CHAPTER 1

1.0 INTRODUCTION

Endometrial carcinoma is the most common malignancy of the female genital tract in western countries and is one of the most frequent malignancies in females.[1–4] In 2019, the predicted occurrence of endometrial carcinoma is roughly 7% in the United States of America. [1–5] Endometrial carcinoma constitutes approximately 4% of all tumours.[4] In South Africa, the most recent published statistics from the National Cancer Registry in 2014 recorded an incidence of 3.32% of uterine malignancies amongst all registered tumours in females.[6]

At present, there is an increase in endometrial carcinoma in the western world.[7–9] This is due to an increase in the use of exogenous hormonal therapy such as Tamoxifen for the treatment for breast carcinoma, the occurrence of increased numbers of females being overweight or obese, in addition to increased longevity globally.[7–10] Tamoxifen demonstrates anti-estrogenic effects in breast parenchyma but paradoxically provides an estrogen rich environment in the endometrium. This provides a fertile soil for possible hyperplasia of the endometrial lining and increases the likelihood of an estrogen dependent tumour developing.[11] Hormonal therapy in the form of estrogen in the absence of anti-estrogenic progesterone, is an additional supply of exogenous estrogen, which may facilitate endometrial proliferation.[11] Increased adiposity in overweight and obese females facilitates increased circulating endogenous estrogen.[11] South Africa currently has a rising percentage of overweight and obese females which may result in a rise in estrogen associated tumours, such as endometrial carcinoma in our country.[12] Polycystic ovarian syndrome as well as certain ovarian tumours may also contribute to increased levels of circulating endogenous estrogen.[11]

Bokhman recommended the dualistic clinicopathologic model of endometrial carcinoma in 1983. This classified endometrial carcinomas into those that were either estrogen dependent

(Type I) or estrogen-independent (Type II) [7, 13–15] The dualistic model whilst rigid, allows for appreciation of the pathogenesis of endometrial tumours and is still used today during histopathological evaluation.[13] However, there are times when histological classification into Type I or Type II is challenging for the histopathologist. Use of this classification system for predicting a clinical outcome is less than ideal and is therefore not used for staging of tumours or risk assessment; as tumour relapses are noted in up to 20% of patients diagnosed with Type I tumours; whereas relapses may not occur in as many as 50% of patients with Type II carcinomas.[13]

Endometrioid endometrial carcinomas (EEC) or Type I tumours histologically resemble normal endometrium and have a tendency to occur in premenopausal or perimenopausal females and are usually low FIGO grade carcinomas that often occur in a background of endometrial hyperplasia and in a setting of raised estrogen. [2,7 15,16] Such tumours are often confined to the uterus and as such, most patients are cured after hysterectomy.[2,15,16] Non-Endometrioid Endometrial Carcinomas (non-EEC) or Type II tumours include serous carcinomas, carcinosarcomas and clear cell carcinomas. These tumours develop independently of an estrogenic background and usually develop in older, non-obese females. [2,17] These tumours have a comparatively worse outcome than Type I neoplasms.[2,15,16,18] Surgical methods alone may result in disease cure for patients diagnosed with early stage endometrioid endometrial carcinomas; whilst some patients may also be treated with adjuvant radiotherapy. In contrast, those with Type II neoplasms are usually subjected to chemotherapy and/or radiation therapy; which are modalities presented to high-staged tumours of both morphological subtypes.[17]There is thus, a need for accurate subtyping of tumours so that careful consideration may be given to possible adjuvant therapy.[17]There are however, situations in which histological distinction of a high-FIGO grade EEC from a serous carcinoma are extremely difficult for a histopathologist to make. It is especially in

such cases that molecular categorization of tumours may allow for a more accurate tumour classification.[17] It is interesting to note that high-FIGO grade tumours have demonstrated poor histopathologist interobserver reproducibility which further lends credence to the notion that molecular classification systems may enhance distinction between tumour subtypes which ultimately have an effect on treatment options for patients.[19] Following surgical staging, presently used endometrial carcinoma risk stratification systems divide patients into three categories with an associated risk of recurrent disease. These systems are based on both pathological and clinical parameters such as tumour subtype, FIGO grade and stage of a tumour, the absence or presence of lymphovascular invasion and the age of a patient. The PORTEC or Postoperative Radiation Therapy for Endometrial Carcinoma, 1 and 2 clinical trials have illustrated that patients assessed as being in the low risk category need not receive vaginal brachytherapy; whereas patients in the intermediate to high risk groups do benefit from such treatment. There are however patients who do receive too much or too little treatment, which further highlights the need for accurate, patient-specific treatment modalities. [20,21]

In 2013, molecular investigations by The Cancer Genome Atlas (TCGA) Research Network ushered in the classification of endometrial tumours into four groups instead of the traditional Type I and II division of endometrial carcinomas.[17]This classification of tumours may provide information at the molecular level which ultimately has a direct bearing on treatment options. The Cancer Genome Atlas Research Network used microsatellite assays, analysis of copy number, and whole genome sequencing and exome sequencing. The four groups comprise: microsatellite instability hypermutated, DNA Polymerase Epsilon or *POLE* ultramutated, copy-number high and copy-number low.[17] Approximately 7% of endometrial tumours tested showed a nucleotide change of Cytosine→Adenosine. These tumours had a high rate of mutations in the exonuclease domain of *POLE*, which encodes a catalytic and

proofreading component of DNA polymerase epsilon, which is necessary for the process of DNA strand replication to begin. The exonuclease proofreading function and the exact inclusion of bases by *POLE* ensures that mutations in the daughter strand are kept to a minimum.[17,19,22] The hypermutated phenotype was demonstrated to arise from inactivation or suppression of proof-reading activities as a consequence of DNA polymerase substitutions.[17,19,22] The *POLE* group of tumours demonstrated mutations in *PIK3CA, PTEN, ARID1A, FBXW7, KRAD* and *PIK3R1*; and were identified as having a better overall prognosis.[17,19,22]The MSI hypermutated group showed mutations occurring at a far greater frequency than those of microsatellite stable tumours. The MSI group demonstrated mutations in *PIK3CA, PTEN, ARID1A, KRAS, PIK3R1* and *RPL22*[17] It is hypothesized that the high rate of mutation results in the formation of neo-antigens thereby stimulating the immune system. This then allows for an increase in anti-neoplastic activity by the body's defences.[22] Microarrays identified recurrent amplifications or deletions which typified the copy number high group, in which chromosomal instability, p53, *PPP2R1A* and *PIK3CA* mutations were present. The fourth category, or copy number low group, were tumours that could not be classified as MSI, copy number high or *POLE* ultramutated.[17,19] Morphologically, the MSI and *POLE* groups demonstrated endometrioid features and the copy number low group comprised FIGO grade 1 and 2 endometrioid endometrial carcinomas. In contrast, the copy number high group were mainly composed of serous carcinomas and a minority of FIGO grade 3 endometrioid endometrial carcinomas. TCGA demonstrated a greater association of the molecular stratification than the morphological features with respect to the clinical outcome.[22] The Proactive molecular risk classifier for endometrial cancer or PROmISE group developed an algorithm to identify the different molecular categories whereby immunohistochemistry for PMS2 and MSH6 should be performed on endometrial biopsies; with those demonstrating an absence of staining being

considered as mismatch repair deficient.[19,23] Those biopsies demonstrating retention of staining are then subjected to POLE testing. Should MMR IHC testing not be available, then the tumours are categorized as "unclassifiable." Tumours that are tested for POLE are assessed as wild-type or are mutated. Should POLE testing be unavailable, the tumours are placed in the unclassifiable category. POLE tumours that are wild-type should subsequently undergo immunohistochemical p53 staining to assess for wild-type staining or mutations which may be either null-mutation or a missense mutation. In cases where p53 staining is not possible, the tumour is unclassifiable.[19,23] This study demonstrated results similar to the work of others whereby POLE mutated tumours demonstrated a better prognosis even in the presence of high-FIGO grade histology.[17,19,23] Classification systems such as this guide clinical decision making such as the need for post-operative adjuvant therapy or in the pre-operative period, may suggest if a lymph node dissection is required.[24] Identification of an MSH6 mutation by IHC with subsequent confirmation of gene mutation has an impact on patient management as patients with such mutations tend to develop tumours at a later age and usually have a relatively lower lifetime risk of tumour development in contrast to other Lynch syndrome mutations. Such patients may then only require tumour-preventative surgeries at later stages.[22]Recently, Cho and co-workers[25] have suggested that p53 and mismatch repair immunohistochemical analysis be performed together with POLE mutational analysis in order to classify endometrial carcinomas into TCGA's 4 groups.[25] Classification into these groups provides insight into the genetic make-up of endometrial carcinomas with possible therapeutic options, but molecular techniques such as sequencing or Polymerase Chain Reaction (PCR) for *POLE* are not accessible to pathologists globally. It is possible that with the development of POLE immunohistochemistry, that this may then provide a useful surrogate for the molecular test and may then facilitate many more anatomical pathology departments around the world to undertake such classification of tumours which will

ultimately be to the benefit of the patient by facilitating a more targeted treatment approach, tailored to the biology of the tumour.

Although there are histological features that may suggest Lynch syndrome, these fare modestly in comparison to molecular testing.[22] These histological features include tumoural lymphocytic infiltration, Crohn-like lymphocytic response, occurrence of the tumour in the lower uterine segment, lymphovascular invasion and poor tumour differentiation or tumour heterogeneity whereby two histologically different tumours are found adjacent to one another but are not intimately associated, with each comprising more than 10% of the tumour volume[22,26]

EEC has been identified as the most common histological subtype in patients with LS.[16,27–32] MSI has been identified in 3 out 4 cases of LS associated endometrial carcinoma and is also noted in up to 30% of sporadic endometrial tumours[30] Other studies however, have stated that there may be a slight increase in LS occurring in non-EEC tumours.[33]

To the best of the investigator's knowledge, studies on microsatellite instability on endometrioid endometrial carcinomas in the South African context have not been published in the scientific literature.

The present study will examine MSI by both immunohistochemistry as well as employ the polymerase chain reaction (PCR) which will allow a comparison of the sensitivity and specificity of the two. In addition, the study intends to ascertain the number of sporadic endometrial carcinomas and will also include a cohort of patients in whom screening modalities suggest LS.

CHAPTER 2

2.0. LITERATURE REVIEW

2.1. INTRODUCTION

Type I and II endometrial tumours demonstrate different molecular pathogenetic pathways as well as different microscopic features.[15–17,30,34] There are, however, occasions whereby histological distinction between the two groups is problematic and challenging. Type I tumours demonstrate microsatellite instability (MSI) and mutations in *PTEN, PIK3CA, KRAS* and *CTNNB1*. [2,16,27,35–37] Type II tumours however have *p53* mutations or *Her2/neu* amplifications.[16] In addition, this group of neoplasms often show loss of heterozygosity (LOH) on multiple chromosomes. [16,17,19,22,27,30,34–37]

2.2. PATHOLOGY OF ENDOMETRIAL CARCINOMAS

2.2.1. MACROSCOPY

Endometrial carcinomas do not have a specific gross appearance. They tend to be polypoid masses within the endometrial cavity (figure 1). Areas of haemorrhage and necrosis may be apparent. It is often difficult to macroscopically assess for the presence or absence of myometrial infiltration.[27,38]

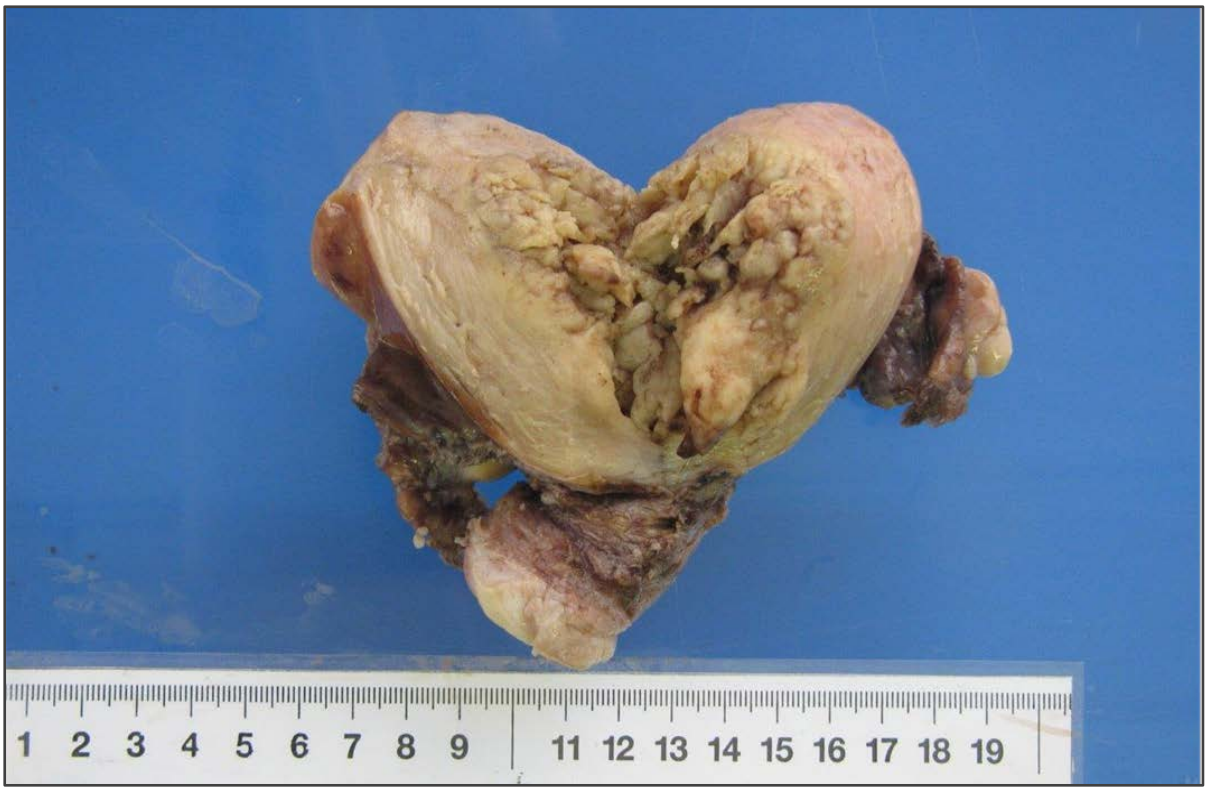

Figure 1. A uterus showing replacement of the endometrial cavity by a fungating tumour.

