Chapter 2

The role of the tumour suppressor gene PTEN in the etiology of endometrial cancer and hyperplasia

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1. Introduction

1.1 Background

Endometrial cancer is the most frequently diagnosed female genital cancer in the western world and it is second only to cervical cancer in the developing world. The incidence worldwide is steeply increasing and so is the number of deaths from endometrial cancer especially in the developing world, as it is frequently not diagnosed early in less developed parts of the world. Various epidemiological studies have also suggested a poorer prognosis for non-European racial groups.

The stage distribution of endometrial cancer is generally very favourable, as it tends to be a symptomatic cancer early in the disease process. The prognosis for the disease is therefore also relatively good although the prognosis per stage is slightly worse than that for cervical cancer. Large differences exist for the prognosis between histological types, differentiation grades and population groups even when corrected for stage.

The relative advantages of different (new) treatment strategies are still hotly debated. Examples of treatments that may offer advantage to some subsets of patients include lymph node dissection, adjuvant radiation, adjuvant chemotherapy and upper abdominal staging.

The classical epidemiological risk factors are listed below in table 2.1. Numerous studies suggest that both endogenous and exogenous estrogen and Tamoxifen play important roles, as does the balance of stimulating versus stabilizing sex hormone (progestogen). Uterine carcinomas are histologically diverse and it should be expected that the pathogenesis of tumour types be different.

Table 2.1: Classical risk factors for endometrial carcinoma

Endogenous hyperestrogenism
Obesity
Polycystic ovary syndrome
Chronic anovulation
Functional ovarian tumours

Exogenous hyperestrogenism
Unopposed HRT
Tamoxifen
Hereditary
Lynch II, hereditary non-polyposis colon cancer
Family history colorectal / endometrial cancer
Other

Endometroid adenocarcinoma is the most common type, with clear cell and serous papillary types much less common and also more aggressive. Endometrial hyperplasia is the ideal benign counterpart for endometroid adenocarcinoma with atypical hyperplasia a proven precursor lesion. These benign and pre-malignant lesions share the etiological factors of endometrial cancer.

Carcinogenesis is a multistep process where genetic lesions are accumulated. These lesions occur mainly in oncogenes and tumour suppressor genes, resulting in the initiation of uninhibited growth and eventually in tumour progression and metastatic potential. Renan (1993) estimated that six genetic mutations are needed for tumorigenesis in the endometrium, based on the age-specific incidence of these cancers.

After some years of study, endometrial carcinoma still displays the highest percentage of PTEN mutations of all tumour types. Additionally, PTEN is the most frequently mutated tumour suppressor gene in endometrial carcinoma (Mutter et al, see also 2.3.4.1.2). It is not known whether this is also true for the South African population as previous reports pointed towards differences between population groups. Some differences between the different local population groups are also expected.

It is not very clear when in the carcinogenetic process these mutations occur and what influence the mutations have on tumour biology and growth patterns. Hormone receptor positivity is another molecular cellular change that correlates strongly with tumour biology and behaviour. There also exists a strong inverse correlation with differentiation grade. It is unknown how PTEN mutation rate correlates with differentiation grade.

In a study comparing 15 Afro-American with 115 European-American endometrium carcinoma patients, Hicks found poorer survival in the first group, which could best be explained by the increased number of poor prognostic factors in the group of Afro-American women (Hicks et al 1997).

Matthews and co-workers conducted a retrospective study on 401 patients to examine the influence of race and histology on outcome. They also found tumour type and stage to be the important predictors of outcome. Again a strong association was found between aggressive histological types and Black race (Matthews et al 1997).

In an important population based study, Plaxe and Saltzstein (1997) reported that Black women develop significantly less low risk tumours and in fact have the same incidence of high risk endometrial cancer. This leads to the bias towards high-risk tumours and poor outcome in Black women. The incidence of these tumours and the outcome in different South African race groups has not been studied.

Bokhman described two different clinical pictures of endometrial cancer and related it to histological features. This classification is discussed below under histology. These two types also display important molecular differences and although these features are not absolute the differences are usable in the clinic and correlates with outcome. These features are summarized in table 2.2.

Table 2.2: Typical clinical and molecular features of type I and type II endometrial cancer

Feature	Type I	Type II
Clinical features		
Etiology	estrogen related	age related
Race distribution	more in European	equal
Histological type	endometroid	other
Differentiation grade	grade 1	grade 3
stage	early	late
prognosis	good	poor

Molecular features		
MSI	positive	negative
PTEN	positive	negative
Her 2/neu	negative	positive
K-ras	positive	positive
P53	negative	positive

1.2 Research quest

In this chapter existing evidence of involvement of the PTEN gene in endometrial carcinogenesis will be explored and interpreted using the knowledge of endometrial carcinogenesis in histology, genetics and molecular medicine. Additionally, involvement of the tumour suppressor gene in subsets of patients with endometrial cancer of different grades and endometrial hyperplasia specimens will be tested.

The hypothesis is that the PTEN gene is intimately involved in endometrial carcinogenesis and may be involved in endometrial hyperplasia.

The research questions as listed in chapter 1 are:

1. What role does PTEN gene mutation and pten protein inactivation play in the etiology of endometrial carcinoma?
2. What is the frequency of PTEN mutations in endometrial cancers and pre-cancers?
3. When in the carcinogenetic process do these mutations occur?
4. How does PTEN mutations correlate with disease stage and grade?
5. How does the involvement of the PTEN gene differ between the different population groups in South Africa?
6. How does the involvement of the gene differ between South African patients and other reported populations?

2. Literature overview

2.1 Genetic changes in endometrial proliferative disorders

2.1.1 DNA repair genes and micro-satellite instability

Micro-satellite instability (MSI) was first demonstrated in colon cancer and endometrial cancer as another genetic alteration that occur in many familial and in some sporadic tumours (Ionov et al 1993; Risinger et al 1993). This anomaly manifests through the alteration of DNA repeats, called micro-satellites. Due to the repeating nature, these repeated sequences are more prone to replication errors, named RER+, which is then recognised by the instability of the microsatellites.

In hereditary non-polyposis colorectal carcinoma, HNPCC, these genetic abnormalities are inherited in a dominant fashion, leading to a germline mutation causing the syndrome (Aaltonen et al 1993). It is now known that inherited mutations in the DNA mismatch repair genes, namely hMLH1, hMSH2, hMSH6, hPMS2, cause HNPCC. Endometrial cancer is the commonest non-colonic cancer in females born with these mutations, causing a lifetime risk of between 22% and 43% (Watson & Lynch 1993; Aarnio et al 1995). Lynch syndrome or HNPCC is the only known familial cancer syndrome that often causes endometrial cancer.

Although MSI is frequent (15% to 34%) also in sporadic endometrial cancer, somatic mutations in the DNA repair genes are not frequent in sporadic endometrial cancers. Instead, it has been shown that the finding of MSI correlates strongly with methylation of the hMLH1 promoter region (Simpkins et al 1999), which is an epigenetic finding, causing inactivation of the hMLH1 gene (Salvesen et al 2000). The result on cellular level is the same as a mutation in the gene causing defects in the DNA repair system (Peiffer et al 1995). Impaired DNA repair may be an important reason for mutations in the tumour suppressor genes to go unchecked and be a pre-cursor to further pre-malignant genetic change.

In endometrial cancer MSI has been found almost exclusively in the endometroid adenocarcinoma histological subtype. No association was found with either differentiation grade or tumour stage by Smid-Koopman in 2002, but others

have shown association with poor differentiation grade and other markers of poor prognosis (An et al 2007).

Some authors have investigated the correlation between PTEN mutations and MSI, but results are not easy to interpret and study size tend to be small (Kanaya et al 2005).

2.1.2 Proto-oncogenes and oncogenes

2.1.2.1 K-ras

Activation of the Ras proto-oncogene family has been detected in a number of malignancies at frequencies depending on the type of tumour. Activation occurs mostly by point mutation and was found most frequently in pancreas carcinoma (~90%) but relatively infrequently in gynaecological cancers (Kofa & Spandidos 1997). In endometrial cancer mutations occur mostly in the K-ras (14%-30%) and sometimes in the H-ras (7%) gene.

Two groups correlated activation of these genes with a poor outcome (Mizuuchi et al 1992; Fujimoto et al 1993), while another group found the opposite (Sasaki et al 1993). K-ras mutations is now frequently quoted to be associated with type I cancers although the evidence is somewhat unconvincing (Cerezo et al 2006). Ras activation was also demonstrated in precursor lesions, suggesting involvement at the early stages of carcinogenesis (Mutter et al 1999).

Turbiner and co-authors found more K-ras mutations in Tamoxifen exposed endometrial cancers and fewer PTEN mutations (2008).

2.1.2.2 HER *2/neu* or c-erbB-2

Since 1991 several groups have studied the involvement of this oncogene in endometrial cancer, by studying over-expression, mutation and amplification. Many attempts have been made to find an association with histological grade, stage and prognosis with varying results. An important study by Macwhinnie and Monaghan (2004) could not show a difference between serous papillary and endometoid histological subtypes.

The majority of published results suggest higher grade, and some also higher stage at diagnosis or worst prognosis in patients with over-expression (Saffari et al

1999; Rolitsky et al 1999; Riben et al 1997). Konopka and co-workers (2004) could not demonstrate any erbB-2 amplification in 43 endometroid adenocarcinomas.

2.1.2.3 Bcl-2

The proto-oncogene Bcl-2 is an inhibitor of programmed cell death. It counteracts the action of p53, which induces apoptosis. Morsi and colleagues studied bcl-2 protein expression in normal endometrium (2000). This group observed cyclical changes of bcl-2 expression in normal endometrium and decreased expression levels in hyperplasia and carcinoma. Several groups have since showed that expression levels correlate negatively with differentiation grade (Geisler et al 1998; Zheng et al 1996).

