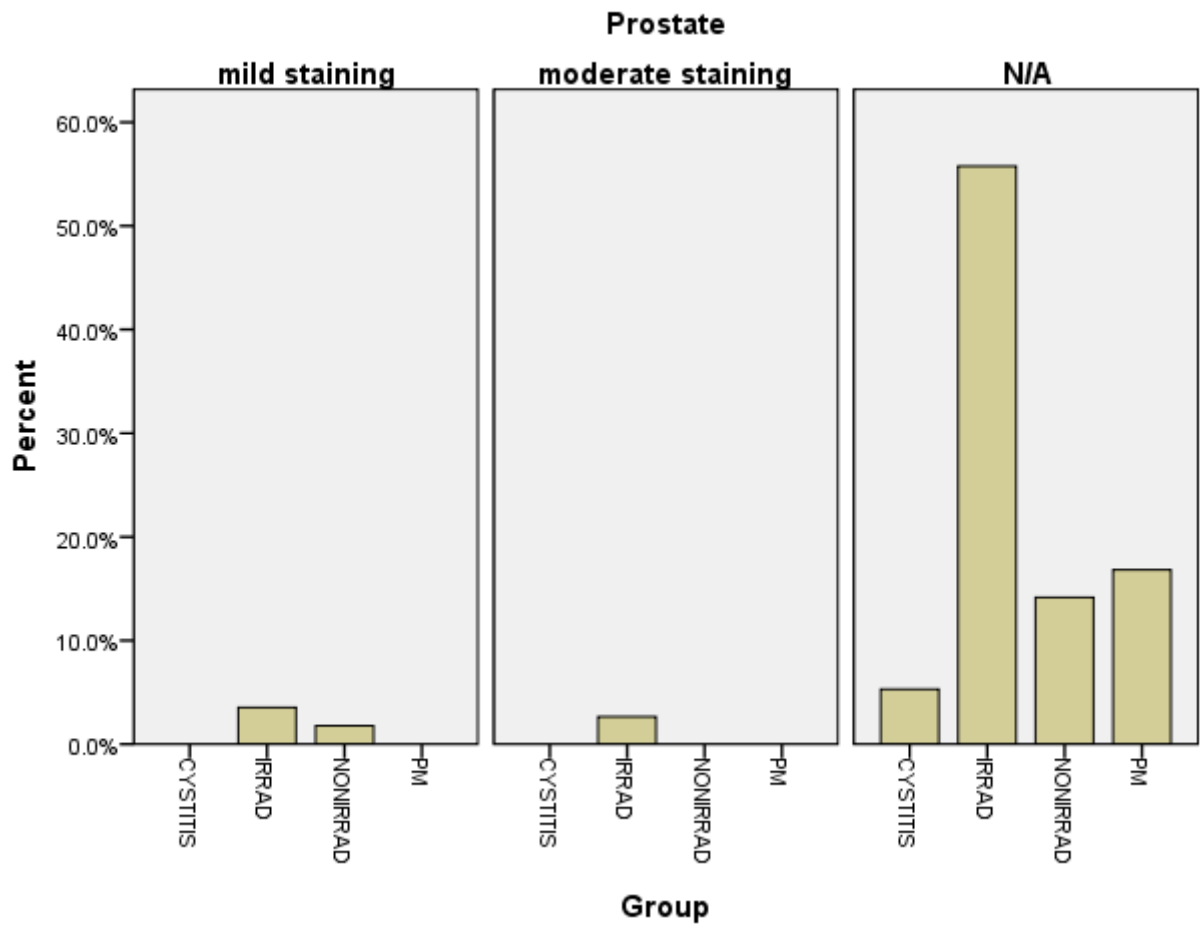
Chi-Square Tests

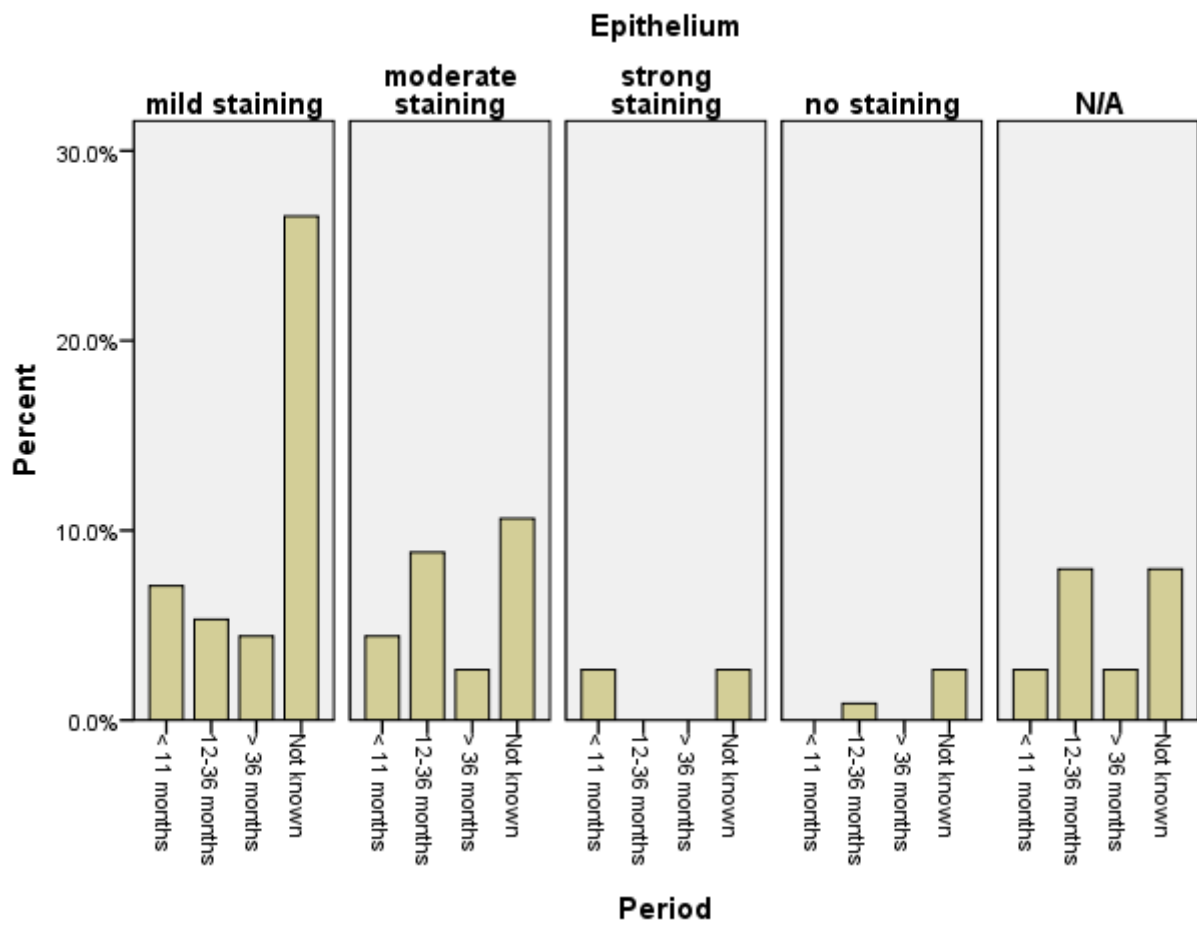
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	30.386 ^a	20	.064
Likelihood Ratio	37.198	20	.011
N of Valid Cases	118		