2.2.2. MICROSCOPY

2.2.2.1. ENDOMETRIOID ENDOMETRIAL CARCINOMA

Endometrioid endometrial carcinoma is the most common histopathological subtype of endometrial carcinoma.[27] The vast majority have an endometrioid appearance with variable degrees of differentiation. Metaplastic changes are often present. The prototypical well differentiated EEC is composed of endometrial glands that are arranged in a back-to-back growth pattern with little intervening stroma. The glands are of variable sizes and shapes and bare similarity to that of proliferative endometrium. The neoplastic glands may contain central necrotic debris within their lumina. A fifth of tumours contain foam cells. Architectural complexity, as demonstrated by a cribriform growth pattern may be seen in higher FIGO grade tumours. High FIGO grade tumours may also exhibit a solid sheet-like growth with little gland formation. Individual tumour cells lining glands are columnar in

shape and have round to ovoid nuclei. The neoplastic cells share a common apical border with neighbouring cells. The nuclei have mild to moderate atypia but may have varied degrees of pleomorphism depending on the grade of the tumour. The neoplastic cells may have prominent nucleoli. Mitotic activity is variable. Numerous apoptotic cells are often seen. The neoplastic glands often contain intracytoplasmic mucin which is demonstrable on a Periodic Acid-Schiff (PAS) stain.[27] Examples of endometrioid endometrial carcinomas are seen in figures 2 to 8.

EECs are graded according to their architecture.[11,27,38] FIGO grade 1 tumours are those that possess 5% or less of solid growth. FIGO grade 2 tumours contain between 6-50% solid growth. FIGO grade 3 tumours contain greater than 50% solid growth. Tumours that contain marked nuclear atypia in more than 50% of the tumour are considered to behave more aggressively and as such warrant an upgrade of the tumour by one FIGO grade.[38]

EECs may develop in a milieu of endometrial hyperplasia or in an endometrial polyp. However, these neoplasms may also arise in an atrophic endometrium.[27]

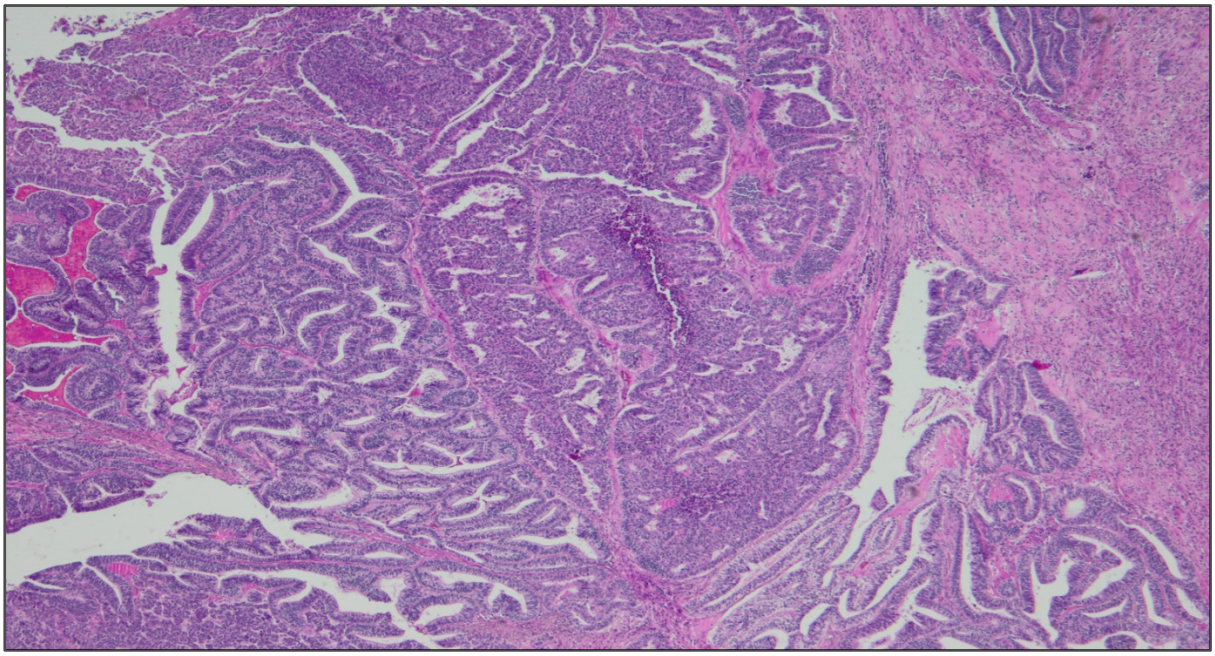

Figure 2. The back-to-back growth pattern of an endometrioid endometrial carcinoma is striking. H&E stained section. Original magnification: 40x

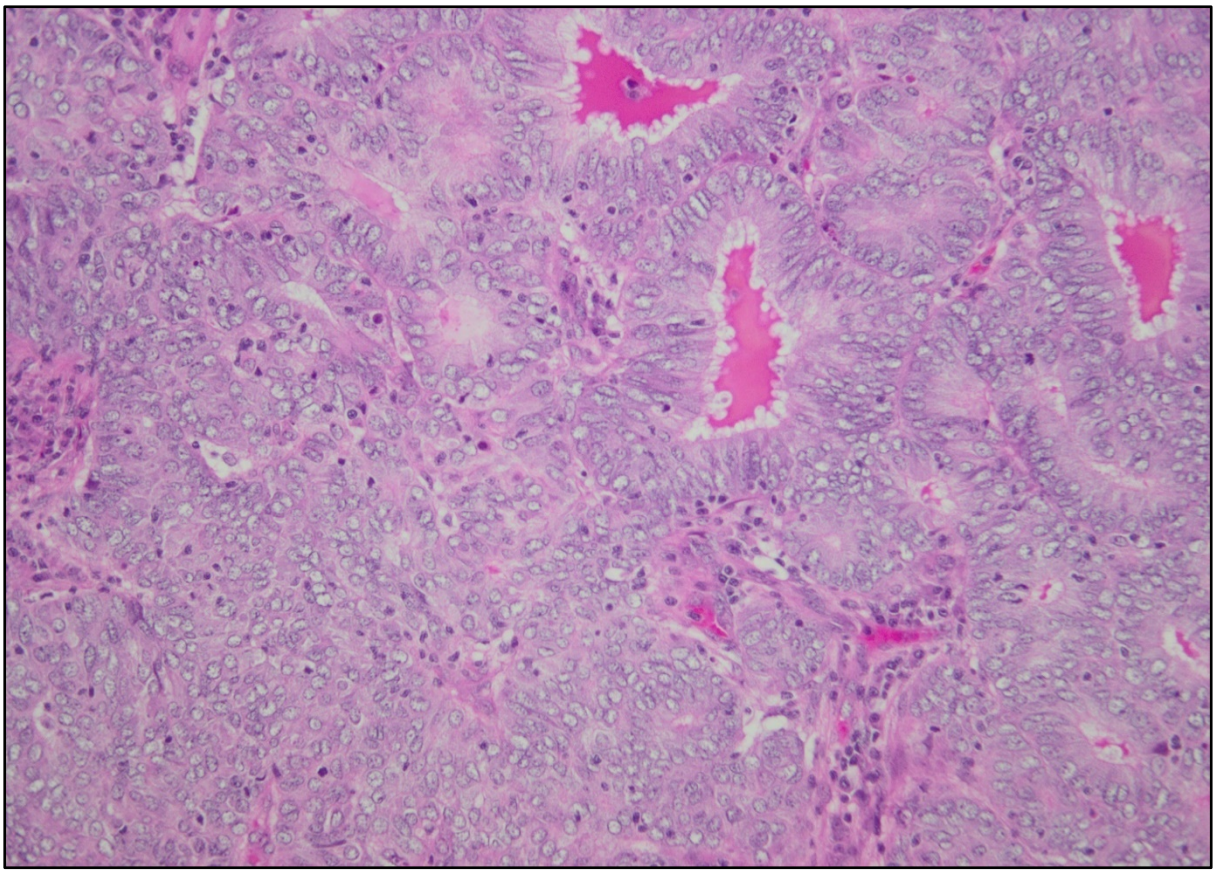

Figure 3. An endometrioid endometrial carcinoma. H&E stained section. Original magnification: 200x

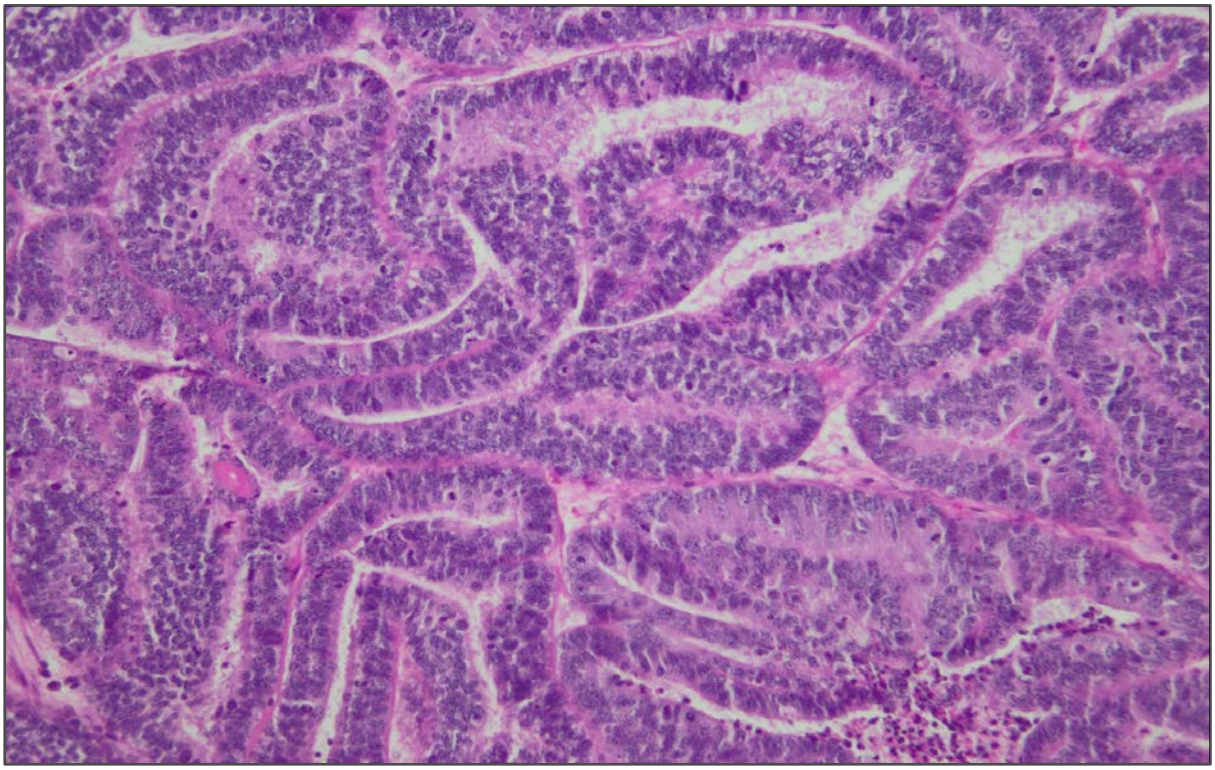

Figure 4. Marked glandular crowding with little intervening stroma is seen in this endometrial carcinoma. H&E stained section. Original magnification: 200x