The correlation of the bcl-2 activity with carcinogenesis is still incompletely expression seems to be important and not the quantity. The function and inhibition of this proto-oncogene remains difficult to study and the various measures of activity difficult to interpret. Levels of expression may correlate with some other measures of aggressive tumour growth but is not an independent prognostic indicator (Peiro et al 2003).

2.1.2.4 C-fms

The proto-oncogene c-fms encodes a transmembrane tyrosine kinase receptor for the growth factor CSF-1 or colony stimulating factor-1. CSF-1 was found to inhibit growth and induce cellular differentiation. Altered expression of this factor and thus of the CSF-1 receptor is found in 50-60% of endometrial carcinomas and has been correlated with high grade tumors (Leiserowitz et al 1993; Smit et al 1995; Kimura et al 1991).

2.1.2.5 C-myc

Proto-oncogene c-myc is an early response gene and is essential in controlling cell proliferation. Mutations in this gene seem to be rare in endometrial carcinomas (Monk et al 1994; Niederacher et al 1999).

2.1.3 Tumour-suppressor genes and onco-suppressor genes

2.1.3.1 P 53

Mutations of the tumour-suppressor gene P53 occur very commonly in human tumours. Wildtype P53 functions as a G1 arrest and such arrest at the G1-S checkpoint creates extra time for DNA repair meganisms. P53 may initiate cell death via apoptosis (Lain 1992) if DNA repair fails. Mutations in the P53 gene can result in a protein with increased stability, leading to a longer half life of the mutant protein. Functionally inactive P53 protein can thus become over-expressed in the tumour cell (Findley 1988). Total loss of P53 expression is a relatively uncommon event, which can result from complete deletion of the P53 gene.

Several groups studied the expression level of the p53 protein in endometrial cancer using immuno-histochemical staining. In endometroid adenocarcinomas, p53 overexpression occurs in 15-55%, but in the more aggressive papillary serous adenocarcinomas 52-95% of tumours show over-expression. Various authors found a significant correlation with high stage tumours (Kohler et al 1992; Ito et al 1994; Lax et al 2000). In serous adenocarcinomas, p53 overexpression seem to occur as an early event with overexpression shown in high and low stages (Geisler et al 1999). This group and others (Kohler et al 1992) demonstrated that p53 overexpression is an independent prognostic marker, using multivariate analysis.

Recently, however, many authors challenged immuno-histochemical staining as a method to evaluate p53-activity. Methods to quantify staining results, cut off levels for defining overexpression and the antibodies used, differ widely between the studies. Importantly, Ito et al (1994) also found that about 15% of the P53 mutations show no immunoreactivity.

Overexpression of p53 seems to be a rare event in pre-malignant hyperplastic endometrium and even in stage 1 endometroid adenocarcinomas. However, in serous papillary adenocarcinomas the precursor lesion, EIC (endometrial intraepithelial carcinoma) is always associated with p53 overexpression if the tumour is p53 positive (Sherman et al 1995), demonstrating involvement early in carcinogenesis.

2.1.3.2 DCC

The tumor suppressor gene DCC (Deleted in Colorectal Carcinoma) plays a roll both in cell growth and cellular differentiation. Three groups have investigated DCC-expression in endometrial carcinoma (Seagusa et al 1999; Enomoto et al 1995; Gima et al 1994). These groups reported mutations and loss of protein expression in 30-50% of endometrial carcinomas without association with stage or grade.

2.1.3.3 Rb-gene

The retinoblastoma gene (Rb) was the very first tumour suppressor gene to be described and it was found to be responsible for hereditary retinoblastoma syndrome (Friend et al 1986; Lee et al 1987). In endometrial cancer alterations in this gene seem to be very rare. Loss of heterozygosity (LOH) at the Rb locus have been demonstrated in 10% of endometrial carcinomas and immuno-histochemistry has been used to demonstrate loss of Rb protein expression by Niemann et al (1997) and Semczuk et al (2000).

2.1.3.4 PTEN

The role of the PTEN tumour suppressor gene in endometrial carcinogenesis has been studied extensively and will be critically analysed and discussed below.

2.2 Histology of endometrial proliferative disorders

2.2.1 Endometrial hyperplasia and precursors of endometrial cancer

2.2.1.1 Endometrial hyperplasia

Endometrial hyperplasia essentially implies overgrowth of endometrium, consisting of endometrial glands and stroma. It is almost exclusively associated with a relative excess of endogenous or exogenous estrogen. Various histological subtypes are identified, according to the degree of cellular and structural differentiation and atypia. Simple hyperplasia (SH) resembles the normal endometrial tissue growth pattern, while complex hyperplasia (CH) has a more complex and thus more abnormal architectural growth pattern.

Both simple and complex hyperplasia can be associated with cellular atypia (SAH and CAH), which seems to be the most important predictor of malignant

potential (Scully et al 1994). Complex hyperplasia with atypia is the most dangerous type, with an estimated risk of simultaneous malignancy of about 20% (Prat 1996). Molecular markers that will predict progression to malignancy with accuracy are still outstanding (Orbo et al 2004).

2.2.1.2 Endometrial intra-epithelial neoplasia (EIN) or carcinoma (EIC)

This precursor of malignancy is the non-hyperplastic precursor lesion associated with serous papillary endometrial cancer (Fox 1992). This lesion is not associated with hyper-estrogenism, and will commonly arise in a background atrophic endometrium (Ambros et al 1995). When computerised morphometric analysis is used, the term is sometimes also used for atypical hyperplasias (Mutter 2000). This is probably warranted in a lesion co-existing with carcinoma in 20% of cases.

Precursor lesions have for the most part the genetic aberrations of the malignant lesion that will typically follow them. This correlation is so strong that markers of genetic abnormality are now used to detect the pre-cursor lesions in the background “normal” tissue. Maia and co-workers (2003) used PTEN and bcl-2 markers to help detect EIN in background epithelium of patients with carcinoma.

In the same way endometrial hyperplasia and specifically atypical hyperplasia (CAH) can be used to study the carcinogenesis of endometroid adenocarcinoma and endometrial intra-epithelial carcinoma (EIC) to study serous papillary adenocarcinoma.

2.2.2 Endometrial polyps

Various scientific findings support the idea of a common etiology or at least some shared etiological factors for endometrial thickening, endometrial hyperplasia, endometrial polyps and cancer. While endometrial hyperplasia presents overgrowth of both components of the endometrium, endometrial polyps are formed by stromal overgrowth covered in a normal fashion by glandular epithelium.

The abundance of literature on tamoxifen and its effects on the uterus includes benign, premalignant and malignant changes of both myometrium and endometrium, but with a very definite emphasis on the latter. The most common

lesions associated with its use are endometrial polyps and cystic hyperplasia. This also supports the idea of a shared etiology.

On a tissue level it can be postulated that endometrial polyps are the most probable benign counterpart of endometrial stromal sarcoma. Endometrial polyps and these models of carcinogenesis were not explored further in the current study but would deserve future attention.

2.2.3 Endometrial cancer

2.2.3.1 Pathogenetic subtypes

Since Bokhman described two types of endometrial cancer (Bokhman 1983), many authors have investigated and confirmed that there are two main etiological or pathogenetic pathways. In the majority of young patients the carcinoma is associated with hyperestrogenism and a better prognosis, while older patients typically have endometrial atrophy, low estrogen levels and more aggressive tumours. Molecular findings support this view and have identified various cellular genetic differences between the groups (Kaku et al 1999, Matias-Guiu et al 2001). Some of these findings were summarized by Ryan and colleagues in 2005.

Concomitant endometrial hyperplasia seems to be the most constant histological differentiator between these two groups (Beckner et al 1985, Deligdisch et al 1985). Tumours developing in a background of hyperplasia display a better prognosis and association with the type of genetic aberrations frequently seen in such better prognostic groups and in well-differentiated tumours (Scully et al 1994, Kurman et al 1994).

It is important that the pathogenetic subtypes were described initially within the group of endometroid adenocarcinomas, as a way to differentiate between two prognostic groups with essentially the same histopathologic tumour type. It is widely accepted that the etiopathogenesis of serous papillary adenocarcinoma overlaps mainly with Bokhman type 2 and arises mainly from atrophic endometrium.

Other non-endometroid carcinomas are rare and the pathogenetic differences are poorly understood (Darvishian et al 2004). Some endometrial carcinomas with

tubular growth may indeed represent serous carcinomas on molecular basis. When the treatment strategies for these tumour types diverge molecular tumour typing will become extremely important.

2.2.3.2 Histological subtypes

Endometroid adenocarcinoma is by far the commonest type, accounting for 80% of tumours. These tumours are subdivided in adenocarcinoma, adeno-acanthoma and adenosquamous carcinoma (Pecorelli et al 1999) according to the presence and type of squamous component. The degree of cellular atypia further divides the group into well, moderately and poorly differentiated groups, which has been convincingly shown to have important prognostic implications and has been included in FIGO staging in 1988.

Non-endometroid carcinomas include papillary serous adenocarcinoma (the most common subtype, accounting for about 10% of cases), clear-cell carcinoma, mucinous adenocarcinoma, adenosquamous carcinoma, undifferentiated and mixed carcinoma (Scully et al 1995, Pecorelli et al 1999). The latter four subtypes are extremely rare and are therefore inadequately studied.

With improved techniques more tumours are shown to be of the mixed subtype. As is the case in carcinosarcoma, these tumours are convincingly shown to be monoclonal with molecular techniques. An and co-workers (2004) showed monoclonality by identifying identical mutations in PTEN and P53 genes in different sections of several mixed tumours.