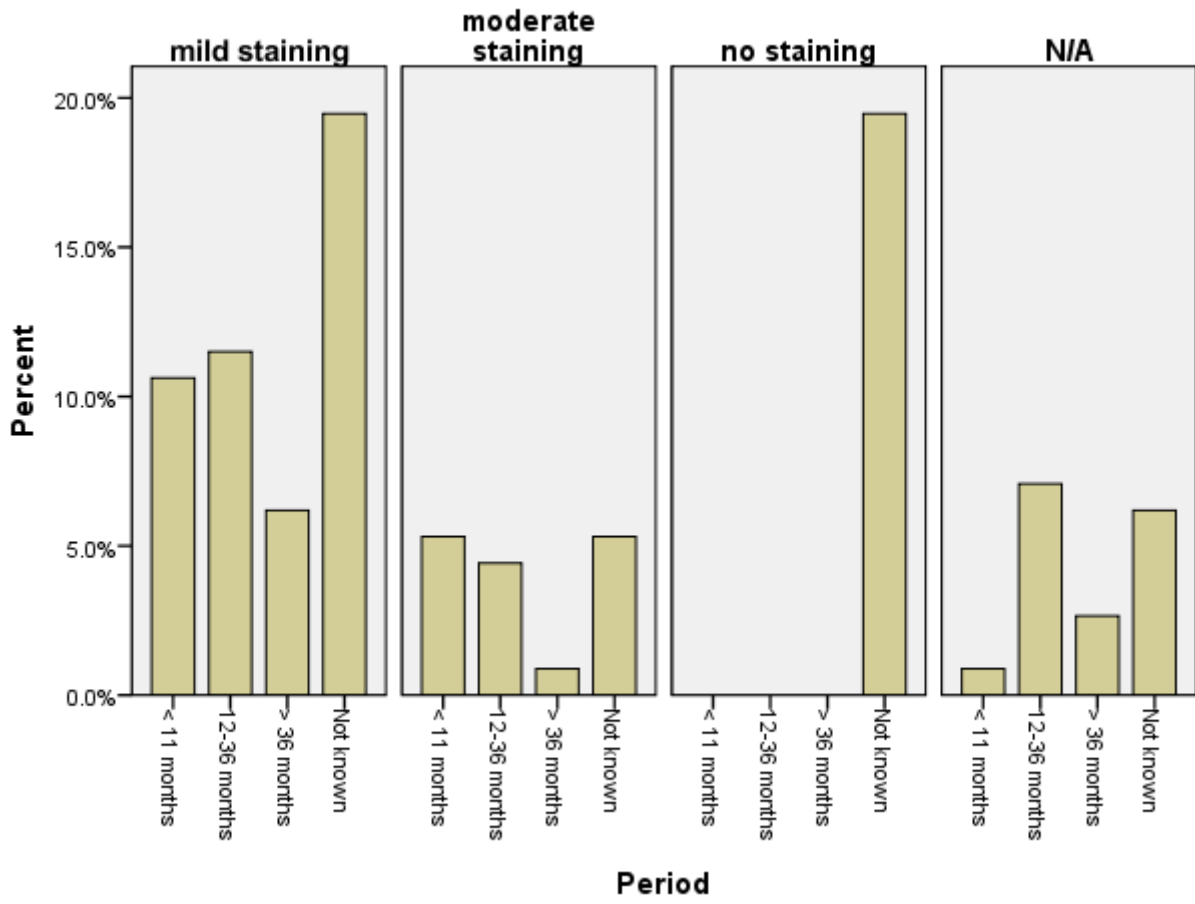
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	47.936 ^a	20	.000
Likelihood Ratio	57.480	20	.000
N of Valid Cases	118		