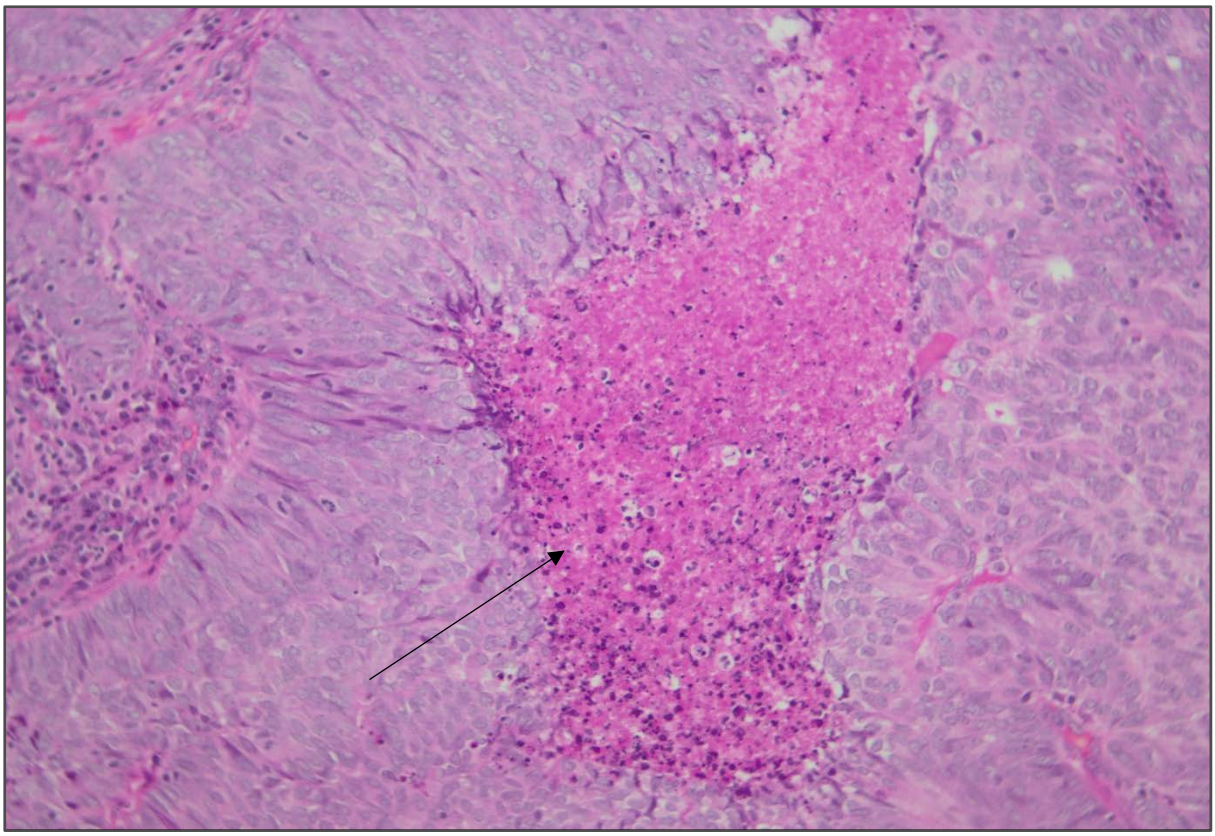

Figure 5. A focus of central necrosis (arrow) is evident in this tumour. H&E stained section. Original magnification: 200x

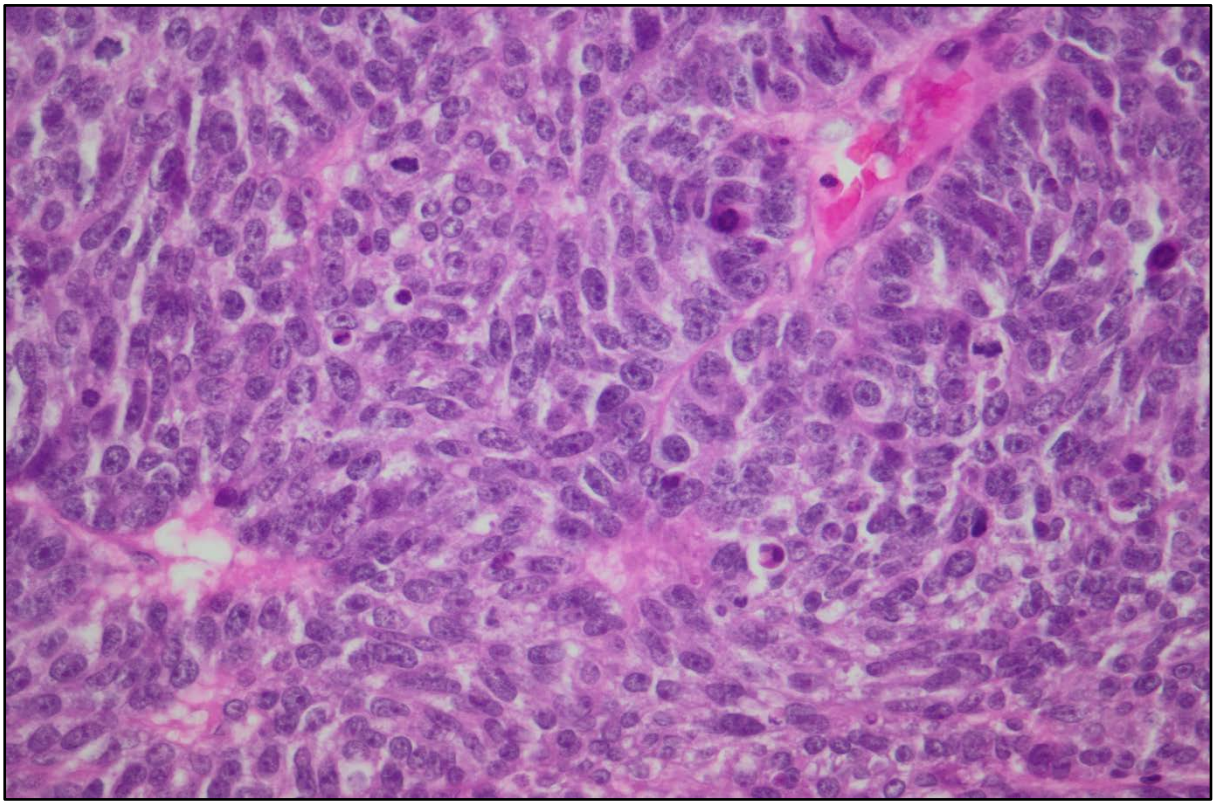

Figure 6. Nuclear pleomorphism is observed in this tumour. H&E stained section. Original magnification: 400x

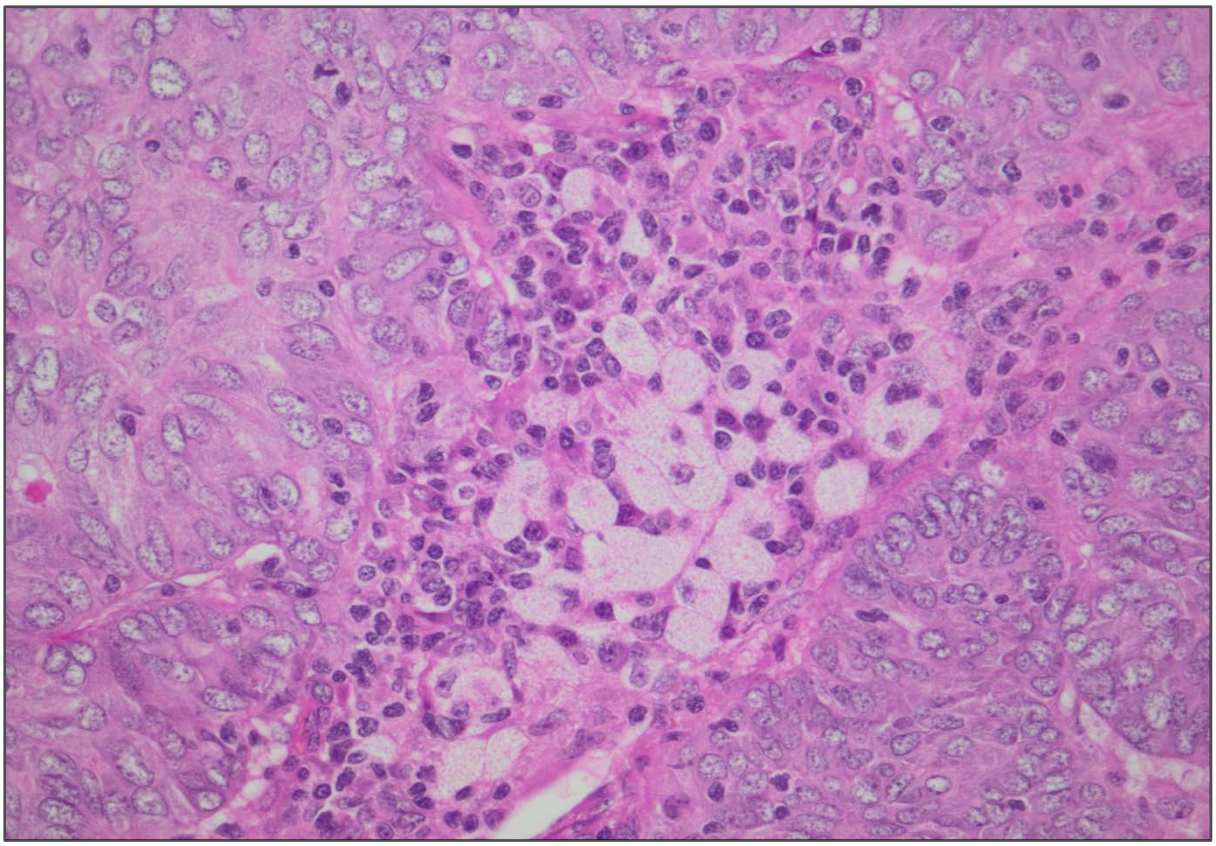

Figure 7. An area containing foam cells is identified. H&E stained section. Original magnification: 400x

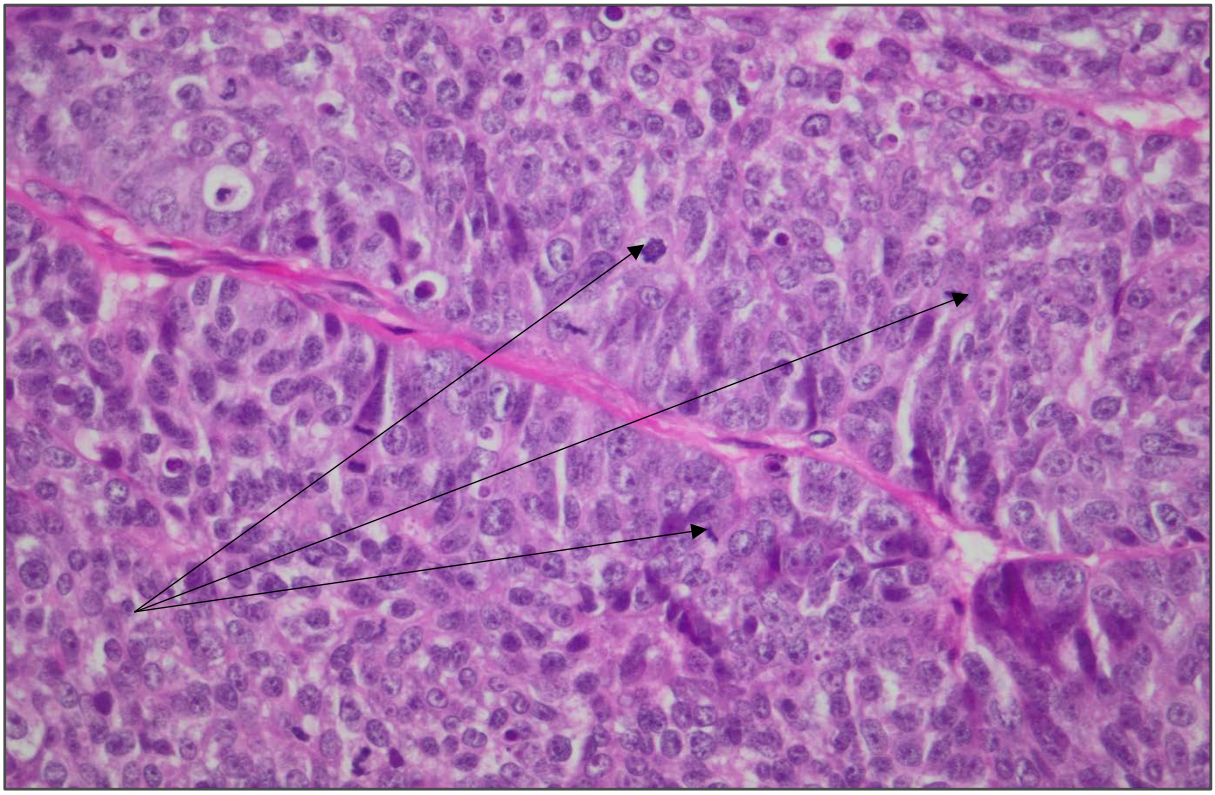

Figure 8. Brisk mitotic activity is demonstrated (arrows) in this endometrial carcinoma. H&E stained section. Original magnification: 400x

2.2.2.2. MUCINOUS CARCINOMA

This is a rare tumour accounting for less than 10% of endometrial carcinomas. Similar to EECs, these tumours develop in an estrogen rich environment and are considered a variant of endometrioid endometrial carcinoma. More than half of the tumour must consist of mucinous cells for such a diagnosis to be rendered. The tumours are composed of glands lined by columnar epithelium with abundant apical mucin that may be pale to slightly basophilic and may be granular. Tumour nuclei show minimal atypia. Mitoses are infrequent. In general, these tumours are well differentiated and have a reasonably good outcome.[38] Figures 9 and 10 show mucinous carcinomas.