2.3 PTEN gene and endometrial proliferative disorders

2.3.1 PTEN in normal endometrium

Pten expression in the normal endometrium changes in response to hormonal variations (Mutter et al 2000). During the proliferative phase pten-protein is expressed in all tissue types, while expression is increased in the early secretory phase and lowered in the late secretory phase. These changes seem to be confined to the functionally active and hormonally responsive layers of endometrium.

Interestingly, Mutter and co-workers also found a proportion of normal, non-hyperplastic, endometrial lining cells already deficient in pten expression. PTEN

mutations were not studied in these cells, thus abnormal pten expression can be due to many factors. These cells probably do not progress to clonal representation, due to exfoliation. The apoptotic effect of progesterone would also induce natural cell death of genetically abnormal cells.

2.3.2 PTEN in endometrial hyperplasia

Should PTEN mutations be found to occur at about the same rate in pre-cancerous proliferative disorders as in endometrial cancer, this finding would suggest early inactivation of the PTEN-gene and thus an important role in carcinogenesis.

In the very limited reports available, PTEN mutations have indeed been demonstrated in endometrial hyperplasia and specifically in higher grades of hyperplasia like CAH. Sun and co-workers (2001) found different rates of PTEN mutations when analysing different subtypes of endometrial hyperplasia. They report an incidence of only 1 in 40 hyperplasias without atypia, but an incidence of 18% in CAH. The same group reported an incidence of 26% in endometroid endometrial cancer.

Kanaya and co-authors (2005) reported PTEN mutations in 5 of 27 (19%) endometrial hyperplasia specimens, all five were all atypical hyperplasia. Both frameshift mutations were associated with hMLH1 hypermethylation and the authors postulate that the latter is the earlier event leading to the PTEN mutation. PTEN mutation is then probably the important pre-cancerous event.

When computerised morphometry was used to identify EIC, this precursor lesion showed a high frequency of PTEN mutation (55%). This series included mostly complex atypical hyperplasia and not the EIC originally described in the absence of hyperplasia as a pre-cursor of papillary serous carcinoma. This important difference explains the high frequency of mutations as this series can then be seen as including mostly true clonal pre-cursor lesions of endometroid adenocarcinoma. In the same series the highest ever PTEN mutation rate of 83% is reported for endometrial carcinoma. These authors could demonstrate an absence of mutations in normal endometrium (Mutter et al 2000).

The group of Levine examined a series of 29 complex atypical hyperplasias and report an incidence of 27% in patients with synchronous cancer and 22% PTEN mutations without synchronous invasive disease (Levine et al 1998). Maxwell et al (1998) could not demonstrate differences in the incidence of PTEN mutations in hyperplasia with and without cellular atypia, finding 19% versus 21%.

It is considered important to study this topic further in the light of limited information and discrepancies in the literature.

2.3.3 PTEN in progression to endometrial cancer

Although evidence points toward increased incidence of PTEN mutations in precursor lesions of higher order, no direct association has been demonstrated between mutation of the PTEN-gene or loss of protein function and the development of an invasive lesion. On the contrary, evidence seems to support the early development of PTEN mutation as a factor supporting uncontrolled growth and associated also with pre-invasive lesions.

Interestingly, small clusters of PTEN deficient glandular cells have even been demonstrated in hyperstimulated endometrium not yet fitting the diagnostic criteria for hyperplasia (Mutter et al 2000). These results also demonstrate the cellular genetic variance of normal endometrium versus the cellular monoclonality of true pre-invasive hyperplasia.

The results of PTEN gene involvement in normal endometrium, as for most normal tissues, have been obtained using immunohistochemical staining for the pten protein, a technique showing very high rates of altered pten levels in endometrial cancer (up to 83%) and hyperplasia (55%). This technique also demonstrates the involvement of pten protein levels in cellular growth abnormalities by other methods than PTEN gene mutation. Pten protein expression can, for example, also be abnormal due to inhibition of upstream growth regulators.

2.3.4 PTEN in endometrial cancer

2.3.4.1 Frequency of PTEN mutations in endometrial cancer

2.3.4.1.1 Germline mutations

As described in chapter 1, PTEN was initially found as a result of the mapping of the susceptibility gene for Cowden syndrome. Germline mutations in the PTEN gene has subsequently also been linked to the Banayan-Zonana syndrome and to Proteus syndrome. It is thought that the latter can also be the result of mosaic germline mutations.

Mice with pten protein knockout develop complex proliferative endometrial lesions pointing to importance in cellular growth regulation in the female reproductive tract (Podsypanina et al 1999).

Despite the association of malignant epithelial and sometimes endometrial cancer with the Cowden syndrome (a cancer predisposition syndrome) (Mutter et al 2000), germline mutations are very uncommon in sporadic endometrial cancer. Black and co-workers (2004) recently published the results of 240 consecutive patients with endometrial cancer in whom they found only one scarce polymorhism (exon 4) and no disease causing germline mutations. This work confirms the results of other workers in the field.

2.3.4.1.2 Somatic mutations

Loss of heterozygosity was found to occur frequently (in about 40%) on the 10q chromosome (LOH 10q) in endometrial carcinomas (Peiffer et al 1995; Kong et al 1997). This led to the search for a tumour suppressor at this locus and the PTEN or MMAC1 gene, situated on 10q23.3, was found to fit the description. Evidence for the co-involvement of multiple other genes at this site has since emerged and continues to be investigated (Nagase et al 1996; Nagase et al 1997; Simpkins et al 1998).

The incidence of somatic mutations in PTEN in endometrial cancer is the highest of any tumour suppressor gene in any primary malignancy analysed so far. The frequencies reported vary from approximately 40% to even 76% and 83% (Nagase et al 1997; Peiffer et al 1995; Risinger et al 1997; Tashiro et al 1997; Mutter et al 2000).

Risinger and co-workers (1997) examined 70 endometrial cancers and found PTEN mutations of 34%, making it at the time the most commonly mutated known gene in endometrial cancer. Tashiro et al (1997) reported a 50% mutation rate in endometroid endometrial cancer. Kurose first described inactivation of both alleles, reporting an incidence of 33%, and confirmed the pattern that defines tumour suppressors for PTEN mutation in endometrial cancer (Kurose et al 1997).

2.3.4.2 PTEN gene inactivation by other methods

Not only genetic change but also epigenetic change can inactivate this important gene. It is now widely recognised that promotor PTEN methylation plays a role in tumorigenesis in some neoplasms. Some tumours have more than one genetic abnormality or "two hits" in PTEN and this may be two mutations or one mutation plus promoter methylation. This finding correlates with abnormality of DNA repair (Salvesen et al 2004) which is often also methylation of the hMLH1 gene.

The relation of genetic changes to pten protein expression is extremely complex. PTEN activity can be tested by mutation analysis, pten protein immunohistochemistry (Kimura et al 2004), and PTEN-antibody 6H2.1. PTEN antibody abnormality is decreased with exon 8 mutations. The latter two are low in about 20% of endometroid tumours, while about 54% will display PTEN mutations.

2.3.4.3 PTEN mutations in endometrial cancer subtypes

Sun and co-workers found a PTEN gene mutation incidence of 26% in endometroid and only 1 in 7 (14%) in non-endometroid tumours (Sun et al 2001). This confirmed the results of Risinger et al (1998) who found 37% in endometroid and 5% in non-endometroid carcinomas and Tashiro et al (1997) who reported 50% in endometroid and 0% in non-endometroid carcinomas.

Simpkins examined 34 endometroid tumours shown to have loss of 10q sequences and found an incidence of 38% PTEN mutations. The numbers in all these studies are small, but the results compare well, supporting the overall accuracy of the findings. This research has not been repeated in South Africa, in African patients or outside the developed world.

2.3.4.4 Association of PTEN with other genetic anomalies

Loss of **P53** function and overexpression of p53 protein product is strongly associated with serous papillary tumour type and with poorly differentiated tumours lacking hormone receptors (Kovalev et al 1998; Koul et al 1997; Boyd 1996). Tumours with PTEN mutations typically lack p53 overexpression, suggesting that the same biological endpoint, namely inhibition of apoptosis, is reached via either of these pathways but typically not via both.

Microsatellite instability or MI (typically defined as tumours with detectable alterations at two or more different microsatellite loci) is strongly associated with PTEN mutations in both endometrial carcinoma and hyperplasia (Levine et al 1998; Tashiro et al 1997). This strong association suggests that PTEN mutations may arise secondary to loss of the DNA repair system, although PTEN mutations can also precede the development of the MI phenotype (Levine et al 1998).

Loss of heterozygosity (LOH) of chromosome 10q is associated in about 40% of cases with PTEN mutation. Many other genes may be responsible for this finding. LOH of many chromosomes are typically associated with poorly differentiated tumours and serous papillary subtype. This finding has a low predictive value and no current therapeutic potential.

Although **K-ras activation** has been demonstrated frequently in precursor lesions and in endometrial cancer, the association with PTEN is unclear. This association will be discussed in more detail in chapter 3.

2.3.4.5 PTEN mutations and the prognosis of endometrial cancer

PTEN mutation is associated with tumour characteristics that generally have a good outcome. The most important is the close association with endometroid histology. PTEN mutation also correlates with the presence of MSI and absence of p53 overexpression, both predictors of good clinical outcome (Risinger et al 1998).

In many other tumours PTEN mutation has been found to predict a worse outcome and higher metastatic potential. Glioblastoma multiforme is one important example of a tumour where PTEN anomalies are frequently associated with late stage and a poor outcome. In endometrial cancer PTEN mutation

correlates with a better rather than worse outcome. This difference in the role of the tumour suppressor in different cancers is poorly understood. It probably depends on the specific sequence of genetic alterations that would lead to tumorigenesis in the specific organ and histological or morphological tumour type.