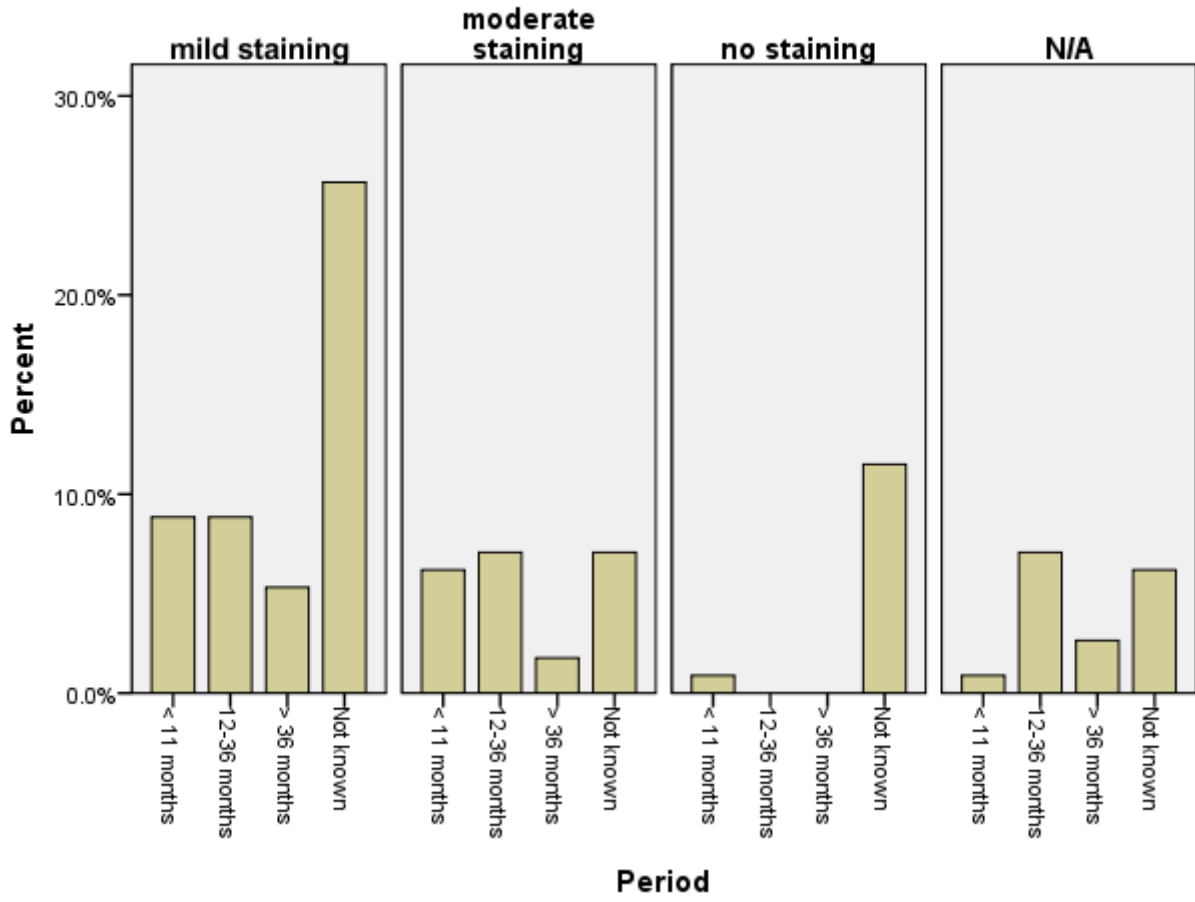


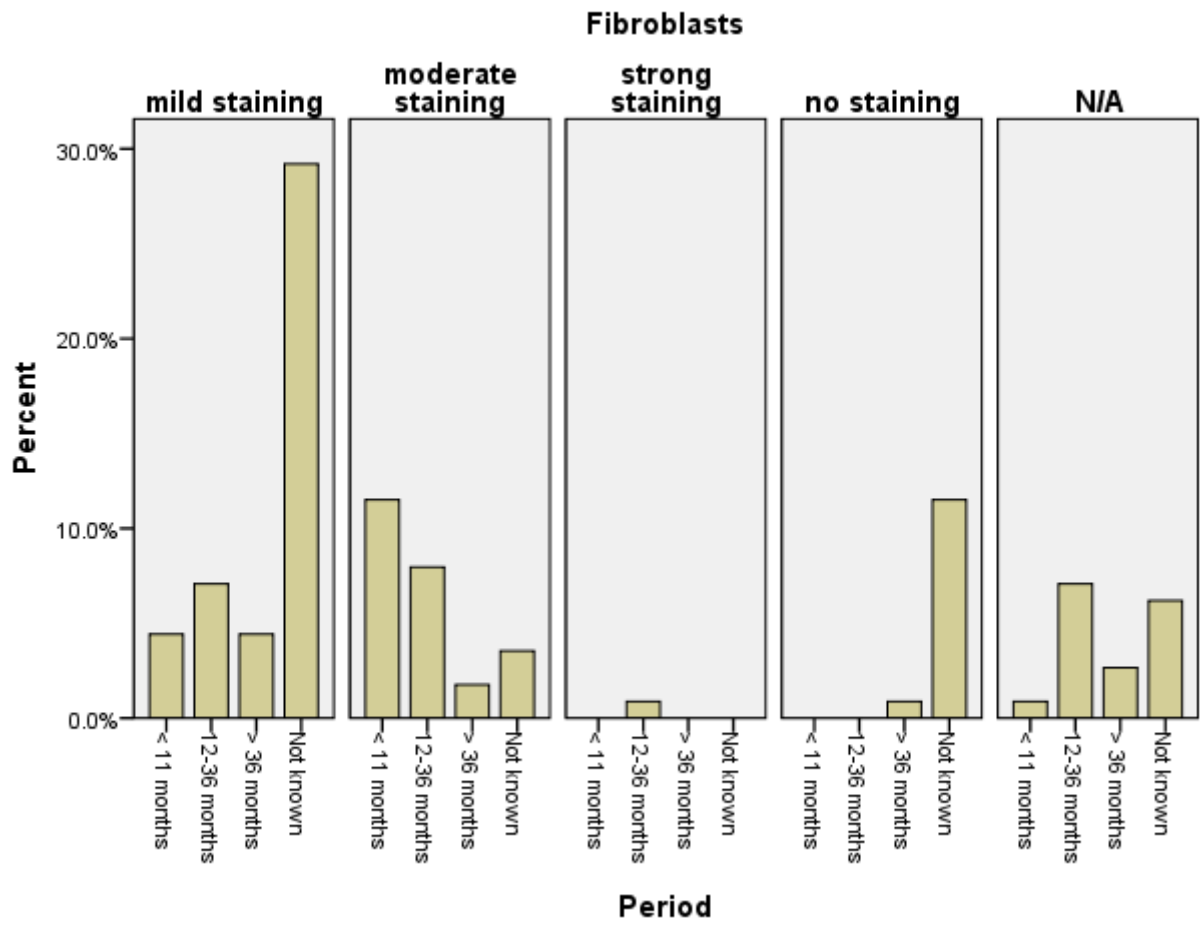


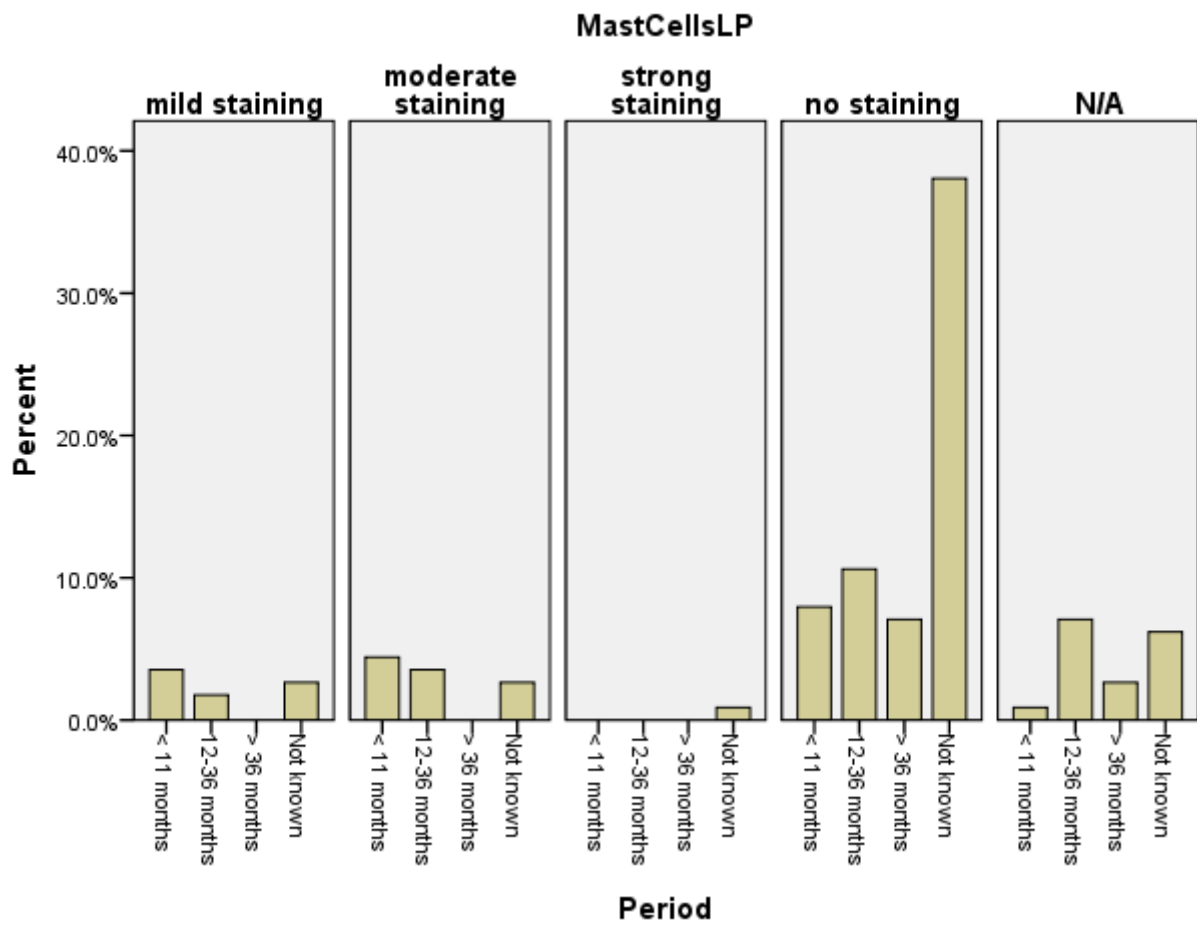
Superficial Endothelial Cells



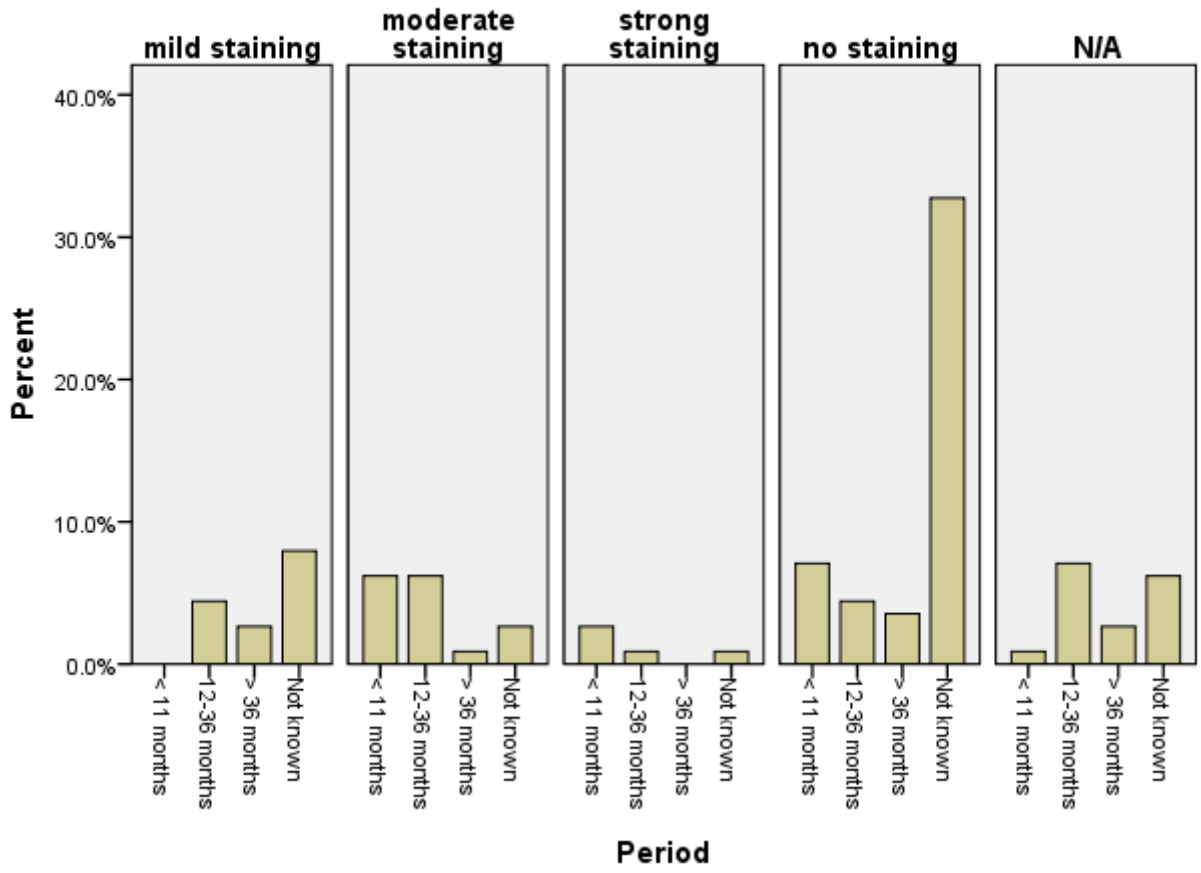
DeepEndothelialCells



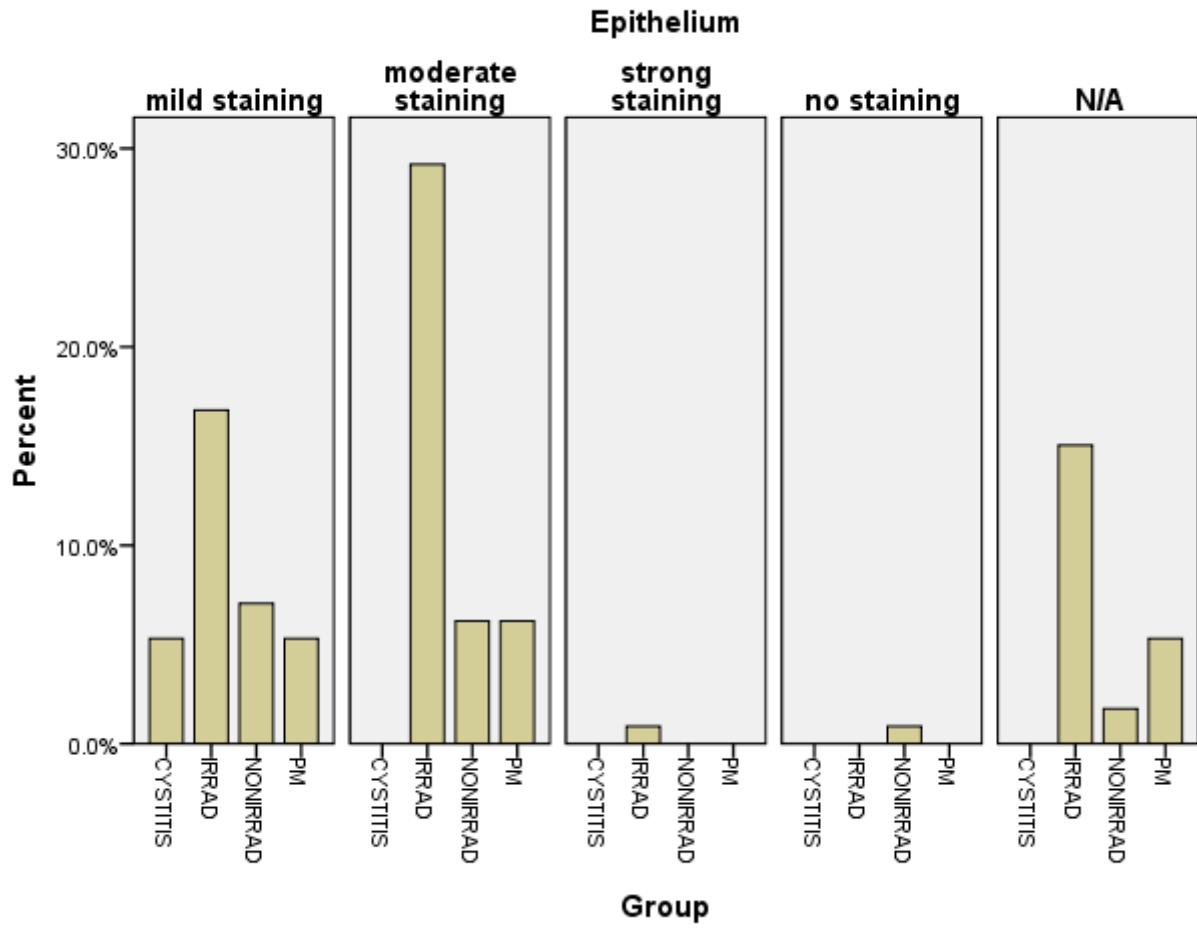




MastCellsMuscle



PDGFR-beta



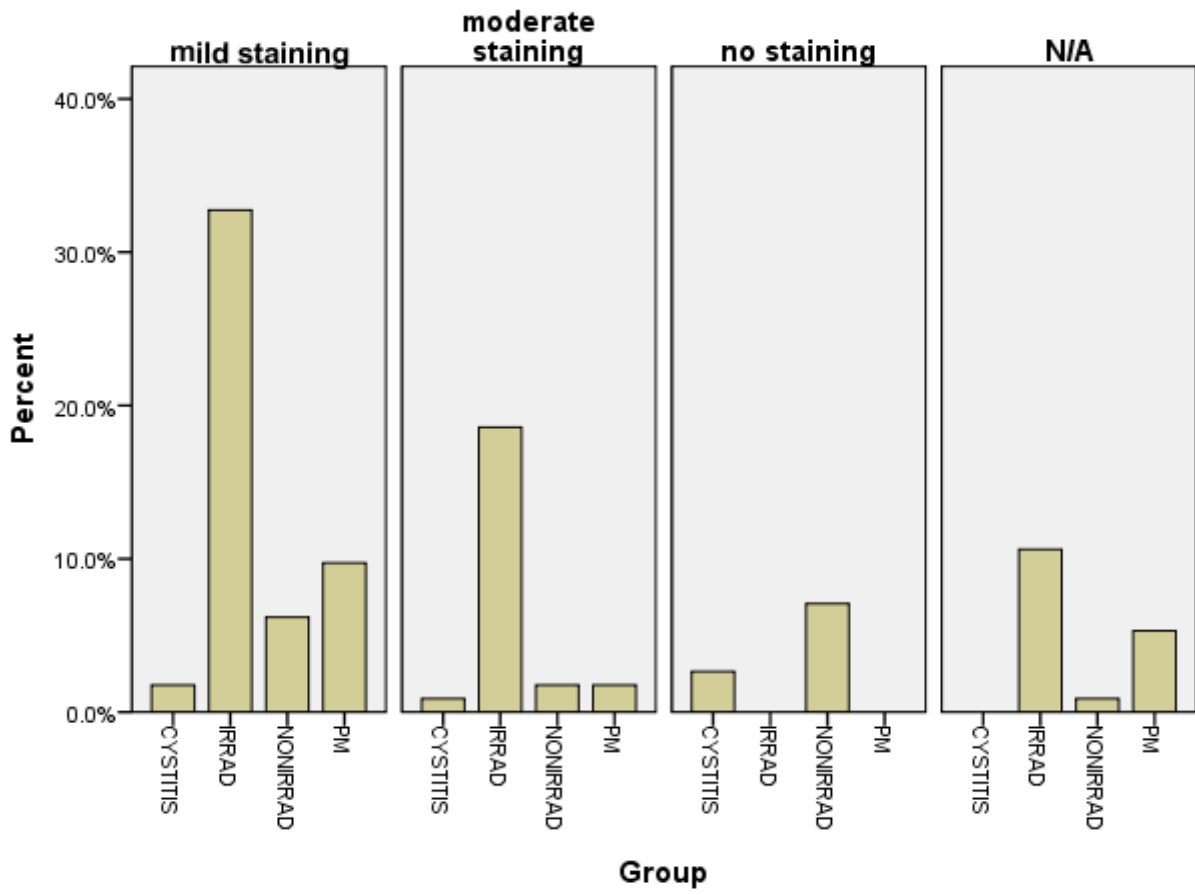
Epithelium Crosstabulation

Group	Epithelium					