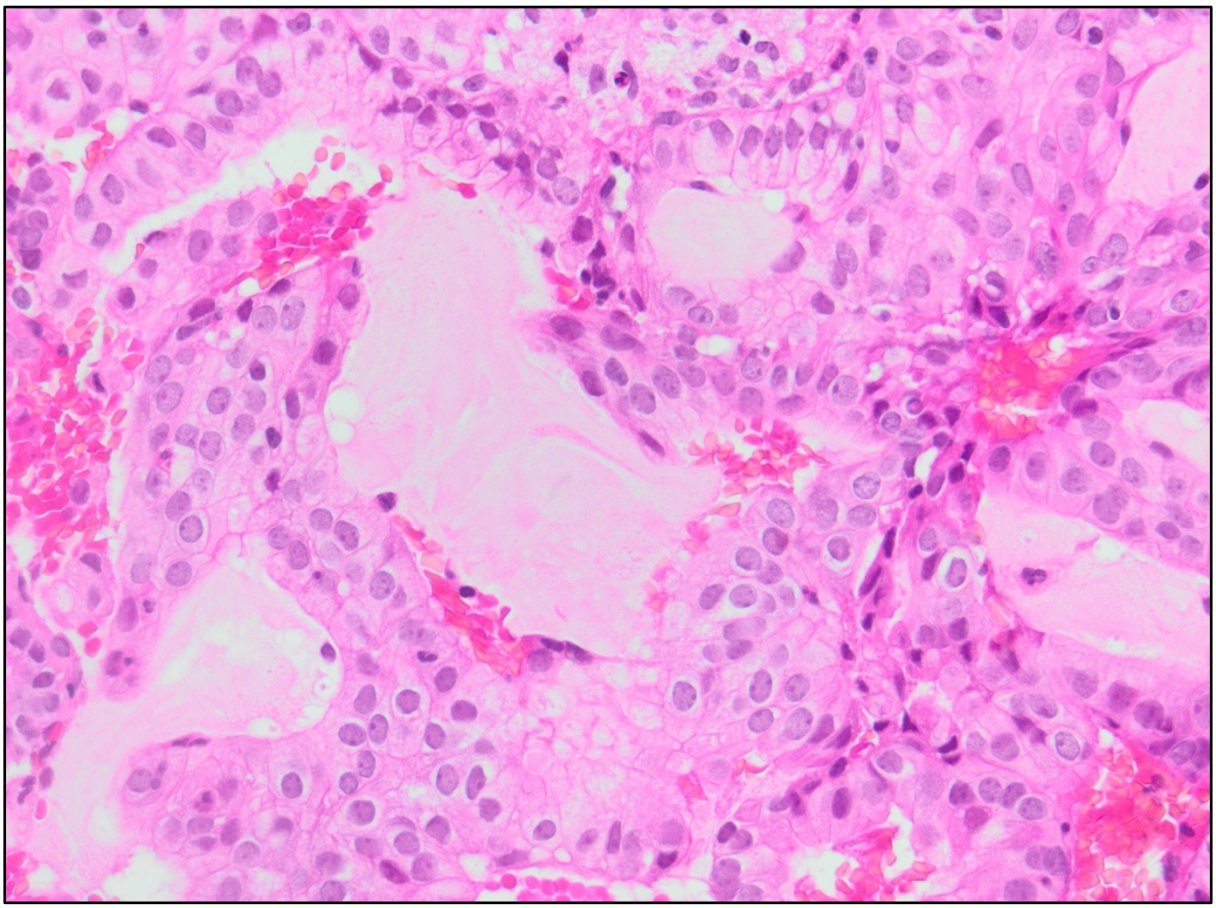

Figure 9. This photomicrograph demonstrates columnar cells with clear apical mucin in a mucinous carcinoma. 2μm H&E stained section. Original magnification: 400x

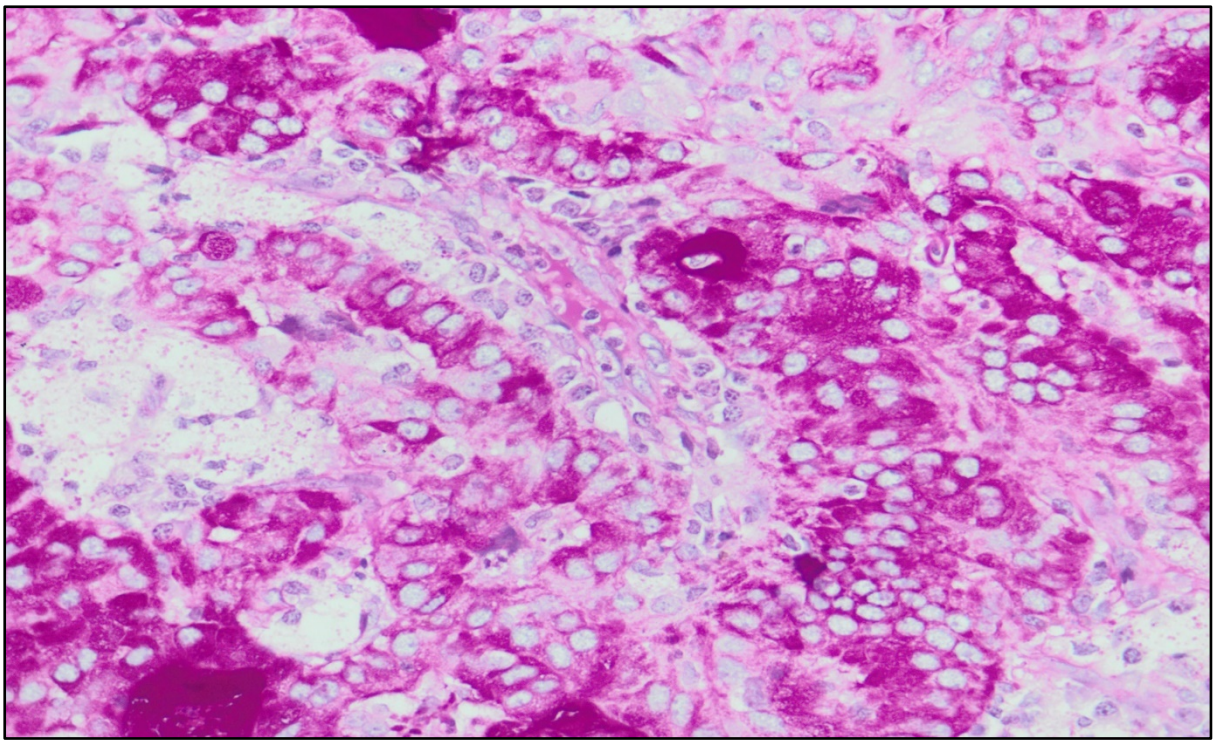

Figure 10. This photomicrograph demonstrates intracytoplasmic mucin within columnar cells of a mucinous carcinoma. 2µm Periodic Acid-Schiff Diastase stained section. Original magnification: 400x

2.2.2.3. SEROUS CARCINOMA

This tumour is the prototypic Type II neoplasm and is histologically characterized by a papillary architecture that may exhibit marked complexity.[27,38] In addition, there may be solid areas or prominent glandular regions. The papillae contain true fibrovascular cores and papillae may be long or short, slender or broad. The lining epithelial cells have a hobnail appearance as a result of apical cytoplasm containing nuclei protruding into a lumen. The nuclei may be vesicular with prominent macronucleoli or may show marked hyperchromasia. Brisk mitotic activity is the norm with such tumours. Atypical mitoses may be apparent.[27,38]

Serous carcinomas in contradistinction to EECs, do not arise in a hyperestrogenic state.[34,38] They may develop in the background of an atrophic endometrium or from serous intraepithelial carcinoma (SEIC) on a polyp.[27,38] Examples of serous carcinoma are presented in figures 11 and 12.

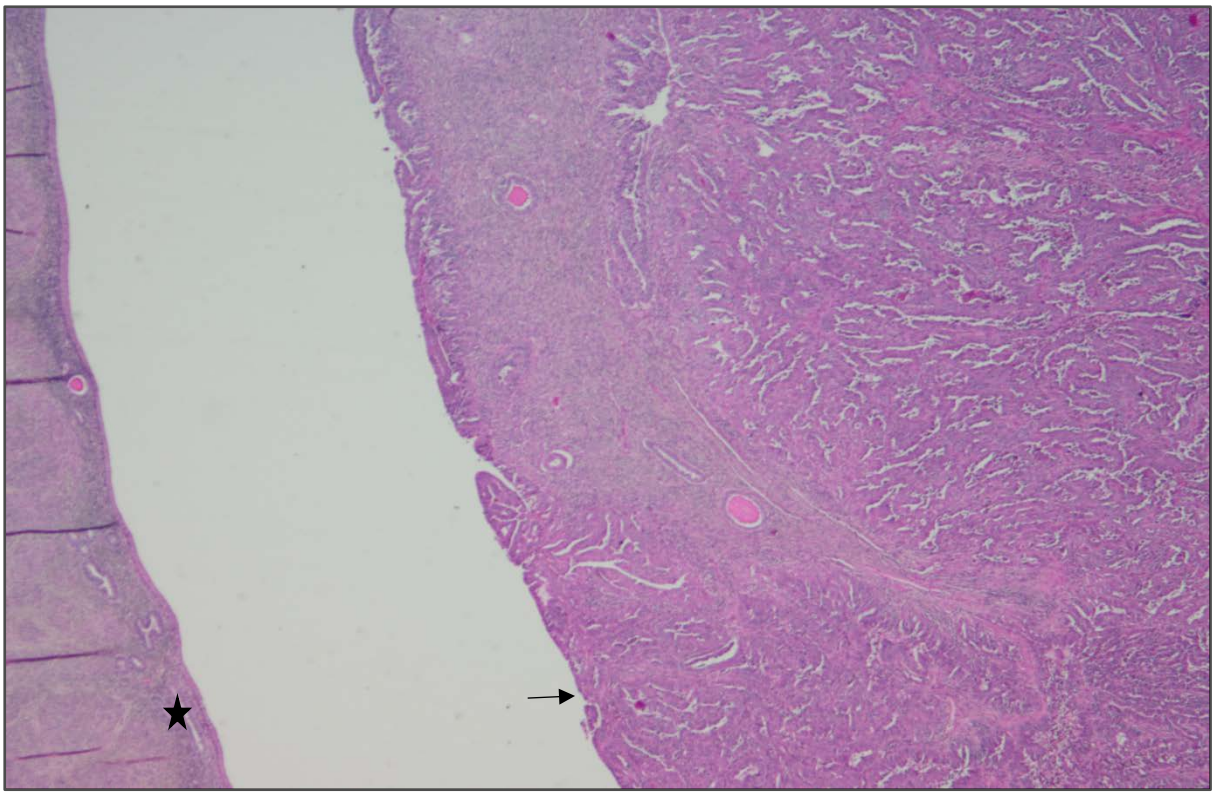

Figure 11. A serous carcinoma is identified on the right arising from intraepithelial carcinoma (arrow). Atrophic endometrium is noted on the left (star). 2µm H&E stained section. Original magnification: 40x