2.3.4.6 PTEN in different population groups and races

Little is known about PTEN mutation rates in different races. It is expected that the total incidence of PTEN mutations in Black women would be lower, because of the lower incidence of low risk, endometroid adenocarcinomas. Large differences in tumour types and tumour incidence exists between different population groups, and a difference in tumour type and also in etiopathogenesis on molecular level should be expected.

This study aims to address this as an important research question.

3. Materials and methods

3.1 Materials

3.1.1 Sampling and clinical material

Purposive sampling was used to select cases in a non-randomized way. This case selection is a non-probability type of sampling suited to this kind of research where cases typical of a category are of interest to the researcher. Forty-eight consecutive cases of endometrial carcinoma were identified in a retrospective review of the gynaecologic oncology clinical filing system. All these patients were surgically treated at the Gynaecologic Oncology Unit at the University of Pretoria during the years 1996 to 1998. Clinical data was available from these files on presentation, treatment, stage and outcome on most patients.

While on a study visit in Utrecht, the Netherlands, I was also allowed to include samples from the University Hospital of Utrecht (AZU). The anatomical pathologist (Dr Daisy Sie-Go) identified twenty-three suitable cases diagnosed from 1996 to 1999. These cases were selected for endometroid histology, all occurred in Caucasians and the clinical and histological characteristics will be explored further.

PTEN mutations have been reported in endometrial hyperplasia before, with a higher frequency in premalignant types. These findings have not been repeated locally and have not been correlated with race. In any study of PTEN involvement in endometrial cancer, it would be important to correlate the findings with that of the benign counterpart. Here we wanted to correlate the findings of the different grades of tumour with mutation frequency including hyperplasia of different aggressiveness on histology.

Slides of paraffin embedded endometrial tissue samples of different types of endometrial hyperplasia were kindly presented to us by the department of Anatomical Pathology, University of Leuven in Belgium. Twelve tissue samples with histological diagnosis were received, originating from ten patients.

3.1.2 Histology reports

Histology results from the original reports were available for all patients. These reports were used in the analysis. All tumours were also reviewed, histological diagnoses were confirmed and the tissues containing tumour and normal tissue were outlined.

The pathologist in Utrecht, Dr Daisy Sie-Go, also reviewed all tumours from her subset. Abnormal areas containing tumour material were marked and the material was then analysed as described below.

Two of the twelve tissue samples of endometrial hyperplasia had the histopathological diagnosis of complex atypical hyperplasia and ten had simple atypical hyperplasia. All the patients were Caucasian.

3.1.3 Tissue for DNA analysis

Paraffin embedded tissue was retrieved from the existing stored blocks of tissue. The first section of each block was stained and studied to confirm the diagnosis and presence of tumour and normal tissue. DNA from tumour and normal tissue was obtained from each case. The details of DNA extraction will be outlined below.

The endometrial hyperplasia samples were received and cut in sections. The first slide was marked to show the areas of hyperplasia reported on the histology. Tissue was obtained directly from the glass plates with a sterile sharp scalpel.

3.2 Methods

3.2.1 DNA extraction

Paraffin embedded tissue previously confirmed to contain either tumour or normal tissue was used. After hematoxylin staining, the pathologist reviewed the histological diagnosis and indicated normal (myometrial) and tumour areas on one slide. Using this slide as a guide, normal and tumour tissue was removed with a sterile blade from five to ten consecutive sections per patient.

Tissue samples were carefully transferred to micro-tubes where it was treated with the extraction buffer (10mM Tris-HCL, pH8.0; 0,45% Nonidet P40; 0,45% Tween-20).

Extraction buffer (200 µl) and 0,2mg/ml Proteinase K (Roche) was added to the tissue. After overnight digestion at 55°C, the proteinase K was inactivated by boiling (5 minutes at 95°C). The DNA solutions were quenched on ice and centrifuged. The supernatant, containing the DNA was transferred to new sterile tubes and used or stored at 5°C.

3.2.2 DNA amplification

PTEN-coding sequences were amplified by polymerase chain reaction using the primers described by Guldberg et al (1997) (Utrecht) and Davies et al (1999) (Pretoria). The nine exons were amplified in ten and eleven sections, with exons five in two sections and eight in one (Utrecht) or two (Pretoria) sections. Intron-based primers were used to minimise the risk of amplifying the processed PTEN pseudogene on chromosome 9, as described by Dahia et al (1998).

The primer sequences, the amplification conditions and product lengths are displayed in tables 2.3 and 2.4.

3.2.3 PTEN mutation analysis

Samples in the Pretoria group were analysed using the SSCP-method and exons displaying aberrant bands were directly sequenced. Materials collected in Utrecht were analysed in Utrecht using the DGGE-method. Direct sequencing was not completed there on the aberrant exons and the results were later confirmed by SSCP and sequencing of abnormalities in the cancer genetics laboratory under supervision of professor EJ van Rensburg.

Although the initial screening for mutations were done using two different methods, all material was eventually screened using SSCP and the methods were found to render comparable results.

3.2.3.1 Denaturing gradient gel electrophoresis (DGGE)

The melting temperature of amplicons varies according to nucleotide composition. Using DGGE, the 5% glycerol and 15-50% urea-formamide gradient simulates the temperature gradient and separates the amplicons.

Under the optimised conditions displayed below (table 2.3), the PCR-products move through the gel until the level of the melting temperature of its lower domain. Migration of the product immediately ceases when this point is reached, with the product then visible on the gel at this point. Mutant DNA exhibits changed migration and usually shows up as double bands: wild type from contaminating normal tissue or from the normal allele plus the altered band of mutant PTEN product.

Ten primer sets, as described by Guldberg et al (1997), were obtained from Eurogentec. Exon five was amplified in two parts. DGGE gels were stained with ethidium bromide and read by UV lamp illumination. Results were photographed using Kodac film.

Table 2.3: Primers and optimised conditions used for amplification and mutation detection with DGGE (Utrecht)

Exon	Primers	Primer sequence (5' → 3')	pcr conditions (35 cycles)	DGGE gradient	Volt-hours at 60°C
1	pten 1F pten 1R	ccgtcctcctttttcttcagccac (gc)-gaaaggtaaagaggagcagcc	30" 95°C 30" 57°C	20%–50%	600

			1' 72°C		
2	pten 2F pten 2R	(gc)ttagtttgattgctgcatatttc cggcgacatcaatatttgaaatagaaaagc	30" 95°C 30" 50°C 1' 68°C add 10% DMSO	20%-50%	600
3	pten 3F pten 3R	tgttaatggtggctttttg (gc)gcaagcatacaaataagaaaac	30" 95°C 30"55°C 1' 72°C (= standard)	10%-40%	500
4	pten 4F pten 4R	(gc)ttcctaagtgcaaaagataac tacagtctatcgggtttaagt	standard	20%-50%	600
5-1	pten 5F1 pten 5R1	(gc)ttttttcttattctgaggttatc tcattacaccagttcgtcc	standard	20%-50%	600
5-2	pten 5F2 pten5R2	tcatgttgccgaaattcac (gc)gaagaggaaaggaaaaacatc	standard	20%-50%	600
6	pten 6F pten 6R	(gc)agtgaaataactataatggaaca gaaggatgagaatttcaagc	standard	20%-50%	600
7	pten 7F pten7R	cgcgcccgaatactggtatgtatttaaccat (gc)tctcccaatgaaagtaaagta	standard	20%-50%	600
8	pten 8F pten8R	gcccgtttttaggacaaaatgtttcac (gc)cccacaaaatgtttaatttaac	standard	20%-50%	600
9	pten 9F pten 9R	gttttcattttaaattttctttc (gc)tggtgttttatgggtcttg	standard	20%-50%	600

Table 2.4: Primers and optimised conditions used for amplification and mutation detection by SSCP method - Pretoria

Exon	Primer name	Primer sequence	Product length (bp)	PCR conditions Temp (°C) MgCl2 (mM)	SSCP conditions (hours at 8W)
1	PTEN 1F PTEN 1R	caagtccagagccatttcc cccacgttctaagagagtga	233	58 2.0	16
2	PTEN 2F PTEN 2R	ttcttttagtttgattgctg gtatctttttctgtggcttag	239	50 2.0	16
3	PTEN 3F PTEN 3R	ctgtcttttggtttttctt caagcagataactttcactta	213	50 2.0	15
4	PTEN 4F PTEN 4R	tataaagattcaggcaatgtt cagtctatcgggtttaagtta	190	50 2.0	15
5A	PTEN 5AF PTEN 5AR	ttgttaattaaaaattcaagag gcacatatcattacaccagt	217	48 2.0	15
5B	PTEN 5BF PTEN 5AR	tgaccaatggctaagtgaa aaaagaaacccaaaatctgtt	248	50 2.0	16
6	PTEN 6F PTEN 6R	cccagttaccatagcaat taagaaaactgttccaataca	275	50 2.0	16

7	PTEN 7F PTEN 7R	ttgacagttaaaggcatttc cttattttggatatttctcc	264	50 2.0	16
8A	PTEN 8AF PTEN 8AR	ttcatttctttttctttttctttt ggttggctttgtctttctt	238	53 2.5	15,5
8B	PTEN 8BF PTEN 8BR	ccaggaccagaggaaac cacatacatacaagtcacaa	235	56 1.5	16
9	PTEN 9F PTEN 9R	agtcatatttgtgggtttt ttattttcatggtgttttatc	268	48 3.0	17

3.2.3.2 Single strand conformational polymorphism (SSCP)

The gene was amplified in eleven pieces, with exons five and eight in two parts. The primer sequences described by Davies et al (1999) were used.