	mild staining	moderate staining	strong staining	no staining	N/A	Total
CYSTITIS	6	0	0	0	0	6
IRRAD	19	33	1	0	17	70
NONIRRAD	8	7	0	1	2	18
PM	6	7	0	0	6	19
	39	47	1	1	25	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	21.390^a	12	.045
Likelihood Ratio	21.562	12	.043
N of Valid Cases	113		

SuperficialEndothelialCells



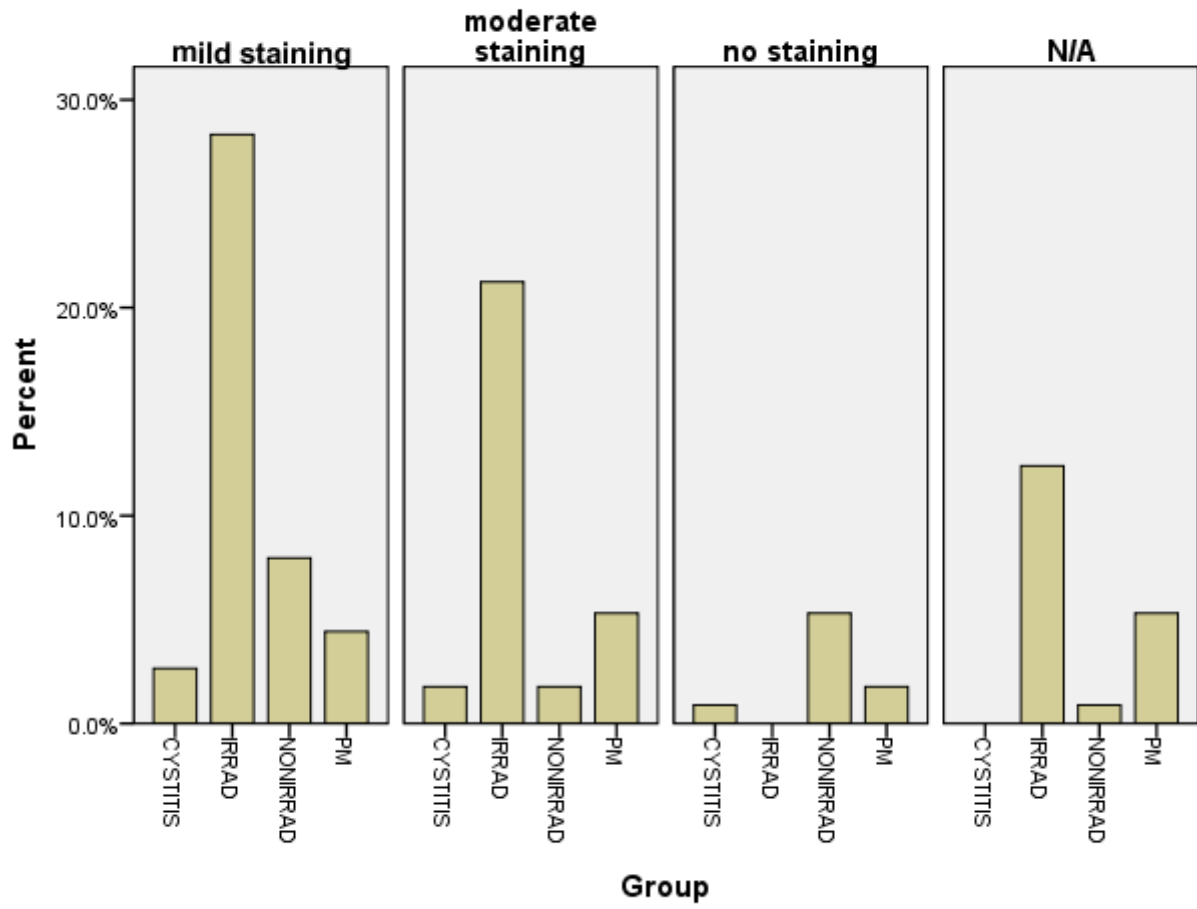
Superficial Endothelial Cells Crosstabulation