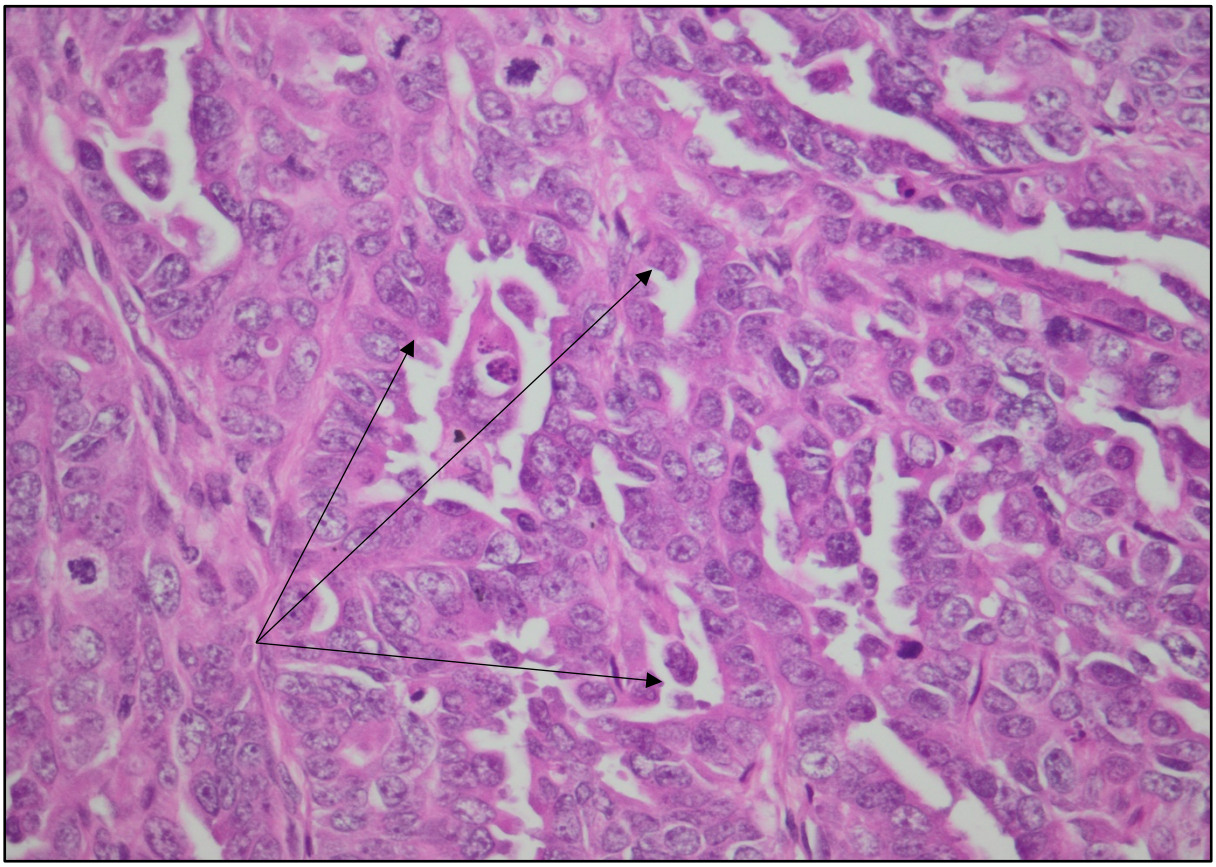

Figure 12. A high-power view of serous carcinoma in which numerous hobnailed cells are identified (arrows). 2µm H&E stained section. Original magnification: 400x

2.2.2.4. CLEAR CELL CARCINOMA

Clear cell carcinoma is an uncommon Type II tumour that has a tubulocystic, papillary or solid architecture (figure 13). The tumour consists of polygonal to hobnailed cells which display marked cytoplasmic clearing. These cells contain copious amounts of glycogen which is demonstrable on a PAS stain and is digested with a PAS-Diastase stain. The nuclei may be variably pleomorphic but there is at least focal high-FIGO grade atypia. Numerous mitotic figures may be identified. Hyaline bodies which are bright eosinophilic extracellular droplets are frequently encountered.[27,38]

These tumours tend to develop in a background of an endometrial polyp or endometrial atrophy.[27,38]

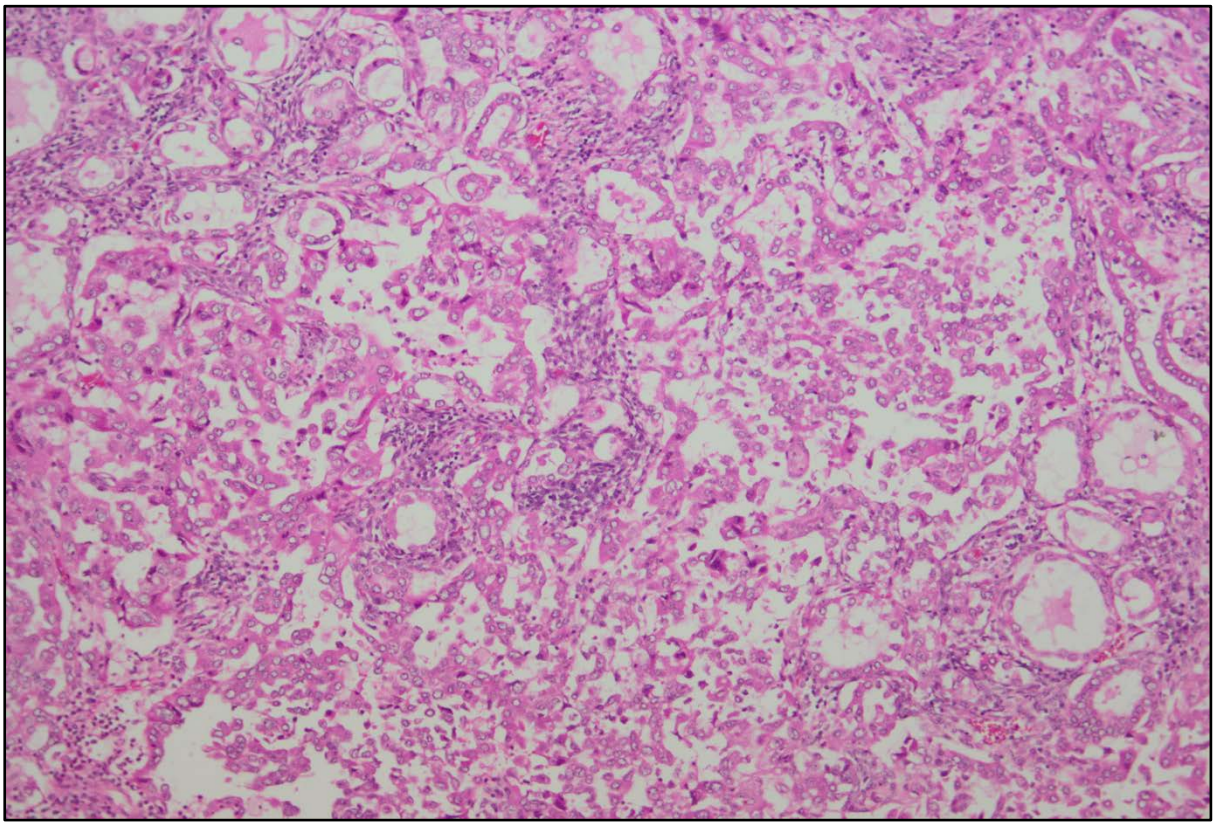

Figure 13. The tubulocystic architecture is well represented in this clear cell carcinoma. 2µm H&E stained section. Original magnification: 200x

2.3. MICROSATELLITE INSTABILITY

MSI is one of the most frequently identified molecular abnormalities in Type I neoplasms and has been detected in the majority of Lynch syndrome or Hereditary Non-Polyposis Colorectal Carcinoma (HNPCC) associated neoplasms.[10,17,18,30,35] Furthermore, up to 30% of sporadic tumours have been found to be microsatellite unstable.[17,38]

Microsatellites are one to five base pair sequences of DNA that are repeated several times. These short-tandem repeats are highly susceptible to changes in mismatch repair and in humans, repeats of CA are the most commonly detected microsatellite.[18,36,37] A fully functional "DNA mismatch repair system (MMR)" identifies replication errors, such as "insertion-deletion loops" as well as "base-base mismatches" that may occur on microsatellite areas and corrects these. [39–43] Coding and non-coding regions of DNA for example, centromeres and telomeres, may have microsatellites.[17,39] In coding regions, single-base mismatches cause point mutations which may be nonsense or missense. Mutations may be silent or deleterious.[41] Deleterious mutations, at a protein expression level, result in negative staining of tumour nuclei immunohistochemically.[41] These deleterious or pathological mutations may be either non-truncating or truncating and it is the latter that may result in "loss of expression" or to expression of an MMR protein that may be easily degraded. The non-truncating mutations have point mutations which result in an amino acid substitution within an MMR protein which then causes a change in its stability or its efficacy. Insertion-deletion loops that are not recognised and corrected for, can result in frameshift mutations.[41] Nonsense mutations may result in extensive microsatellite instability and may result in the onset of neoplasia at an early age. Missense mutations, however, tend to result in varied clinical presentations. Microsatellites occurring in coding sequences are involved in tumourigenesis, as seen in EECs.[17,39,41] Up to 30% of sporadic, non-hereditary cases of

endometrial carcinoma are microsatellite unstable.[30,35] Microsatellite instability is found predominantly in endometrioid endometrial carcinomas. [38,44]

Eukaryotes and prokaryotes have a mismatch repair system.[41] Eukaryotic nomenclature is akin to that utilized in the prokaryote *Escherichia coli*.[41,45]Mutational or "Mut" proteins in bacteria are termed MutS, MutH and MutL. The mutated eukaryotic genes are referred to as "*Mut*". *MutS* homologs include *MSH6, MSH2* and *MSH3*. *MutL* homologs comprise *MLH1*, *PMS1* and *PMS2* (Post-Meiotic Segregation proteins).[41,42] In humans, the letter "h" is the prefix assigned to the genes and proteins e.g. *hMSH6*.[41] A minimum of seven MMR genes have been recognized in humans which include *MLH1, MLH3, MSH6, MSH3, MSH2, PMS1* and *PMS2*.[46] To date, *MutH* homologues have not been identified in humans.[46] MMR genes are divided into major and minor groups.[42] The major genes include *MLH1* and *MSH2* and the minor genes comprise *PMS2, MSH3, MSH6* and others. There is interplay between the major and minor genes which work as heterodimeric complexes; with the minor genes assisting in stabilization of major genes, whilst the minor genes are reliant on their major gene "binding partners" for protein expression.[42,43]

MSH2 and *MSH6* bind together forming a heterodimer, "*MutS*α" which recognizes mismatches in base pairs.[46] It is involved in single base pair substitution repair in addition to restoration of single base pair insertion-deletion mutations. Furthermore, it instigates apoptosis.[41,45] *MSH2* and *MSH3* form a complex, *MutS*β, that detects and repairs short deletions or insertions. [41,46] *MSH2* is necessary for the function of both these complexes, but *MSH3* and *MSH6* have functional overlap and are thus considered somewhat redundant.[46] *MLH1* and *PMS2* combine to form the *MutL*α complex which combines with *MutS* complexes to excise and reconstitute any unusual or abnormal DNA.[41] MLH1, MSH2, PMS2 and MSH6 proteins together with the proliferating cell nuclear antigen (PCNA), DNA

polymerase (Polδ or Polε), exonuclease-1 (EXO-1), a ligase and replicating factors RFC and RPA, act to repair mismatch points on the daughter strand of DNA.[46] In brief, the predominant phases in MMR activity includes the following:

1. Identification of mismatch
2. Inclusion of replicating factors, enzymes and nucleotides
3. DNA strand separation
4. Removal of the abnormal daughter strand
5. Re-synthesis of DNA

If any of the four proteins, namely MLH1, PMS2, MHS2 and MSH6, are functionally inactive, mismatch repair will not occur.[46]

In situations where there are extensive genomic abnormalities that are not amenable to correction by the MMR proteins, MMR proteins induce apoptosis to ensure that genomic material is conserved.[41] Mutations in MMR proteins result in less apoptosis thus favouring cellular immortality and thus neoplasia.[41]

2.4. ADDITIONAL FUNCTIONS OF MMR PROTEINS

MMR proteins have been shown to have additional functions in tumourigenesis.[32]These newly identified functions include:

1. Signalling of DNA damage from exogenous toxins. This results from interplay between *p53, p63* and *p73* and the *MutL*α and *MutS*α complex. In response to the noxious stimulus, MLH1 then operates together with an element of the "BRCA1 associated surveillance complex" known as MRE11, to facilitate cell cycle regulation and apoptosis.
2. Inhibition of "reparative recombination of non-identical sequences". [46]

3. Facilitation of meiotic cross-over. MMR proteins prevent anomalies caused by mismatches, deletions and insertions during chromosome recombination. The MMR proteins involved in this process include MLH1, MLH3 and PMS2.
4. Somatic hypermutation with resultant immunoglobulin divergence and expansion. This process is orchestrated by the *MutSα-MutLα* complex in concert with proteins Polμ ("DNA polymerase 'error prone'") and "activation induced cytidine deaminase" or (AID)[46] Deficiencies of MutSα have been found to correlate with T-cell neoplastic change.[46]
5. An increase in triple repeat sequences such as CGG and CTG, that are responsible for certain neurodegenerative disorders such as Fragile X-Syndrome and Huntington's disease. The exact means by which this is achieved has, to date, not been fully elucidated. It is thought that whilst *MutSβ* combines these nucleotide repeats, repair is not possible due to "looping conformations" of these expanses. It is also thought that the dysfunction of *MutSβ* may protect against the "intergenerational instability" that results from the triple repeat sequence extensions that underlie the familial anticipation of the disease.[46]
6. Alteration of micro-RNA's. *MutLα* has been found to interact and bind with primary pri-miRNA's, to convert these to precursor or pre-miRNA's. This ultimately effects translation of target mRNA's.[46]

These newly discovered roles suggest that MMR deficiency results in resistance of repair in addition to affecting apoptosis in response to DNA damage. These are a consequence of compromised MMR functionality together with diminished activity of other cellular mechanisms.[46] Thus whilst MMR proteins are responsible for DNA repair, it may be seen that they also play a part in other mechanism of tumourigenesis.