PCR products were diluted 1:10 with denaturing buffer, denatured at 95°C (5 minutes), quenced on ice and 3 µl product was loaded on a 0.5X Mutation Detection Enhancement (MDE) gel. The gels were run at 8 Watts, 14-20 h in 0,6X TBE buffer and read after drying using exposure to medical X-ray film (Fuji).

3.2.3.3 Sequence analysis

Direct DNA sequencing was performed on all samples displaying abnormal SSCP or DGGE patterns. Sequenced samples were diluted and heat denatured. Three microliter was loaded on a 6% denaturing polyacrylamide gel. Electrophoresis was performed in 1X TBE buffer at 60 Watts and results read as for SSCP gels.

4. Results

4.1 Clinical data

Of the 48 initial patients, 14 were Caucasian, one was Indian and 33 were African. Mutation analysis was completed for 47 patients who had enough suitable tissue material for DNA analysis. The age distribution of these 47 patients is shown in table 2.5 below. The majority of African patients were in the age category 60 to 69 (11/32), while the Caucasian patients had an almost bimodal pattern with six under the age of sixty and eight (the rest) over the age of seventy. The one Indian patient was 47 years at diagnosis.

The age distribution according to race is displayed in figure 2.1 below. All 23 patients in the Utrecht subset were included in this dataset.

Of the original subset, 8/14 (57%) Caucasian patients were older than 70, while 10/48 (21%) African patients were older than 70. This tendency towards higher age in the Caucasian racial group did not reach significance. The age distribution of Caucasians in the Utrecht group is similar to the African group.

Table 2.5: Age distribution of women at diagnosis per racial group.

	African (%)		Caucasian Pretoria (%)		Caucasian Utrecht (%)		Indian (%)	
< 50	2	(6)	3	(21)	2	(9)	1	(100)
50 - 59	9	(28)	3	(21)	6	(26)		
60 - 69	11	(34)	0	(0)	8	(35)		
70 - 79	6	(19)	7	(50)	6	(26)		
80 +	4	(13)	1	(7)	0	(0)		

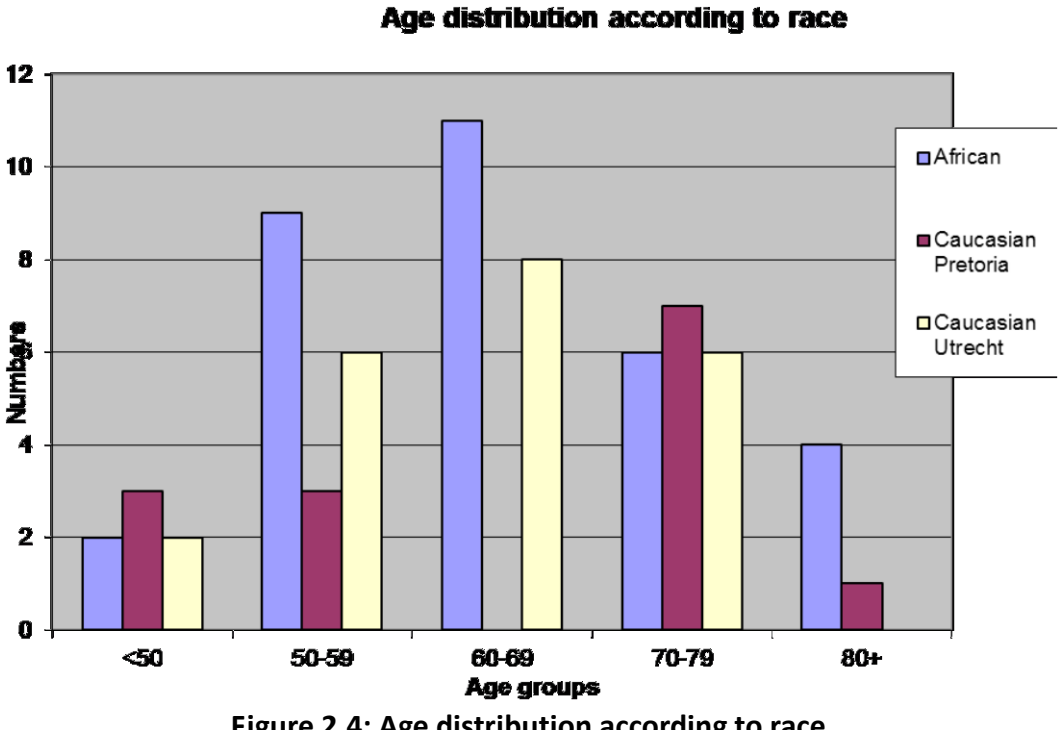

Figure 2.4: Age distribution according to race

FIGO stage distribution using the 1988 staging classification (Prat et al 1996) was known for 65 of 70 patients. Of the 37 Caucasian patients, the large majority (76%) had stage 1 disease, but only 50% of the 32 African women was diagnosed in stage 1. In the African group 28% had stages 3 and 4 disease, while only 11% of Caucasian women had such advanced disease.

The stage distribution and the percentage stage distribution according to race are displayed in table 2.6 and figures 2.2 and 2.3 below.

This important tendency towards higher stage correlates with other studies and with other gynaecological neoplasms studied in the local population. It has been ascribed to a multitude of factors, including poor socio-economic conditions, poor access to health care and lack of (health care) education. There is also the unstudied possibility of more aggressive cancer biology.

Table 2.6: FIGO stage distribution of women per racial group.

	African (%)		Caucasian Pretoria (%)		Caucasian Utrecht (%)		Indian (%)	
Stage 1	16	(50)	10	(71)	18	(78)	-	
Stage 1a	3	(9)	3	(21)	0	(0)		
Stage 1b	8	(25)	4	(29)	11	(47)		
Stage 1c	5	(16)	3	(21)	7	(30)		
Stage 2	3	(9)	1	(7)	3	(13)		
Stage 3	7	(22)	2	(14)	1	(4)	1	(100)
Stage 4	2	(6)	0	(0)	1	(4)		
Unknown	4	(13)	1	(7)	0	(0)		