Group	Superficial Endothelial Cells				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	2	1	3	0	6
IRRAD	37	21	0	12	70
NONIRRAD	7	2	8	1	18
PM	11	2	0	6	19
	57	26	11	19	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	50.869^a	9	.000
Likelihood Ratio	45.580	9	.000
N of Valid Cases	113		

DeepEndothelialCells



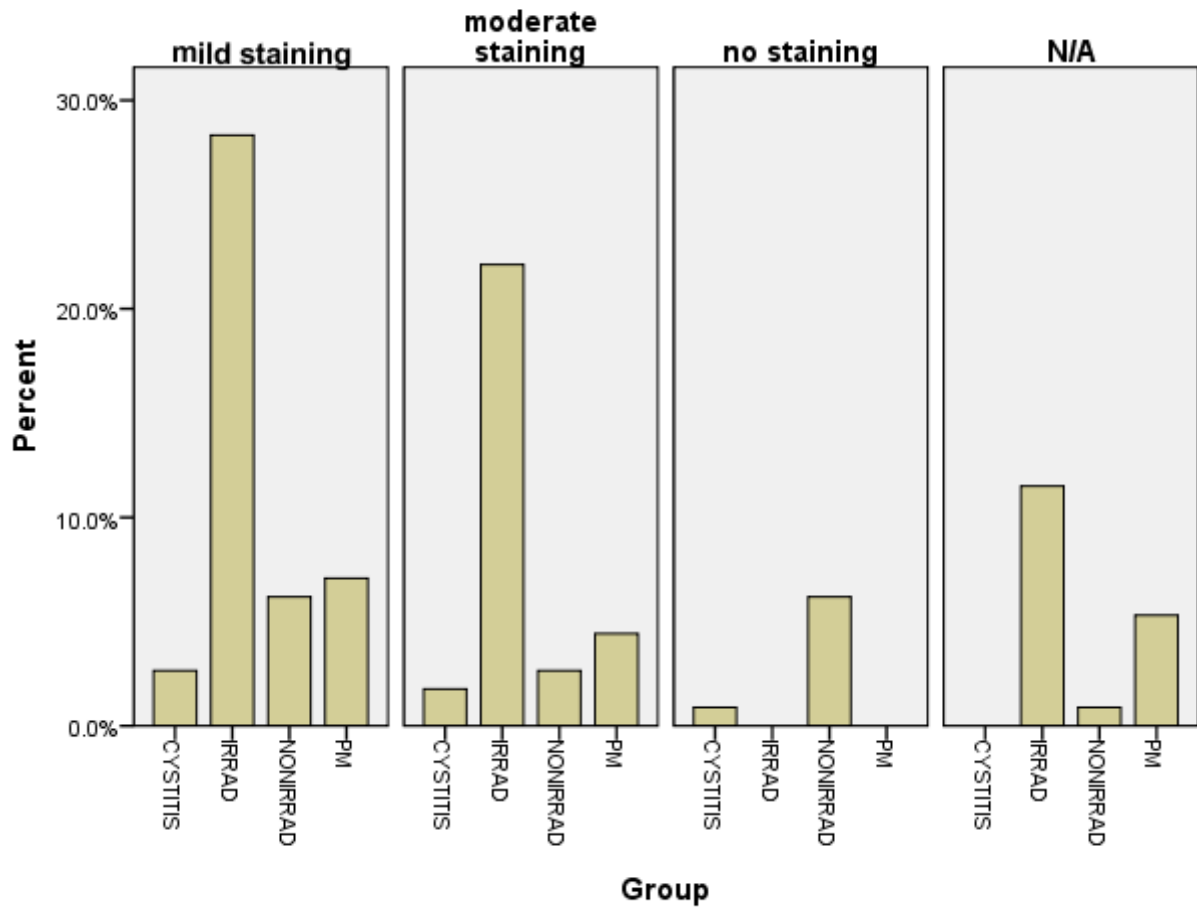
Deep Endothelial Cells Crosstabulation

Group	Deep Endothelial Cells				Total
	mild staining	moderate staining	no staining	N/A	
CYSTITIS	3	2	1	0	6
IRRAD	32	24	0	14	70
NONIRRAD	9	2	6	1	18
PM	5	6	2	6	19
	49	34	9	21	113

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	29.614^a	9	.001
Likelihood Ratio	30.851	9	.000
N of Valid Cases	113		

LargeVessels

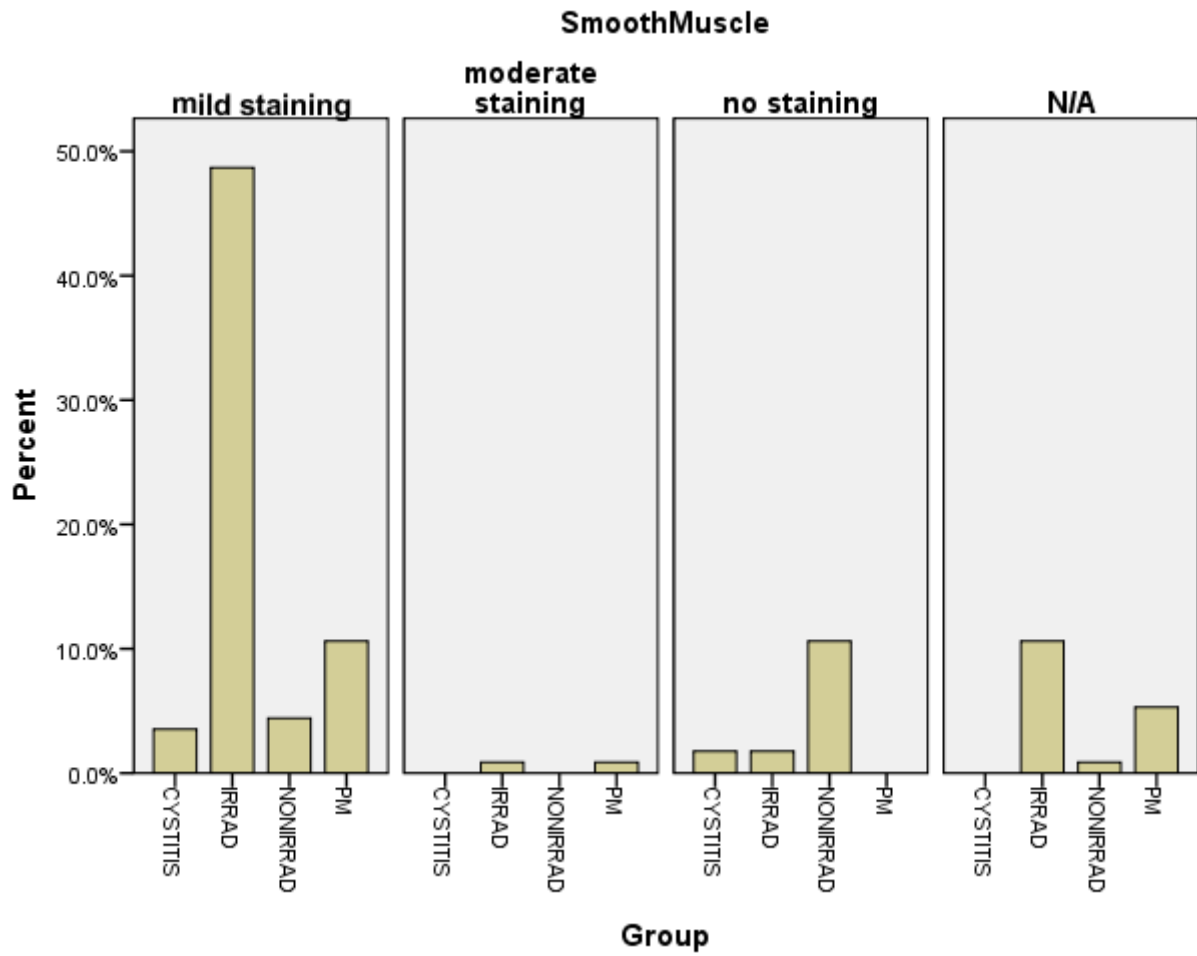


Large Vessels Crosstabulation

Group	Large Vessels				
	mild staining	moderate staining	no staining	N/A	Total
CYSTITIS	3	2	1	0	6
IRRAD	32	25	0	13	70
NONIRRAD	7	3	7	1	18
PM	8	5	0	6	19
	50	35	8	20	113