2.5. LYNCH SYNDROME

Lynch syndrome (LS), which is also known as Hereditary Non-Polyposis Colorectal Carcinoma, (HNPCC) is an autosomal dominant syndrome caused by mutations in the DNA MMR system that predisposes affected individuals to colorectal, endometrial, ovarian, gastric, pancreaticobiliary and urothelial carcinomas in addition to brain and skin tumours.[41–43,45–48] Lynch syndrome is one of the most common inherited tumour syndromes in humans.[48] Commensurate with Knudson's two-hit hypothesis, in order for LS to clinically manifest, both alleles must be inactivated.[48] LS carriers have one wild-type allele and one mutated, germline allele (first hit) in any of the mismatch repair genes. [33] An insult (the second hit) to the wild-type allele in LS carriers by means of mutation, hypermethylation or loss of heterozygosity results in a breakdown of the DNA mismatch repair machinery, with subsequent escalation of microsatellite instability that ensues which raises the odds in favour of malignant change.[33]Prior to tumorigenesis occurring, sporadic tumours are afflicted by a hit in both alleles of somatic neoplastic progenitor cells. It has been suggested that either hit or both hits may be inherited or may be because of epigenetic events. Both hits tend to be genetic in LS associated neoplasms and for the first hit, may be due to point mutations or can be ascribed to nucleotide changes. The second hit then results from gene changes or loss of the second or wild-type allele.[48] An epimutation, which is abnormal inhibition of an active gene occurs as the first hit. This is followed by the second hit by means of promoter hypermethylation. In contrast to mutational inactivation, promoter hypermethylation is a more common form of gene inactivation.[48,49] It has been shown that colorectal carcinomas that are sporadic in nature, are a result of promoter hypermethylation of the MLH1 promoter region.[48]

Up to 50% of females with LS may present with endometrial carcinoma as their first neoplasm and are at risk of synchronous or metachronous tumours.[43] Identification of patients

with LS is of importance as this allows for continuous surveillance of various possible tumours in the affected individual; and provides the prospect of genetic counselling and possible genetic testing together with surveillance and investigation for their immediate relatives.[31,43] The Amsterdam Criteria I and II and Bethesda Guidelines[26,50] may predict the possibility of an individual having LS, but these require extensive knowledge from patients with regard to their own malignancies; in addition to having knowledge of tumours diagnosed in family members. As such, these may not be ideal criteria to identify possible LS patients.

2.5.1. THE HISTORY OF LYNCH SYNDROME

LS was first identified in a family in 1913.[42,48] Thereafter, a patient was suspected of having Lynch syndrome based on a positive family history and clinical presentation. Eighty years later the first locus that predisposed to LS was identified; with numerous mutations now having been identified. Recently hypermethylation, a common epigenetic event has been instrumental in tumourigenesis.[48]

Hereditary Non-Polyposis Colorectal Carcinoma was the name provided to distinguish this tumour syndrome from Familial Adenomatous Polyposis (FAP) syndrome, in which there is an early age of onset and the development of numerous colonic polyps.[51] The presence of a brain tumour in the form of glioblastoma together with colorectal carcinoma was termed Turcot Syndrome; whereas sebaceous neoplasms and colorectal carcinomas were called Muir-Torre syndrome.[48,51]

The Amsterdam criteria was the first set of criteria for HNPCC which was developed in 1991, by researchers and clinicians. According to this set of criteria, HNPCC was identified in individuals who had three close family members in two or more generations with colon carcinoma, with a minimum of one of these diagnoses having been rendered below the age of 50 years but did not have FAP. This, with at least one of these diagnoses having been made

under the age of 50.[51] The Amsterdam I criteria was recorded to have a sensitivity of 60% and specificity of 70%. [52] Extracolonic LS tumours were soon identified and were included in the Amsterdam II criteria in 1999.[51] A sensitivity of 72% and specificity of 78% was documented for use of the Amsterdam II criteria. Despite the Amsterdam II criteria including non-colonic tumours such as endometrial carcinoma, this set of criteria did not consider patients who had their sentinel malignancy being in the female genital tract. Furthermore, the possibility of small family bloodlines were not given due consideration.[52] Thus, the Amsterdam II criteria was not entirely useful to individuals who were diagnosed with malignancies of the female genital tract.[52]

However, it was noted that the Amsterdam criteria were unable to detect many patients with LS; at which time the Bethesda Guidelines were developed in an endeavour to detect cases of LS that would have been missed by the Amsterdam I and II criteria. (Table 1)[26,46,50,53–55] The Bethesda and Amsterdam I and II criteria were intended to detect patients with colonic tumours, thus making assessment of the efficacy of such criteria for female genital tract malignancies, difficuly.[52] The current Bethesda guidelines are the best clinical tool used to assess the possibility of an individual having a hereditary tumour syndrome.[52]However, the Bethesda Guidelines have poor sensitivities in cases where patients have endometrial carcinomas as their main tumour, and in cases where patient family bloodlines are limited.[52]

Table 1. Amsterdam I and II Criteria and Revised Bethesda Guidelines. [53,54]

Amsterdam I Criteria
1. Histologically diagnosed colorectal carcinoma in a minimum of three relatives, one of whom is a first-degree relation of the others, once Familial Adenomatous Polyposis (FAP) has been excluded.
2. At least two generations have colorectal carcinoma
3. A minimum of one of the relations must have been diagnosed with colorectal carcinoma under the age of 50 years
Amsterdam II Criteria
1. A minimum of three relatives with Hereditary nonpolyposis colorectal carcinoma associated carcinomas such as colorectal carcinomas, endometrial carcinoma, tumours of the ovary, small bowel, ureter or renal pelvis, stomach,

pancreaticobiliary tract, skin or brain, once Familial Adenomatous Polyposis (FAP) has been excluded.
2. A minimum of two generations are involved
3. A minimum of one of the syndrome associated neoplasms is diagnosed before the age of 50 years
Revised Bethesda Guidelines
1. Colorectal carcinoma diagnosed under the age of 50
2. Synchronous or metachronous colorectal carcinoma or other Lynch syndrome associated neoplasm irrespective of age
3. Colorectal carcinoma with MSI-high histopathological features such as Crohn-like lymphocyte response, mucinous or signet ring cell morphology under the age of 50 years
4. Colorectal carcinoma in at least one first degree relation under the age of 50 years
5. Colorectal carcinoma diagnosed in at least two first or second-generation relation with HNPCC associated tumours irrespective of age.

Lynch syndrome has been named after Dr. Henry T. Lynch who diagnosed many patients with this tumour syndrome. Lynch syndrome is currently the term used in preference of HNPCC; as HNPCC does not include all non-colonic tumours and importantly, LS is diagnosed on the basis of a molecular mutation in any of the mismatch repair genes.[50,51,53] In contrast, HNPCC is a clinical diagnosis rendered to those fulfilling Amsterdam I or II criteria.[54] Furthermore, mismatch repair defects do not underlie all family history cases of HNPCC.[51]

2.5.2. LYNCH SYNDROME SCREENING TESTS

2.5.2.1. IMMUNOHISTOCHEMICAL MISMATCH REPAIR

Undertaking immunohistochemical testing on formalin fixed paraffin embedded (FFPE) tissue is a fairly easy first step in the possible identification of patients with LS. This method employs antibodies for the MMR proteins MLH1, MSH2, PMS2 and MSH6. In about 1996, the first of these antibodies that was made available for commercial use, was MSH2.[56] Immunohistochemistry is a test that is accessible to many histopathologists and allows for a rapid, relatively economical route of assessing possible MMR defects. [54]It must be borne in mind that this is a screening test and is not diagnostic of LS. It has been shown that up to

33% of endometrial carcinomas are negative by immunohistochemistry for one of the MMR proteins.[26] Only completely negative staining of tumour nuclei in the presence of valid internal controls such as lymphocytes, stromal cells and endothelial cells is regarded as a negative result.[26] As such, retained mismatch repair is diagnosed even if there is faint, focal positive nuclear signalling.

Whilst IHC is fairly consistent in identifying mutations that cause truncation of a protein or protein degradation, it is unable to discriminate abnormal proteins that come about from missense mutations, versus those arising from wild-type peptides.[56] Many MSH2 mutations are truncating and as such MSH2 mutant tumours are expected to be negative for this immunohistochemical stain. Nevertheless approximately 33% of MLH1 mutations are missense mutations that may produce proteins that are functionally inoperative but maintain their antigenicity. As such, a false positive stain is identified by immunohistochemistry. In addition, there may also be false retention of staining in cases of MLH1 large in-frame deletions and protein-truncating deletions. The process by which this results, is not entirely known at present. [56]Another source of false positive staining may be ascribed to the second hit that inactivates the normal allele. The second hit may produce a protein that is not functional but is able to bind to the immunohistochemical antibody, which thus results in a positive nuclear signal. This is supported by the finding of individuals who have identical MLH1 mutations but have varied immunohistochemical staining patterns.[56]

Interaction of the four MMR genes and proteins is exemplified in tumours that lose MLH1 expression as there is simultaneous loss of PMS2 and likewise, a loss of MSH2 is associated with a loss of MSH6. [42,43,56] This is due to *MLH1* and *MSH2* being the major, obligatory partner of the respective complexes. [56] Abnormalities in either *MLH1* or *MSH2* may end in degradation of the protein dimer with subsequent loss of the major or obligatory component as well as the minor component.[56] An exception to this is seen in cases of germline mutations

of *PMS2* or *MSH6* which result in loss of expression of only the affected gene without concomitant loss of MLH1 or MSH2.[42,43] This is likely due to the fact that functions of these minor, secondary proteins may be counteracted for by other proteins such as PMS1, MLH3 and MSH3.[47,56] As such, loss of only PMS2 or MSH6 suggests an underlying germline mutation in the respective gene.[43] However, it has been suggested by Kato *et al*[47] that occasional *MLH1* mutations may only result in loss of PMS2 expression immunohistochemically with retention of MLH1 staining.[47] It may then be inferred that in cases of loss of only PMS2 that the possibility of abnormalities of *MLH1* may not be entirely excluded.[47] Shia describes the potential for seven or more mononucleotide repeats occurring in the coding sequence of *PMS2*, *MSH6* and *MSH2* which may endure additional mutations that can then bring about protein loss that is different to the usual patterns described previously.[56] Such occurrences are however not common. An example of this would be unexpected negative staining of PMS2 IHC in a patient with *MSH2* mutation. This would result in negative staining of both MSH2 and PMS2. The rarity of this may be explained by the fact that secondary mutations tend not to occur during tumourigenesis but instead may take a foothold in developed neoplasms.[56]

2.5.2.2. MICROSATELLITE INSTABILITY BY POLYMERASE CHAIN REACTION (PCR)

Assessment of microsatellite instability by PCR warrants examination of both neoplastic and non-neoplastic tissue from an individual. As such, paraffin embedded tissue blocks containing areas of tumour and normal tissue are selected by a histopathologist prior to microdissection.[33] There are currently a number of markers that may be utilised in MSI testing. A National Cancer Institute sponsored workshop held in 1997 recommended the Bethesda panel which comprises five microsatellite loci. These loci include BAT25 and BAT26 which are mononucleotide repeats as well as the following dinucleotide repeats: D17S250, D5S346 and D2S123.[33] In 2002, a panel consisting of five mononucleotide repeats

namely, NR-21. NR-22, NR-24, BAT25 and BAT26 was introduced.[33,57]An additional panel containing five mononucleotide repeats (MONO-27, NR-21, NR-24, BAT25 and BAT26) is presently also available for use.[33] It is believed that the mononucleotide markers are superior to dinucleotide markers for identification of MSI due to greater sensitivity[26,33] Furthermore, it has been suggested that use of mononucleotides may negate the need for normal tissue comparison. Nevertheless, most laboratories continue to utilise both tumour tissue and normal tissue to compensate for the possibility of polymorphisms in an individual.[33]