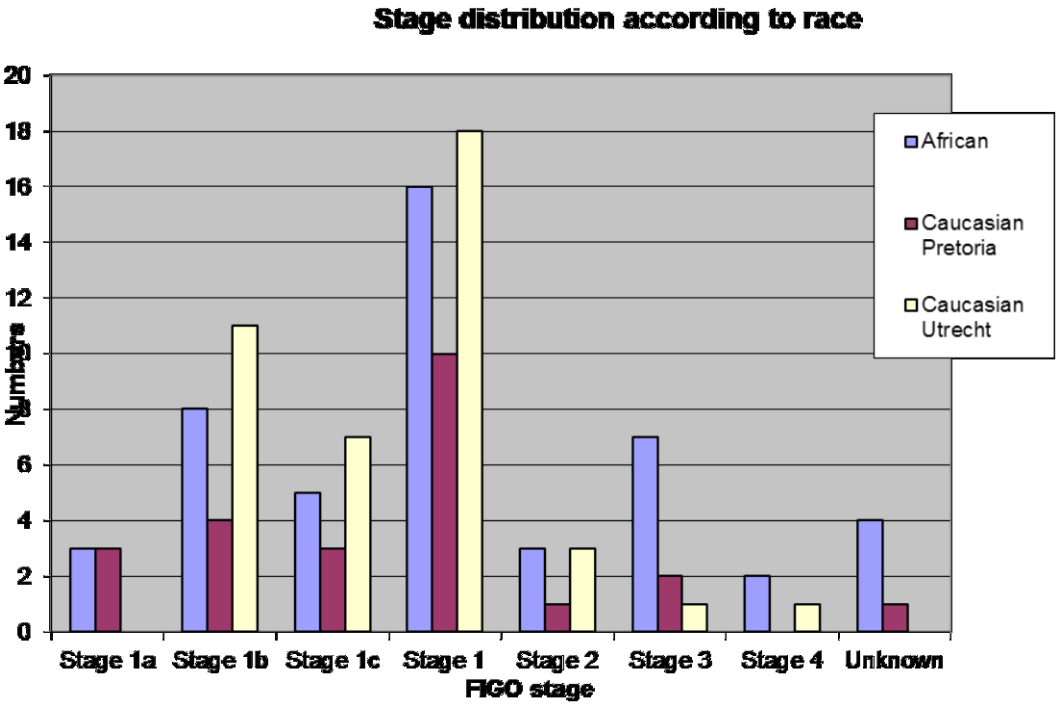

Figure 2.5: FIGO stage distribution according to race

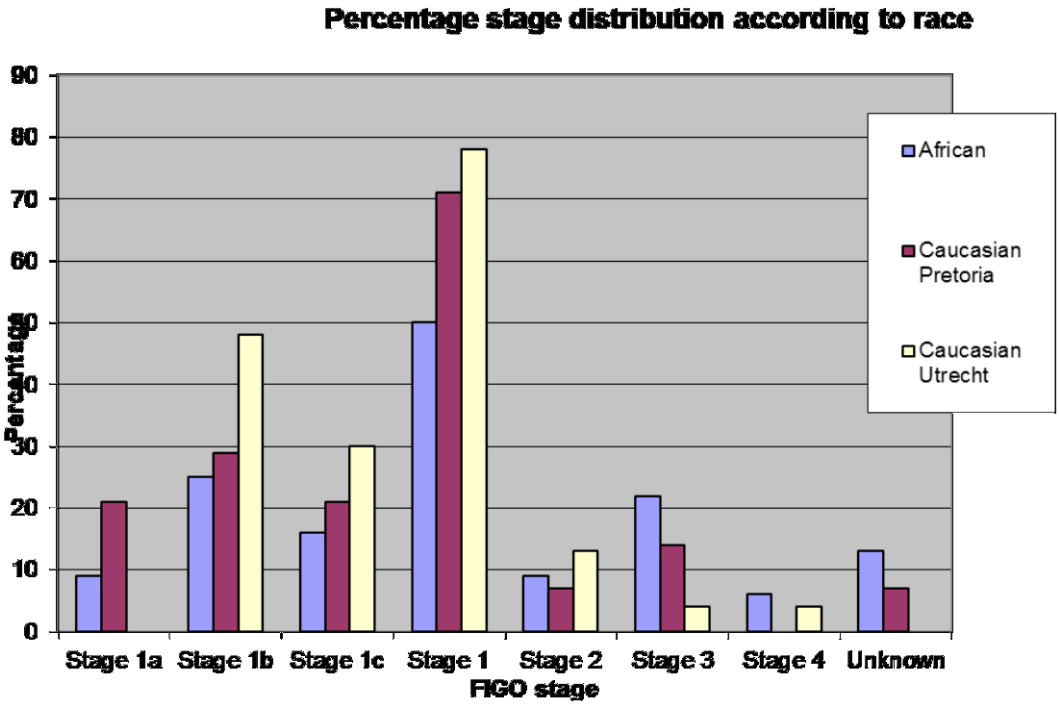

Figure 2.6: Percentage FIGO stage distribution according to race

4.2 Histology data

4.2.1 Histological type

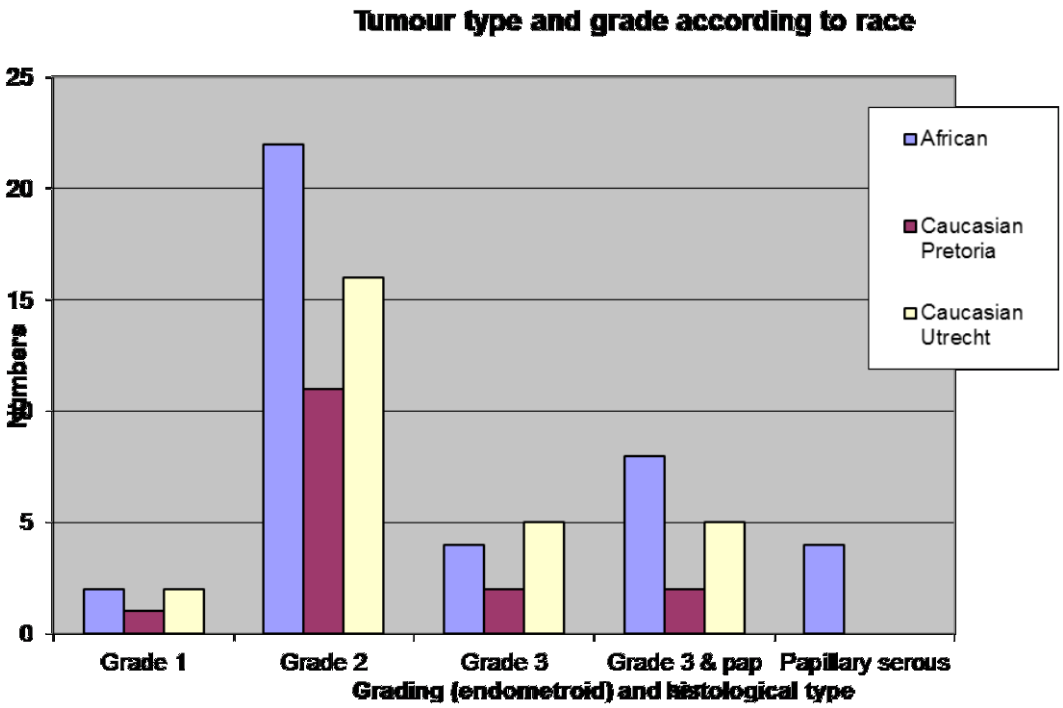

Figure 2.7: Histological type and grading distribution according to race

Four of the 48 patients had papillary serous tumours, while the rest, 44, had endometroid adenocarcinomas of various differentiation grades. The differentiation grades and tumour types of the study population are shown below in table 2.7. In African women, four of 32 tumours (13%) had papillary serous histotypes, while no Caucasian woman had this subtype. Both table 2.7 (below) and figure 2.4 display the tumour type as well as the differentiation grade according to race.

4.2.2 Histological grade

In this group of patients there was a strong tendency towards more poor prognostic pathological types in African women. Seven of the 32 (13%) African women had poorly differentiated tumours and two of 14 (14%) Caucasian women had grade 3 tumours. In all racial groups grade two cellular differentiation grade dominated.

Table 2.7: Histological grade and tumour type per racial group.

	African (%)	Caucasian Pretoria (%)	Caucasian Utrecht (%)	Indian (%)
Grade 1	2 (6)	1 (7)	2 (9)	

Grade 2	22	(69)	11	(79)	16	(70)	1	(100)
Grade 3	4	(13)	2	(14)	5	(21)		
Grade 3 & papillary serous	8	(25)	2	(14)	5	(21)		
Papillary serous	4	(13)	0	(0)	0	(0)		

4.3 Mutation screening

4.3.1 Denaturing gradient gel electrophoresis results

Eighteen tumours with normal myometrium as controls were analysed. All these analyses were repeated on SSCP and all abnormal patterns on SSCP were directly sequenced.

Representative examples of the DGGE results are shown in figures 2.5 to 2.7. The mutation screening was repeated because the sequencing was not completed in the Utrecht laboratory and the Pretoria laboratory did not use and trust the DGGE screening results. Too many results were obtained that was difficult to interpret. However, only two mutations were found in this subset with SSCP that was not predicted by the DGGE screening, both in exon 7.

The results of the DGGE screening tests on tumours and normal tissue of the first 18 Utrecht patients are shown in table 2.8.

Table 2.8: DGGE screening test results: tumours (T1-18) and normal tissue (germline DNA) (N1-18) in the Utrecht subset

	Ex 1	Exon 2	Exon 3	Exon 4	Ex 5(1)	Ex 5(2)	Exon 6	Exon 7	Exon 8	Exon 9
T 1	normal	normal	normal	normal	normal	?	normal	normal	normal	normal
T 2	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
T 3	normal	normal	normal	normal	unsure	unsure	?	normal	normal	unsure
T 4	normal	normal	normal	unsure	normal	normal	normal	normal	shift	normal
T 5	shift	normal	normal	normal	normal	normal	normal	normal	normal	normal
T 6	shift	normal	normal	normal	shift	shift	normal	normal	normal	normal
T 7	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
T 8	normal	normal	normal	normal	shift	shift	unsure	shift	normal	normal
T 9	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
T 10	normal	unsure	normal	normal	normal	shift	normal	normal	normal	normal
T 11	normal	normal	normal	normal	?	shift	?	normal	normal	normal
T 12	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
T 13	normal	normal	normal	normal	shift	normal	normal	normal	?	normal
T 14	normal	normal	normal	normal	normal	normal	normal	normal	shift	normal
T 15	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
T 16	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal

T 17	normal	normal	normal	normal	shift	normal	normal	?	normal	normal
T 18	normal	normal	normal	normal	normal	normal	normal	normal	shift	normal
N 1	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
N 2	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 3	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 4	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 5	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 6	normal	normal	normal	normal	normal	normal	?	?	normal	normal
N 7	normal	normal	normal	normal	normal	normal	?	normal	normal	normal
N 8	normal	normal	normal	normal	normal	normal	normal	normal	shift	normal
N 9	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 10	normal	normal	normal	normal	normal	normal	normal	?	normal	normal
N 11	normal	normal	normal	normal	normal	shift	normal	normal	normal	normal
N 12	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 13	normal	normal	normal	normal	normal	normal	normal	normal	?	normal
N 14	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 15	normal	normal	normal	normal	?	normal	normal	normal	normal	normal
N 16	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
N 17	normal	normal	normal	normal	normal	?	normal	normal	normal	normal
N 18	normal	normal	normal	normal	normal	normal	normal	normal	?	normal

shift = definite shift seen on gel
unsure = probable shift
? = uninterpretable result, possible shift
Grey = mutation confirmed

4.3.2 Single strand conformational polymorphism results

Twenty seven of the forty seven tumours of the Pretoria subset showed abnormal migrating on SSCP gels. All were endometroid adenocarcinomas, while no shifts occurred in the papillary serous carcinomas. Shifts were observed in all of the nine exons.

Unfortunately in five of the tumours that displayed shifts, mutation analysis could not be completed as DNA amplification for sequencing was inadequate. Mutation analysis will therefore be reported on the thirty seven remaining endometroid adenocarcinomas, plus the eighteen carcinomas of the DGGE group and the remaining five carcinomas in the Utrecht group that were not screened by DGGE.

Two endometrial hyperplasia samples showed an abnormal migrating pattern on the SSCP gel and were directly sequenced to confirm mutation.

4.4 Sequence analysis

4.4.1 Disease causing mutations

In the Pretoria patients the following was found:

Although shifts occurred in all exons, mutations were not confirmed in exons 2, 4 and 9. A total of 32 mutations were confirmed, 24 different mutations. Three mutations were of unknown significance and one polymorphism was found in four samples.

All these mutations were confirmed to be somatic with none found in the corresponding normal tissue. Only the mutations considered to be disease causing will be discussed further. These results were previously reported, interpreted and discussed in depth by Jamison (2004).