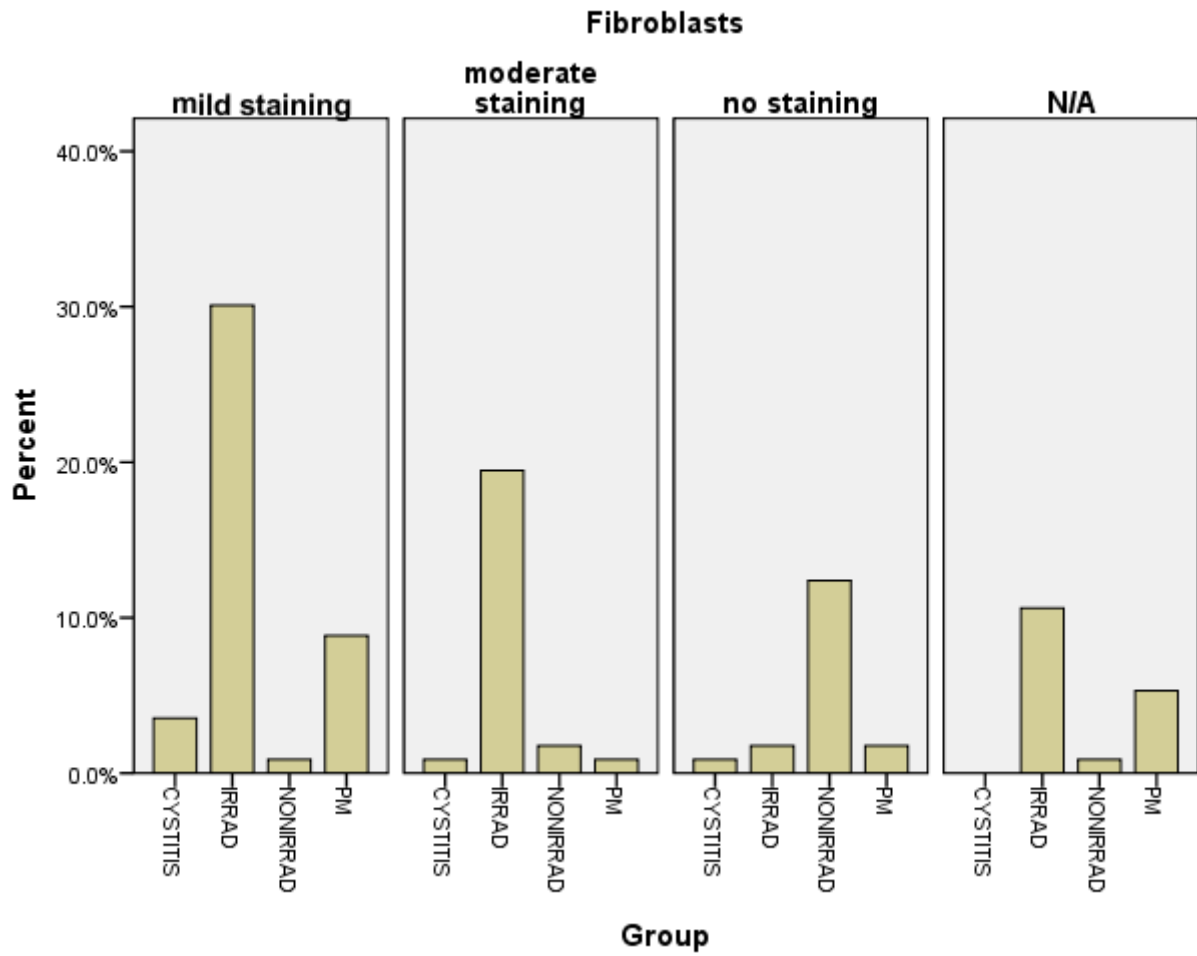
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	39.522^a	9	.000
Likelihood Ratio	33.728	9	.000
N of Valid Cases	113		



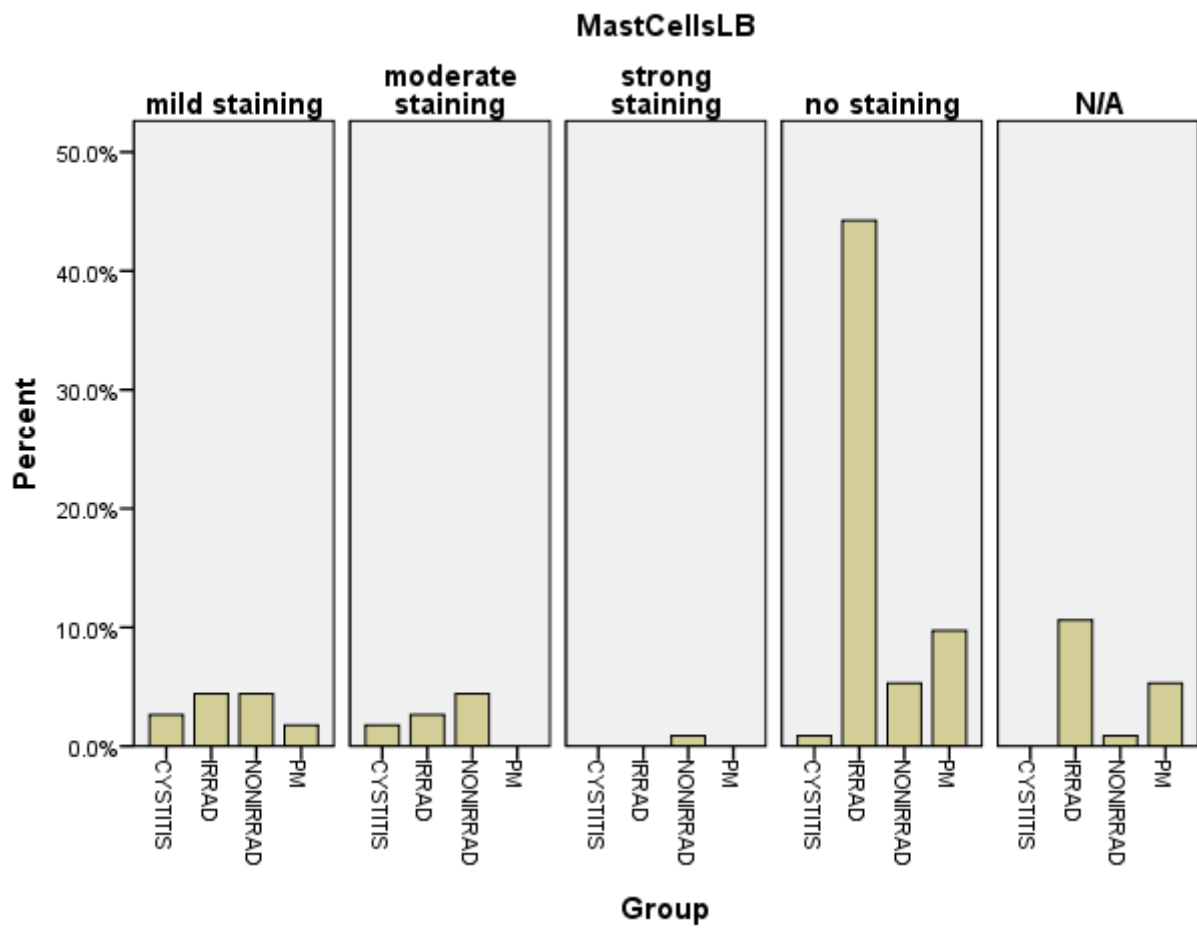
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	74.926 ^a	15	.000
Likelihood Ratio	62.680	15	.000
N of Valid Cases	118		