The deletion or insertion of repeated nucleotides with resultant shortening or lengthening of DNA sequences typify microsatellite instability.[58] After PCR, the fragment sizes are examined using gel electrophoresis. Varied sizes of tumour PCR products are compared to the patient's normal tissue.[32] Tumours showing an allelic shift in one of the markers out of a panel of (20%) are construed as having a low frequency of microsatellite instability (MSI-L); whereas an allelic shift in two or more (or 40%,) are considered as having a high frequency of microsatellite instability or MSI-H.[26,47,57,58] Tumours showing no allelic shifts are viewed as being microsatellite stable (MSS).[26,33,57,58]

MSI PCR assesses functionality of MMR genes. Therefore, MSI PCR may detect MSI cases which are regarded as mismatch repair proficient and demonstrate nuclear positivity on an immunohistochemical protein level but are in fact due to missense mutations in MMR genes. Thus, such a mutation would not be detected immunohistochemically but can be identified by PCR.[59]

2.5.2.3. MLH1 PROMOTER HYPERMETHYLATION

Epigenetic events encompass DNA methylation, histone alteration, remodelling of microRNA and chromatin.[48] During methylation, a methyl group is added to cytosine nucleotides in CpG islands, which are foci rich in CpGs, within the gene promoter region

such as for MLH1.[32,60] The methyl groups attached to cytosine nucleotides spread into the DNA strands' major groove thereby inhibiting DNA transcription by preventing the annealing of transcription factors to DNA.[32] Chromatin compaction and resultant suppression of gene expression is due to methyl groups that attract proteins which bind methyl groups to them.[32]

Three quarters of microsatellite unstable endometrial carcinomas have been shown to arise from hypermethylation of the *MLH1* promoter region with silencing of the *MLH1* gene.[26] A combination of IHC MMR testing, MSI by PCR and hypermethylation studies may indicate the possibility of LS. Deficient MLH1 or PMS2 staining on immunohistochemistry with concurrent MSI-H by PCR and negativity for *MLH1* promoter methylation suggest the possibility of LS.[26] In contrast, MLH1 or PMS2 negativity on immunohistochemistry, together with MSI-H by PCR and positivity for *MLH1* hypermethylation allude to the patient having a sporadic tumour and not Lynch syndrome.[26]

2.5.2.4. BRAF MUTATIONS

In addition to *MLH1* promoter hypermethylation, mutations in *BRAF* V600E are in support of sporadic occurrence of a colorectal tumour. [61] BRAF is one of three subtypes of the RAF proteins that have serine-threonine kinase activity and are implicated in mitogenesis. [62,63] BRAF forms part of the RAS/RAF/MAP/ERK pathway.[62] RAF activity is usually kept in check by RAS. Mutations in BRAF however, result in constitutive activation of this pathway, with unbridled proliferation of cells, and eventual neoplasia, as is seen in colorectal carcinoma.[62,63] BRAF and KRAS mutations have been found to be mutually exclusive. The exons that have been found to harbour mutations in *BRAF* are exon 15 codon V600E and exon 11.[62] It has been demonstrated that mutations of the V600E codon result in ten times the tyrosine kinase activity compared to wild-type BRAF.[63] Up to one third of MSI sporadically

occurring colorectal carcinomas and three-quarters of colorectal carcinomas bearing mutations in the promoter region of *MLH1* have been shown to have BRAF V600E mutations.[63] As such, BRAF has been suggested as the "target gene of abnormal MMR".[63] The role of *BRAF* mutations has however, not been well established in endometrial carcinomas and in the studies that have been performed, conflicting results have been obtained with Feng[62] having reported *BRAF* mutations in up to 21% of endometrial carcinomas whilst a review by Metcalf *et al*[61] reports *BRAF* mutations occurring in 4% of endometrial carcinomas. Stewart however, reports *BRAF* mutations occurring in just 1% of endometrial carcinomas.[43] Therefore it has not been entirely established whether mutations in *BRAF* play a role in tumourigenesis of endometrial carcinoma.[63]

2.5.2.5. LYNCH SYNDROME DIAGNOSIS

MMR IHC and MSI by PCR, hypermethylation studies and BRAF mutational assessment are screening tools used in the work-up of Lynch syndrome. LS is diagnosed upon identification of germline mutations in one of the mismatch repair genes by sequencing.[33,53]

2.5.3. CLINICAL CORRELATIONS

LS is autosomal dominant but with variable expression.[51] However, germline mutations in affected MMR genes have been linked and associated with certain phenotypes (table 2).[64] The clinical phenotype is dependent upon the amount of MMR gene and protein such that heterozygosity for an MMR gene may give rise to Lynch syndrome, Homozygosity however, culminates in Constitutional Mismatch Repair Deficiency Syndrome or CMMR-D. This is a recessive, uncommon condition occurring in individuals who inherit abnormal alleles from both parents.[48] The sex of a person and the specific gene affected impact on the type of Lynch syndrome associated tumour that may arise.[51]There is a risk of up to 60% of developing endometrial carcinoma as the first tumour in females afflicted by Lynch syndrome and these

individuals have a greater lifetime probability of developing endometrial carcinoma in contrast to colonic carcinoma.[32] *PMS2* and *MSH6* genes have decreased penetration relative to the other genes, but have been identified as being the cause of more LS cases than detected previously.[51]

The majority (70 - 90%) of LS cases arise from mutations in *MLH1* or *MSH2*; whilst the remaining 10-30% of Lynch syndrome result from *PMS2* or *MSH6* mutations.[51] Approximately 3% of LS arise from *EPCAM* gene mutations. This gene is positioned upstream of *MSH2* and is involved in cell proliferation, cell signalling and cell adhesion. By virtue of its position, deletions in the 3' end of *EPCAM* cause hypermethylation of the *MSH2* promoter.[51]

It has been demonstrated that mutations of specific genes associated with Lynch syndrome have an increased likelihood of culminating in certain tumours.[51] This is illustrated by tumour development under the age of 45 years, a high degree of penetrance and microsatellite instability occurring in patients with mutations of *MLH1* and *MSH2*. These clinical parameters are indicative of aggressive disease.[46] *MLH1* and *PMS2* are major MMR genes which are responsible for stability of their heterodimeric complexes. As such, a loss in either of these may cause destabilisation of the binding partner and the complex as a whole.

Studies have identified that *MLH1* and *PMS2* mutations have the greatest variety of LS-associated tumours.[51] Individuals with *MSH2* mutations have a high likelihood of developing endometrial, gastric and ovarian carcinomas in comparison to people who have *MLH1* mutations. In contrast, mutations in *MSH2* and *MLH1* have a similar likelihood of colorectal carcinoma development.[46] It has been shown that males who have a mutation of *MSH2* have the highest risk of developing different tumours. [51] With regard to endometrial carcinomas, the frequency of *MLH1* mutations is 24-40% whilst for *MSH2* it is 50-66%. [32]

PMS2 mutations are associated with an early age of tumourigenesis, and have been identified in Turcot syndrome in which multiple colonic polyps occur together with glioblastoma.[46] Development of glioblastomas is thought to be associated with increased mutations in oncogenes and tumour suppressor genes which are expressed at a higher level within cerebral parenchyma.[46] It is interesting to note that *PMS2* mutations have been linked to a lower incidence of colorectal carcinomas and endometrial carcinomas, with a mutational frequency under 5% for endometrial carcinomas[47,51] MMR mutational frequency for *PMS2* in endometrial carcinomas is below 5%.[32] Individuals with PMS2 mutations may develop colorectal carcinomas that are microsatellite unstable but this may occur at an older age or these patients may develop microsatellite unstable colorectal tumours with no family history of neoplasia.[64]

In contrast to individuals with *PMS2* mutations, those with *MSH6* mutations have a high risk of developing endometrial carcinoma. Individuals with mutant MSH6 have a lower likelihood of colorectal carcinoma development.[33,41,51] Different studies have demonstrated varying results on the incidence of *MSH6* mutations in endometrial carcinomas, as some researchers have shown that *MSH6* is identified at lower frequencies than *MSH2* whereas other authors have indicated that *MSH6* is the most commonly occurring mutation in endometrial carcinomas.[33,65] Mutations in *MSH6* are strongly linked to endometrial carcinogenesis and have been shown that *MSH6* mutations results in a risk of developing endometrial carcinoma between 64-71%, whilst an *MSH2* mutation has a relative risk of endometrial carcinogenesis of 4-50%.[33] Females with mutated *MSH2* or *MSH6* thus have a high risk for endometrial carcinogenesis and have a 44% overall estimated lifetime risk.[51] It is currently believed that a mutation of *MSH6* significantly raises an affected female's risk for development of endometrial carcinoma and furthermore, raises her lifetime risk to 70%.[32,50] Females who

have mutant *MSH6* genes tend to present with endometrial carcinoma at an age of over 50 years.[26,54,64,66,67]

The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) embarked on a classification of MMR variants according to how pathogenic each is.[68]This classification was centred on different assays utilised as well as "variant and family characteristics".[68] The International Agency for Research on Cancer utilises a five-FIGO graded classification system which has associated clinical recommendations; and it is this classification system that was accepted and implemented by the InSiGHT. Class 1 indicates a "non-pathogenic" variant which implies that the test result was normal and that there is in essence no identifiable mutation.[54,68] Class 2 variants suggest that there is probably no pathogenicity and like Class 1, may be assumed to have no mutation present. Class 3 is considered a "variant of unknown significance" which necessitates in-depth molecular analysis in order for a degree of pathogenicity to be rendered. Class 4 is a likely pathogenic variant and Class 5 is indicative of a pathogenic variant. Class 4 and 5 are telling of an underlying mutation that necessitates active surveillance in addition to screening of family members.[54,68] Studies have shown that most Class 4 and 5 variants are due to nonsense and frameshift mutations. Non-synonymous missense mutations; which are nucleotide mutations that culminate in a change in the sequence of amino acids in a protein, are responsible for most Class 3 variants. Variants in introns and non-synonymous missense mutations result in Class 1 and 2 variants. In addition, Class 1 and 2 variants may have synonymous missense mutations which are mutations in exons whereby one nucleotide is substituted for another without a resultant change in amino acid sequence.[55] Based on data collected by InSiGHT, pathogenic mutations (Class 4 and 5 variants) have been found to make-up the bulk of variants in *MLH1*, *MSH2* and *PMS2* MMR genes, whereas the non-pathogenic variants namely Classes 1 and 2 constitute the minority. Class 3 variants of uncertain significance were found to comprise the

majority of variants in the *MSH6* gene.[68]Table 2 summarises the clinical phenotypes and mismatch repair mutations.

Table 2. Mismatch repair mutations and clinical phenotypes.[64]

Mutation	**Phenotype**
MLH1 mutation, heterozygosity	LS; colorectal predominance; extra-colorectal carcinomas are less common than with mutations of *MSH2*
MSH2 mutation, heterozygosity	LS; greater occurrence of extra-colorectal carcinomas
PMS2 mutation, heterozygosity	LS; May be associated with numerous colonic polyps; decreased likelihood of carcinoma.
MSH6 mutation, heterozygosity	LS; Increased likelihood of endometrial carcinomas, tumours may show low level of microsatellite instability
EPCAM deletion, heterozygosity	LS; MSH2 expression is silenced; usually lower risk of extra-colorectal carcinomas. However, if the deletion occurs in close proximity to the MSH2 gene, there is an increased likelihood of endometrial carcinoma
MLH1 epigenetic, monoallelic	LS; Phenotypic expression is much like that in carriers of *MLH1* mutation. Most of these epigenetic phenomena occur de novo, with only a minority occurring as a hereditary condition
Mutation in any of the four MMR genes, biallelic	CMMR-D Syndrome; early childhood onset of haematological malignancies, genito-urinary, colorectal carcinomas, glioblastomas and neurofibromatosis

2.5.4. EXPANSION OF LYNCH SYNDROME TUMOURS

The Amsterdam I criteria only included colorectal carcinomas, whereas the Amsterdam II criteria were expanded to include endometrial, small bowel, ureter and renal pelvis, ureter based on information suggesting that these tumours had the greatest likelihood of occurrence amongst all LS tumours.[48]The assortment of LS associated tumours has subsequently increased and varies according to geographic locations; for example; Korean families have a

greater incidence of gastric carcinomas and pancreatic malignancies but have lower frequencies of endometrial carcinomas in comparison to people who are of Dutch descent.[48] In addition, studies have demonstrated a high incidence of breast carcinomas in Brazilian families in contrast to people of other races and ethnic groups.[48] This suggests that the development of LS tumours is influenced by the environment of an individual.