In the Utrecht patients the following was found:

Fifteen mutations were found in 13 of the 23 tumours, with tumours T3 and T11 harbouring two mutations each. No mutations were found in the normal tissue, proving these to be somatic. Of the mutation positive tumours, two were adenosquamous type and four were poorly differentatied tumours. These mutations were also not associated with early stage as one patient with stage 4 disease had two mutations in exon 5. Two mutations of unknown significance were found in T10 and T19.

The distribution of disease causing mutations over the gene is shown in table 2.9 and compared with previously reported frequency. These mutations will be discussed per exon below.

In the endometrial hyperplasia subset of patients the following was found:

Both of the abnormal patterns on gel were confirmed to be mutations in exons 7 and 3 when sequenced. Two mutations were thus found in the group of ten patients with atypical hyperplasia examined (20%). Another mutation of unknown significance was found in HYP9, atypical hyperplasia. Both were observed in the samples with simple atypical hyperplasia, while no mutations were found in the two samples with complex atypical hyperplasia.

Table 2.9: Frequency of disease causing mutations in Pretoria subset according to exon distribution

Exon / Intron	Number of different mutations	Number of tumours with mutation	% of endometroid tumours with mutation	% of all mutations in this exon	Involvement of this exon (%) as previously published*
Exon 1	4	4	11	17.4	7
Exon 2	-	-	0	0	7
Exon 3	1	1	3	4.3	6
Exon 4	1	1	3	4.3	3
Exon 5	5	9	24	39.1	21
Exon 6	1	1	3	4.3	5
Exon 7	3	3	8	13	19
Exon 8	3	4	11	17.4	28
Exon 9	-	-	0	0	0.5
Total	17	23	54 **	100	~100

* Previously published findings estimated from Konopka et al 2002
** Some tumours harboured more than one mutation

Mutation analysis in Pretoria subset

Exon 1

Four disease causing mutations were detected in exon 1, occurring at codons 5, 6, 7 and 17. All four were truncating mutations; two were frameshift mutations causing early termination and one each G-to-T and C-to-T mutations were detected.

Exon 3

The mutation in exon 3 was a C-to-A missense mutation in codon 59.

In the sample numbered EHYP9, a missense mutation was also found. The mutation occurred in exon 3 and the significance is unclear. It could not be determined whether this mutation is germline or somatic and to what extent it would influence DNA replication and protein production. This is a novel synonymous mutation, not previously reported and is considered to be possibly disease causing.

Exon 4

In the Intron 4 a transition G-to-A mutation was detected of which the predicted effect is that of exon skipping.

Exon 5

As previously reported exon five was a mutational hotspot, especially at codon 130 where we found mutations in not less than eight tumours. |Three tumours shared a frameshift mutation (389delG), which would result in termination of the protein at codon 133. Three other tumours had a transition mutation at 388, two had C-to-T and one C-to-G transition, with predicted arginine-to-glysine and arginine-to-stop effects.

Another mutation was found in codon 149 where a C-to-T transition occurred resulting in a predicted truncation of the protein.

Exon 6

In intron six, another mutation was found that would probably result in exon skipping, namely a A-to-C mutation.

Exon 7

Two mutations were detected in exon 7, namely at codon 234 a deletion G resulting in frameshift and at codon 246 a transition C-to-T

Intron 7 again displayed a deletion G mutation, resulting in exon skipping.

In sample numbered EHYP5 (hyperplasia), a nonsense mutation was found in exon 7. The mutation, c.766→T, is presumed to be a somatic mutation although non-tumour DNA was not available for analysis. This mutation will result in protein dysfunction and is considered disease causing.

Exon 8

In exon 8 three frameshift mutations were found in four specimens, one deletion A (codon 288), one insertion A (codon 323) and two patients with deletion ACTT in codon 319.

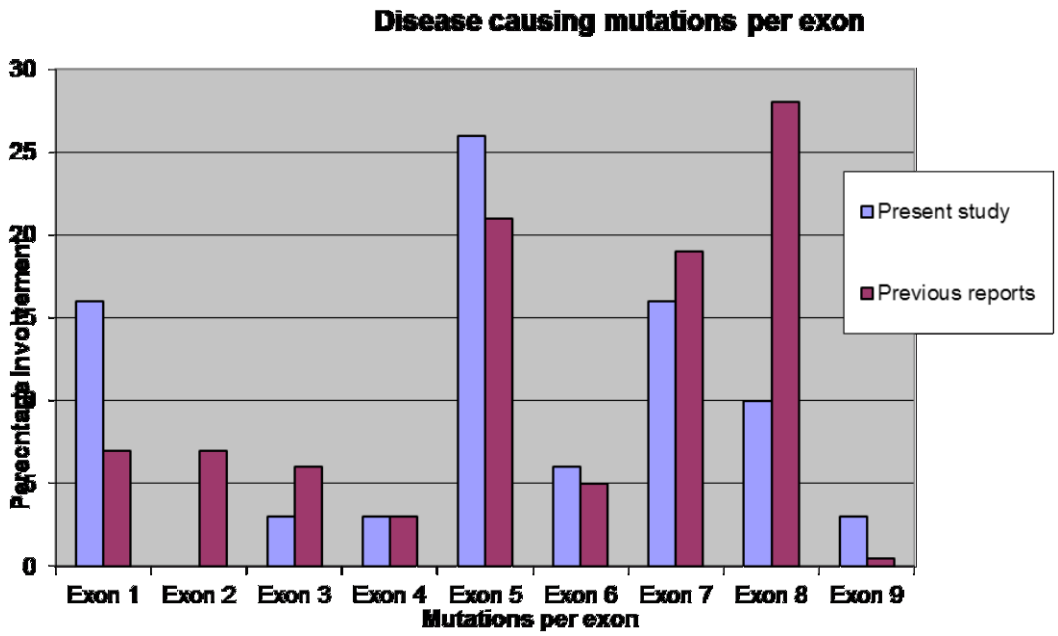

Figure 2.8: Disease causing mutation per exon in Pretoria and Utrecht groups together compared to previous reports

Mutation analysis in Utrecht subset

Exon 1

Two disease causing mutations were found occurring at codons 7 and 26. These were a truncating mutation, G-to-T (demonstrated also in one of the Pretoria group patients) and a frameshift mutation terminating the protein at 53.

Exon 5

Seven mutations were demonstrated occurring in six patients, with T11 having two mutations within this exon. There were four frameshift mutations at codons 130, 135, 142 and 143 resulting in early termination and three transition mutations. The latter occurred at codons 129 and 130 and were G-to-C, C-to-T and G-to-A transitions.

Exon 6

One missence mutation were detected in codon 173, namely a G-to-A transition.

Exon 7

Three mutations were confirmed in exon 7 in the Utrecht subset. These were one frameshift mutation at codon 221 and two identical transitional mutations at codon 233 resulting to a arginine to stop effect.

Exon 8

One of the frameshift mutations detected in the Pretoria group was also present in T4 of the Utrecht dataset (insertion A in codon 323).

Exon 9

Finally a frameshift mutation was detected in codon 346-347 in exon 9. In this tumour CTTC was deleted resultion in early termination.

Table 2.10 Frequency of disease causing mutations in Utrecht subset of endometrial carcinomas according to exon distribution

Exon / Intron	Number of different mutations	Number of tumours with mutation	% of tumours with mutation	% of all mutations in this exon	Involvement of this exon (%) as previously published *
Exon 1	2	2	8.7	13.3	7
Exon 2	-	-	0	-	7
Exon 3	-	-	0	-	6
E / I 4	-	-	0	-	3
Exon 5	7	**6	26	47	21
Exon 6	1	1	4	7	5
E / I 7	3	3	13	20	19
Exon 8	1	1	4	7	28
Exon 9	1	1	4	7	0.5
Total	15	**14	61	~100	~100

* Previously published findings estimated from Konopka et al 2002
** Some tumours harboured more than one mutation

4.4.2 Mutations of unknown significance and polymorphisms

Seven tumours of the Pretoria subset harboured mutations of which the significance is unknown or that are probably non-significant or known polymorphisms. Five of these mutations occurred in tumours shown to harbour at least one other disease causing mutation, while the polymorphism was the only genetic change in tumour END1 and in hyperplasia EHYP9. In tumour END14 a missense mutation was detected as the only genetic anomaly. It is uncertain whether this mutation affects the action of the pten protein and contributes to disease. These mutations are listed in table 2.11 below and will not be discussed further.

It is of importance to note that in this study only PTEN gene mutations were addressed. We did not investigate the incidence of pten-protein aberrations in any way. As discussed protein expression can be measured by semi-quantitative immunohistochemistry or pten protein function.

Table 2.11: Mutations of unknown significance and polymorphisms in endometrial carcinomas

Exon / Intron	Nr of tumours	Nucleotide change	Codon	Interpretation
Exon 1	1	c.44G to T	15	Arg to Iso
Exon 1	1	c.44G to C	15	Arg to Thr
Exon 2	4	IVS2-13delGTTT	N/A	Polymorphism
Exon 2	1	c.97delATT	33	Ile del
Exon 2	1	c.136delTACAGG AACAATATT	46-50	Tyr Arg Asn Asn Ile del
Exon 6	1	c.526delTAT	176	Tyr del

4.5 Correlation between clinical and molecular results

4.5.1 Correlation between PTEN gene mutations and clinical findings in the endometrial cancers

There was no difference found in PTEN mutation frequency according to race when only the endometroid adenocarcinoma group is considered. Including all the tumours, there was a tendency towards a lower frequency of PTEN mutations in African women. Table 2.12 displays the pathogenic mutations per race and stage group.