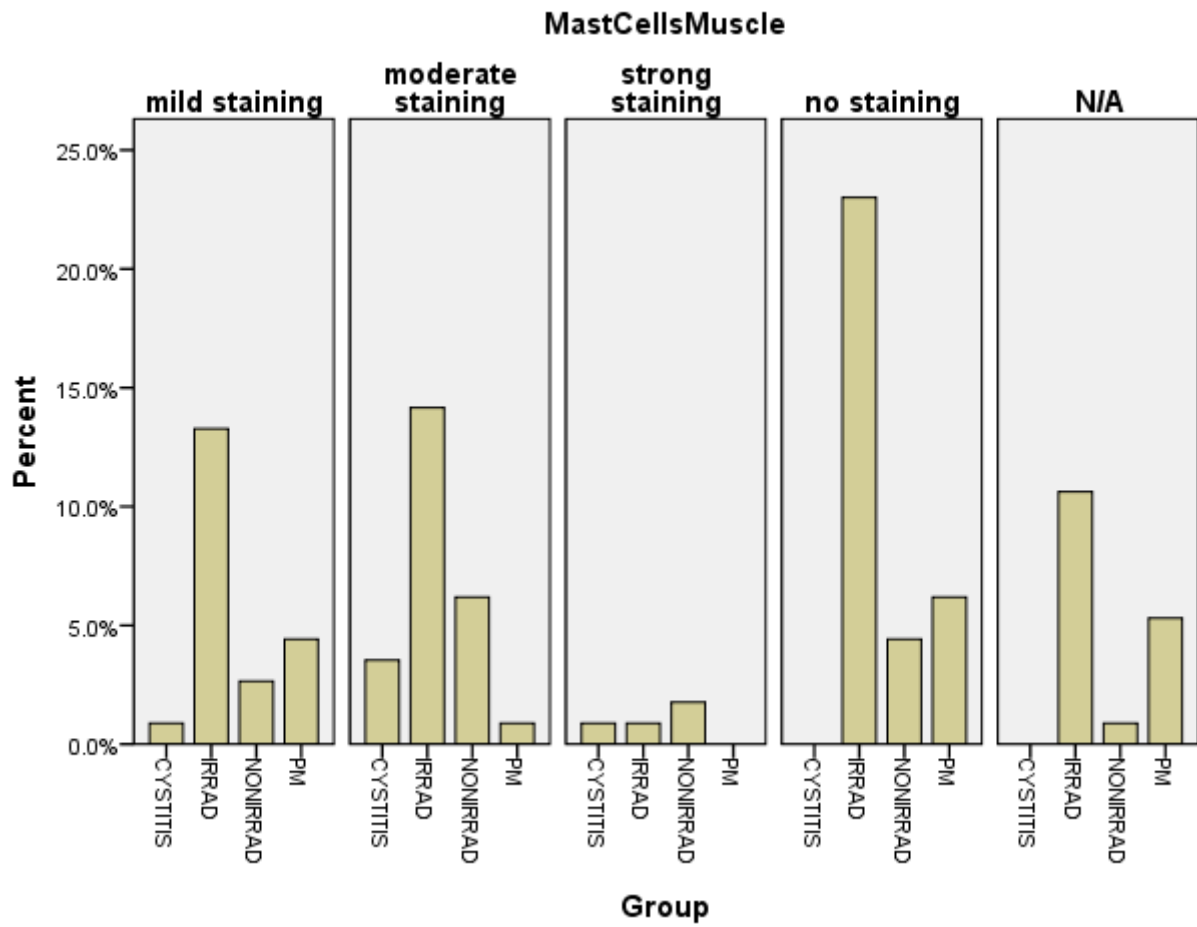
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	74.333 ^a	15	.000
Likelihood Ratio	74.176	15	.000
N of Valid Cases	118		



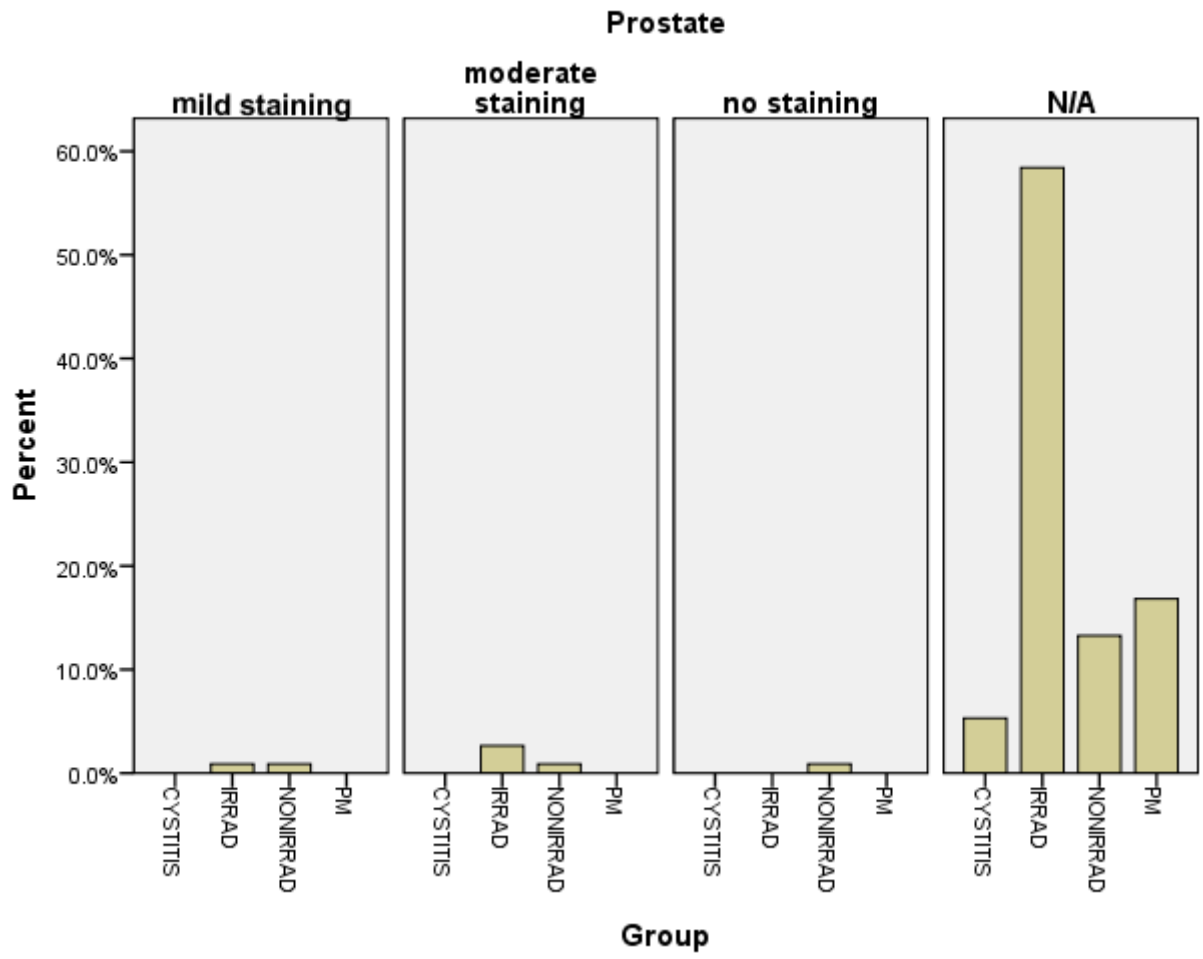
Chi-Square Tests

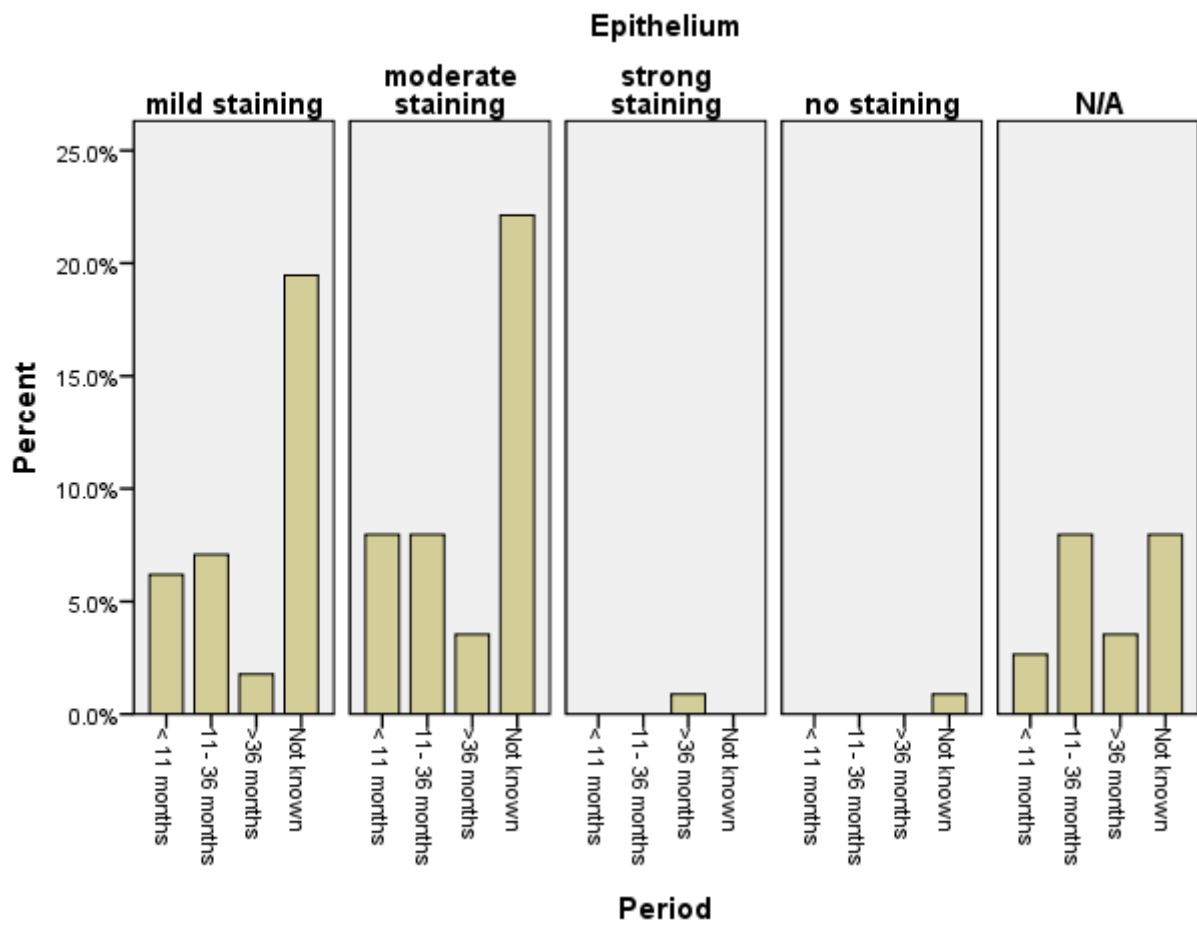
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	57.403 ^a	20	.000
Likelihood Ratio	52.839	20	.000
N of Valid Cases	118		



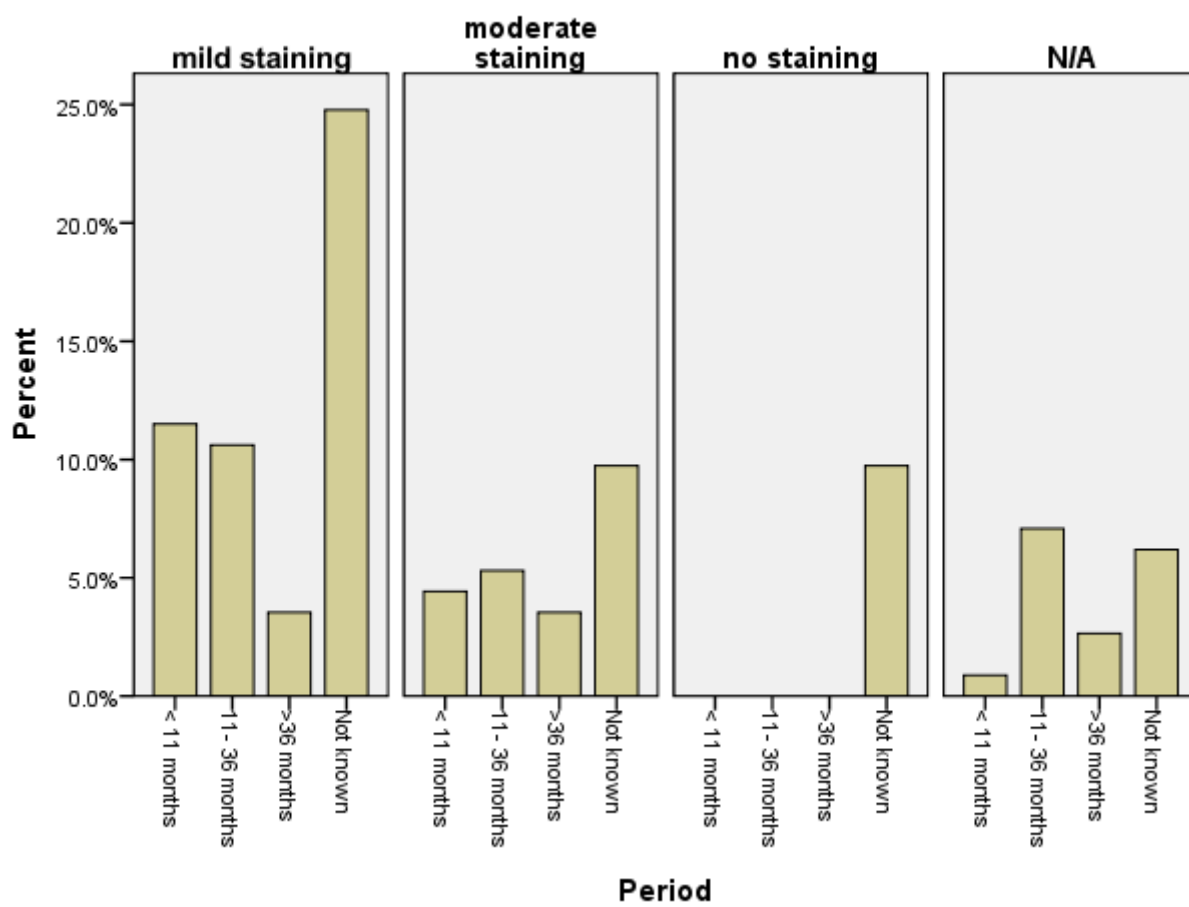
Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	47.909 ^a	20	.000
Likelihood Ratio	48.697	20	.000
N of Valid Cases	118		

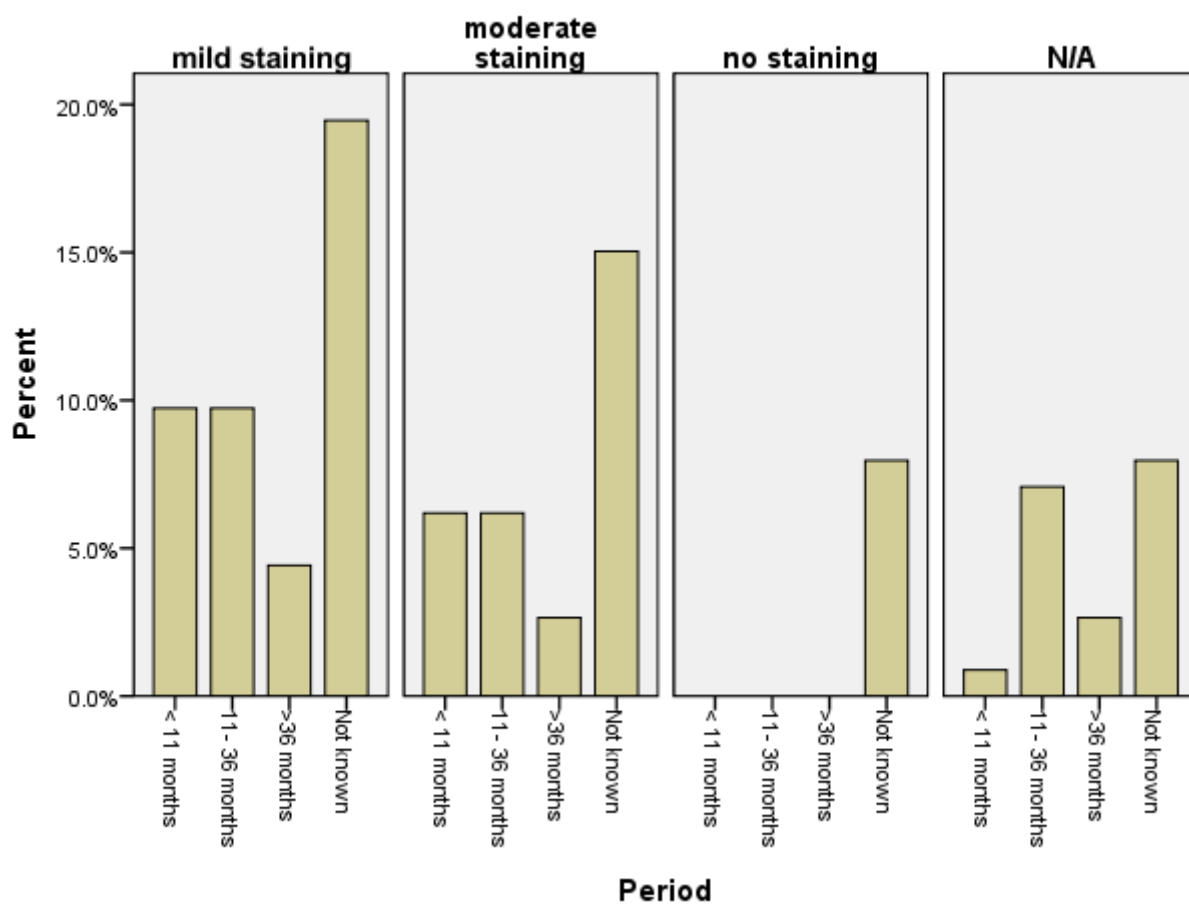




Superficial Endothelial Cells



DeepEndothelialCells



Fibroblasts