Interestingly all four tumours with more than one pathogenic mutation (three with two and one with three) occurred in African patients. When all mutations are considered, a tendency towards more mutations in tumours of a higher FIGO stage was also noted. This finding was not reported before. Most previous series contained limited numbers of patients with higher stage disease. It is possible that these genetic abnormalities accumulate with time or that tumours with more severe pten protein dysfunction will tend to be more aggressive and thus be diagnosed in a later stage.

If the latter theory were true, one would expect also a correlation with histological grade. This was not demonstrated in the current study, although it was

previously suggested. The number of mutations per FIGO stage of endometroid cancers is shown in figure 2.6.

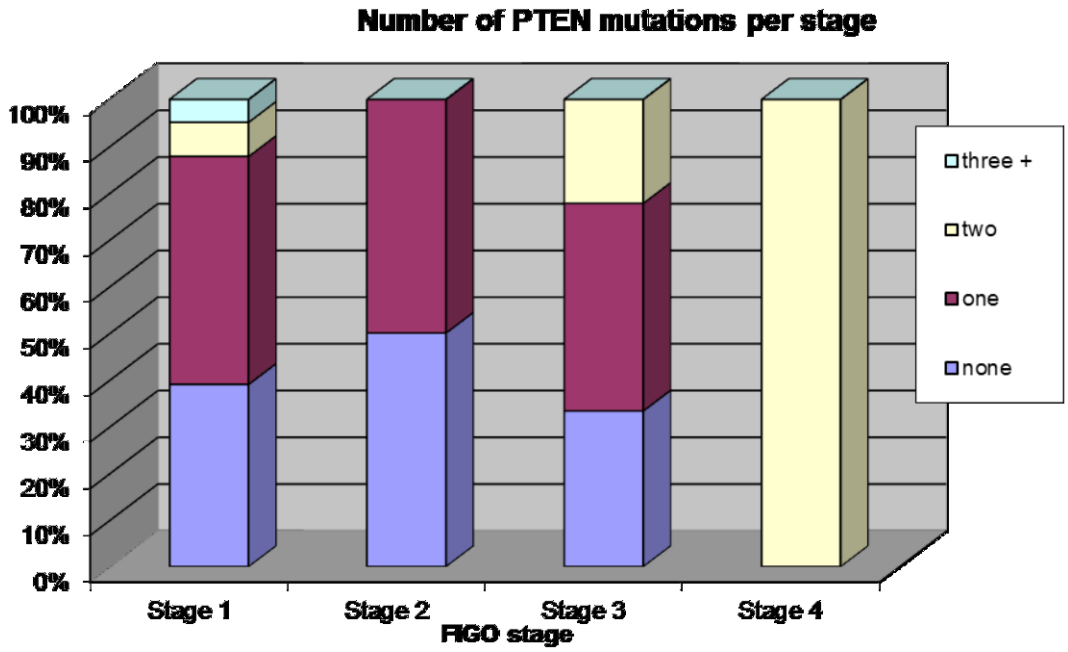

Figure 2.9: The number of PTEN mutations per FIGO stage in endometroid cancers

PTEN mutation positivity did not correlate with any other clinical parameter in this group of patients. The mean age of mutation positive patients was 62,5 years (range 43 to 92) and for mutation negative patients it was 65 years (range 45 to 84).

4.5.2 Correlation between PTEN gene mutations and histology findings in the endometrial cancers

In this group of patients pathogenic PTEN mutations were found in simple atypical hyperplasia (1/10) (10%) and in endometroid adenocarcinomas (20/37) (54%) but not in four papillary serous endometrial carcinomas (0%). Two of three adenosquamous carcinomas displayed one mutation each.

Due to the small sample size, it was not reliable to correlate the pathological subtypes with the presence of PTEN mutations.

PTEN mutations did not correlate with differentiation grade as reported before, but was found with the same frequency in all grades of tumour. This interesting finding is shown in table 2.12.

Table 2.12: Pathogenic PTEN mutations shown according to race and tumour grade (Pretoria)

Race	Differentiation grade	Number of patients *	Tumours with mutations	Percentage with mutations**
TOTAL	Atypical hyperplasia	10	1	10
	Grade 1	5	4	80
	Grade 2	45	23	56
	Grade 3	11	7	64
	Papillary serous	4	0	0
	Total malignancies	**65**	**34**	**52**
AFRICAN	Grade 1	2	1	50
	Grade 2	20	10	50
	Grade 3	4	2	50
	Papillary serous	4	0	0
	Total malignancies	**30**	**13**	**43**
CAUCASIAN PRETORIA	Atypical hyperplasia	10	1	10
	Grade 1	1	1	100
	Grade 2	9	4	44
	Grade 3	2	1	50
	Total malignancies	**12**	**7**	**58**
CAUCASIAN UTRECHT	Atypical hyperplasia	0	0	-
	Grade 1	2	2	100
	Grade 2	16	9	56
	Grade 3	5	4	80
	Total malignancies	**23**	**15**	**65**

* Number with completed mutation analysis
** Only pathogenic mutations included

5. Interpretation and discussion

5.1 Endometrial hyperplasia

Finding PTEN mutations even in simple hyperplasia, confirms the role that inactivation of the protein by genetic mutation plays in the etiology of this disease. This also confirms that this genetic event can occur early in the carcinogenetic pathway.

In the current study it seems PTEN mutations can also occur later in carcinogenesis as the incidence was much higher in cancers than in the hyperplasia. This incidence was reported to be higher if a strict definition of precursor lesions was used, but these results have not been confirmed by other groups or by the current study.

5.2 Endometrial cancer

It was previously reported that PTEN mutations tended to be more common in well-differentiated tumours than in tumours with higher nuclear and architectural grading. In the present study we did not find such a tendency although all grades were well represented in our sampling.

We could confirm the result of previous (small) studies that reported PTEN not to be involved or mutated in papillary serous tumours. This important finding once again confirms that histological type demonstrates molecular differences underlying the processes of tumour genesis.

We found that the frequency of PTEN involvement was the same in all the races when only endometroid cancers were considered. When the serous papillary cancers were added, the frequency of PTEN mutations in African patients was lower.

It appears thus that the frequency of PTEN mutations are not determined by the race, but rather by the tumour type. In the same way that aggressive tumour types are relatively more common in African patients (due to a relative under-representation of endometroid carcinomas), PTEN mutation negative tumours

may be more common in African women (due to an underrepresentation of endometroid PTEN positive tumours).

When the number of mutations per tumour is considered, some interesting and unique findings were made. We found multiple mutations in the PTEN gene in nine tumours and multiple disease causing mutations in six. Seven of these patients were African, while only two of the Caucasian patients in this subset had more than one mutation, one of these patients had stage 4 disease.

This finding suggests that previous clinico-pathological findings of more aggressive tumour behaviour could possibly be explained by more severe inactivation of the pten protein. It is unproven but possible or even probable that more mutations will cause worse impairment of pten protein function. This finding correlates exactly with the findings of aggressive tumour types in African women. The number of mutations per FIGO stage is displayed in figure 2.7 and the FIGO stage distribution per number of mutations in figure 2.6 (paragraph 4.5.1). Although the numbers are small, these column charts demonstrate the tendency towards a higher stage with more mutations.

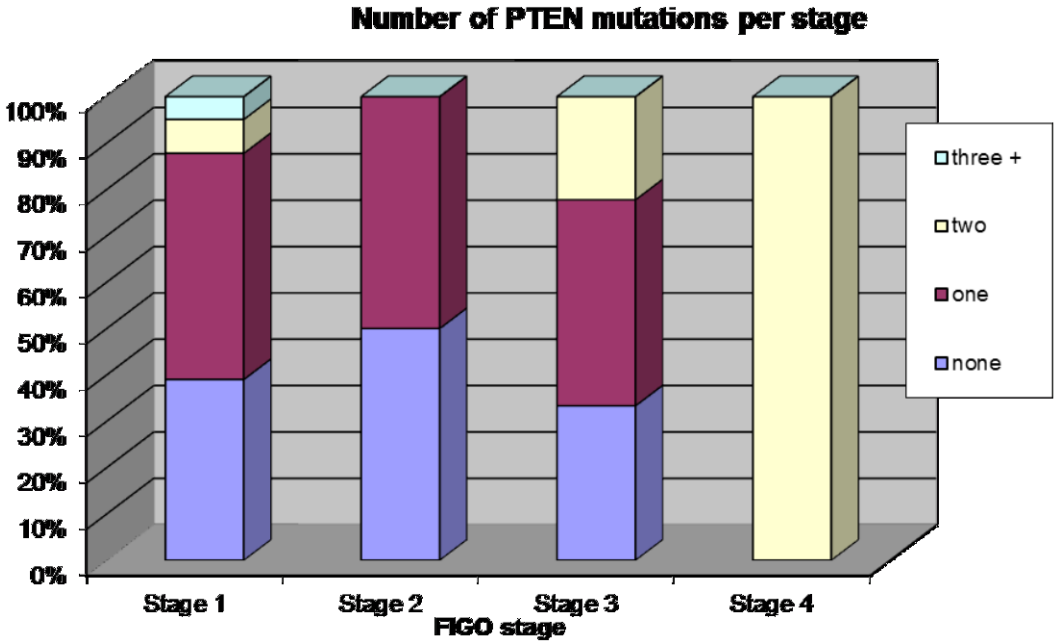

Figure 2.10: FIGO stage distribution per number of PTEN mutations

5.3 Limitations and recommendations for future research

This study was limited to mutation analysis and no attempt was made to study pten protein levels or activity. It would be interesting to correlate gene mutations to protein expression and protein activity.

This study could not demonstrate sufficiently when in the carcinogenetic pathway (early or late) the mutations in the PTEN gene occur. It would be potentially useful to continue this study of the chronology of carcinogenetic mutations in future projects. The findings regarding racial differences should also be confirmed in future studies.