LS tumours are required to fulfil the following criteria:

1. The tumours should occur because of the genetic aberration that the patient has.
2. The lifetime relative risk of the tumours should be greater in individuals who harbour the mutation in contrast to the rest of the population

Research into the incidence of breast carcinomas in patients with LS mutations have been inconsistent thus raising concerns on the link between LS and breast carcinomas based on the first point above.[48,51] There have been contradictory findings in a study that examined the incidence of breast carcinoma in patients with LS.[51] However, there was a study undertaken on patients with breast carcinomas who had MMR gene defects, together with those who did not have the mutation as well as in those who had spontaneous tumours.[48] This study demonstrated that patients with MMR defects developed their tumours at a comparably younger age than patients in whom MMR genes were intact. This lends credence to the notion that MMR mutations do have a role in carcinogenesis in LS.

The alternate pathways resulting in microsatellite instability and inactivation of MMR genes in LS compared to sporadic carcinomas highlights differences in the molecular make-up of the tumours. This is exemplified by the identification of *BRAF V600E* mutations in up to 50% of sporadically occurring microsatellite unstable colorectal carcinomas. This is not seen colorectal carcinomas arising in Lynch syndrome.[48]

There is emerging information on the occurrence, age of onset and associated risks of development of prostatic carcinoma in LS.[51] It has been found that an individual's lifetime risk for prostatic carcinoma may be increased by up to five times. It is not currently known whether LS associated prostatic carcinomas are more aggressive or arise at a younger age.[51]

2.5.5. MORPHOLOGY OF LS ENDOMETRIAL CARCINOMAS

Whilst colorectal carcinoma has been demonstrated to exhibit various microscopic features such as occurrence in the proximal or right colon, mucinous carcinomas or signet-ring cell carcinomas, tumour-infiltrating lymphocytes and a Crohn-like response whereby lymphoid follicles are seen in close proximity to nests of tumour cells; the microscopic features of endometrial carcinomas that are associated with LS are not as well established.[32] The tumours tend to occur more often in the lower uterine segment.[33,38] Most endometrial carcinomas in LS have an endometrioid morphology but may show poorly differentiated, de-differentiated or undifferentiated areas.[26,51] The de-differentiated areas comprise sheets of large round to oval cells that have prominent nucleoli.[26] In these areas, glandular formation is absent.[68]The dedifferentiated areas are seen adjacent to better differentiated regions, but are not admixed. Each component must comprise a minimum of 10% of the overall tumour volume.[31,69] The de-differentiated and undifferentiated carcinomas have been identified in cases of either germline or sporadic *MLH1/PMS2* mutations.[29,69] Tumour infiltrating lymphocytes are often identified in LS endometrial carcinomas. It has been suggested that a count of 42 lymphocytes per ten high powered field be identified as it has been shown that this is seen far less commonly in non-methylated sporadic endometrial carcinomas. Identification of an abundance of tumour infiltrating lymphocytes is considered a pointer toward a tumour having generalised MMR mutations and is seen in tumours that have germline mutations as well as those that have undergone sporadic hypermethylation.[69] The density of the lymphocytic

infiltrate may vary depending on the type of MMR mutation in each case. In addition, large aggregates of lymphoid cells are seen at the tumour interface on scanning magnification.[26]

2.5.6. LYNCH-LIKE SYNDROME

Patients who have mismatch repair deficient tumours detected immunohistochemically or who may demonstrate MSI by PCR but have no detectable germline mutation are known to have "Lynch-like Syndrome".[53,70] It is challenging to distinguish patients who have Lynch syndrome from those who have Lynch-like Syndrome; as Lynch-like Syndrome patients also have microsatellite unstable neoplasms.[71] Tumours associated with Lynch-Like Syndrome show negativity for MLH1 or any of the mismatch repair immunohistochemical markers as seen in true Lynch syndrome. Furthermore, Patients with Lynch-like Syndrome tend to develop tumours at a fairly similar age to patients with true LS. In contrast however, patients with Lynch-like syndrome do not have an identifiable germline mutation.[71,72] There are three possible hypotheses for this.

Three potential explanations for this have been considered. The first possible explanation is that there may be bi-allelic *MLH1* hypermethylation or other genomic abnormalities aside from MMR mutations that result in the development of microsatellite unstable neoplasms. The second possible explanation is that there may be germline mutations in MMR genes that are not identified by current detection methods and the third, is that there may be unidentified gene mutations that result in microsatellite instability besides MMR genes.[71] It is conceivable that all three mechanisms may have a role to play in Lynch-like Syndrome as they are not mutually exclusive processes.[71]

The first possibility has been investigated by Mesenkamp *et al*.[70] who suggested that in approximately half the number of MMR deficient tumours that were negative for *MLH1* promoter hypermethylation and negative for germline mutations of MMR genes, that somatic

mutations could be detected in *MSH2* or *MLH1*.[70] Boland has similarly noted that somatic mutations may also occur in *MSH6* and *PMS2*.[73] Mesenkamp and co-workers[70] have demonstrated that two somatic mutations may have a bearing on MMR gene expression.[70] They have also suggested that Lynch-like syndrome patients develop tumours at an earlier age than sporadic tumours that are microsatellite unstable. Furthermore, they showed that the average age of tumour diagnosis in patients who had two somatic mutations was essentially the same as those who were affected by germline mutations in *MHS2* or *MLH1* but younger than in patients who developed sporadic tumours.[70,71,74,75] The implication of this in patients who have two somatic mutations, in addition to patients with Lynch-like Syndrome in the absence of two identifiable mutations, is that such individuals may indeed have Lynch syndrome.[70,71] Thus the treating physician should continue to investigate for possible tumour development in such patients.

The second possible explanation is a plausible one as presently exonic regions of these genes are sequenced and commonly occurring deletions of *MSH2* and *MLH1* are detected. It has been shown that mutations in *EPCAM* were the reason behind methylation of neighbouring *MSH2*. Therefore, it is possible that new mechanisms may be identified which have a bearing on MMR genes. At present, both promoter and intronic regions do not usually undergo mutational analysis and as such an understanding by researchers and scientists of such regions is by no means complete. Thus, the possibility of mutations yet to be discovered within MMR DNA is a very reasonable one. This could result in a re-categorisation of patients from Lynch-like to Lynch syndrome.[71] Sourrouille *et al* [76] have demonstrated the occurrence of somatic mutations on both alleles resulting in inactivation of MMR genes.

The third potential explanation is, however, unlikely, as the mismatch repair system has been extensively explored and aside from *EPCAM* mutations, there are no other identifiable germline mutations.

Whilst identification of sporadic tumours and Lynch syndrome and its associated high prevalence of tumourigenesis is important; the present distinction between LS and Lynch-like Syndrome is not as definitive. MMR gene inactivation is a result of various processes such as somatic mutation of MMR genes, germline mutation, in addition to hypermethylation as a form of epigenetic events. It is therefore plausible that these may all occur in Lynch-like Syndrome. Moreover, it is possible that Lynch-like Syndrome patients have a mixture of abnormalities between those noted in sporadic tumours in addition to those identified in Lynch syndrome. It is currently believed that with further investigations of patients with Lynch-like Syndrome that better treatment options may be developed.[71]

Mesenkamp and co-workers[70] have suggested that patients with tumours showing somatic mutations without evidence of hypermethylation or germline mutations should not be referred to as having Lynch-like Syndrome. Furthermore, such individuals and their families may follow surveillance programmes according to their family history. As a consequence, the required surveillance for extra-colonic tumours is removed and furthermore reduces the requirement for earlier surveillance colonoscopies.[70]

2.6. ADDITIONAL MOLECULAR PATHWAYS IN ENDOMETRIOID ENDOMETRIAL CARCINOMAS

2.6.1. THE *PTEN* PATHWAY

PTEN is a tumour suppressor gene that encodes the enzyme phosphatidylinositol phosphatase which dephosphorylates phosphatidylinositol-trisphosphate (PIP3). *PTEN* acts to antagonise the phosphatidylinositol 3-kinase and Protein Kinase B (PI3K/AKT) pathway. [16,77] *PTEN* dephosphorylates PIP3, which is a product of PI3K. *PTEN* is located on chromosome 10q23.3 which is a region that undergoes loss of heterozygosity in numerous carcinomas including up to 40% of endometrial carcinomas.[16,77] Lowered *PTEN* activity allows for

greater cell proliferation together with increased cell survival as a consequence of altering pathways of signal transduction.[16,77] Inactivation of *PTEN* may be due to different mechanisms such as promoter hypermethylation, mutation or loss of heterozygosity at 10q23. Loss of functionality of both alleles is a pre-requisite for *PTEN* inactivation. Cases of haploinsufficiency require that only a single mutation be present to result in *PTEN* inactivation.[77]

Up to 55% of endometrial hyperplasias, either atypical or without atypia, have been found to contain PTEN mutations. This suggests that loss of *PTEN* is an early occurrence in the pathogenesis of endometrial carcinoma.[30,77] Salvesen *et al*[78] and Risinger and co-workers[79] have shown that endometrial tumours containing *PTEN* mutations had an endometrioid morphology, showed a lower histological FIGO grade and stage. These tumours had a better overall outcome. More recently, however, studies have suggested that *PTEN* promoter hypermethylation may be linked to a higher stage of disease and that only mutations outside exons 5 to 7, portend a better outcome.[16] It has been suggested that positivity on a PTEN immunohistochemical stain signifies a better prognosis for patients with advanced endometrial carcinoma who undergo post-operative chemotherapy. In contrast, however, PTEN negativity and AKT phosphorylation may herald a worse outcome in patients with endometrial carcinoma. It has also been suggested that concomitant PTEN and p53 immunohistochemical abnormalities occur as late events in the development of endometrial carcinoma but that independent p53 and PTEN aberrations suggest early occurrences.[77]

Between 60-86% of microsatellite unstable endometrioid endometrial carcinomas harbour *PTEN* mutations.[30,77] Prat *et al*[77] have documented that in up to 44% of microsatellite unstable endometrial carcinomas, there are two short coding mononuclear repeats. This therefore, alludes to mismatch repair deficiencies resulting in *PTEN* mutations.[77]

2.6.2. THE PI3K PATHWAY

Function of the Phosphatidylinositol 3-kinase (PI3K) pathway is coupled to functioning of *PTEN*. The PI3K-AKT pathway is involved in cellular growth, cell proliferation, cell survival and cellular growth.[34] PI3K is a heterodimeric enzyme and is composed of a regulatory subunit (p85) in addition to a catalytic subunit (p110).[27,30,77] The *PI3K* gene, coding for the p110α catalytic subunit PIK3CA, is situated on chromosome 3q26.32.[27,30,77] Enhanced PIK3CA activity is noted as a consequence of amplification of this region.[77] Many tumours have shown PIK3CA increased activation or mutations which are oncogenic.[16,77] Up to 36% of endometrial carcinomas have demonstrated PIK3CA mutations; with most of these having been identified as missense mutations in exon 20.[77] Mutations of both PIK3CA and *PTEN* have been noted in one out of four endometrial carcinomas.[27,77] Most mutations occur in the helical domain (exon 9) and kinase domain (exon 20).[27,35] They may however, also occur in in exons 1 to 7. PIK3CA mutations in exon 20 correlate with poor histological prognostic factors such as high tumour FIGO grade as well as lymphovascular and myometrial infiltration.[3,27]

Binding of PI3K to a growth factor receptor kinase culminates in the formation of PIP3 which subsequently activates several other pathways. These pathways direct cell growth, proliferation, cell survival or apoptosis as well as cell motility and adhesion.[77] Such regulation therefore promotes cell proliferation whilst apoptosis is inhibited.[77]

Tissue specific PIK3CA mutations occur in endometrial carcinomas with a greater occurrence of mutations taking place in the C2 and ABD domains of p110α. It has been found that in endometrial carcinomas, p85α, which binds the C2 and ABD domains, undergoes somatic mutation at a greater rate in comparison to other tumour types. These therefore suggest that there is a definite promotion of carcinogenesis in endometrial tissue.[34]

Unlike *PTEN* mutations, PIK3CA mutations are an uncommon finding in atypical hyperplasia and are thought to be a later occurrence in endometrial carcinogenesis.[34]

PIK3CA mutations have not been identified to have any correlation with microsatellite instability or *CTNNB1* abnormalities. Moreover, it has been shown that there is mutual exclusivity between PIK3CA mutations and K-RAS mutations.

Mammalian target of rapamycin or mTor, is downstream in the PI3K-PTEN-AKT pathway. When *PI3K* or *PTEN* mutations occur, mTor becomes activated.[35] In tumours such as endometrial carcinomas which may have *PTEN* inactivation, mTor inhibitor pharmacological agents may have therapeutic benefit.[35]

2.6.3. *RASSF1A*

RASSF1A is the KRAS effector which is a tumour suppressor that is instrumental in preserving genomic stability, arresting the cell cycle and controlling apoptosis.[16] *RASSFIA* has an inhibitory effect on growth signals which is lost during carcinogensis.[27] As such, loss of *RASSF1A* function brings about an increased signalling of the pathway.[16] Promoter hypermethylation which has been identified in endometrial carcinomas amongst other tumours, results in loss of *RASSFIA* function.[16,27] Hypermethylation of *RASSF1A* has been detected in normal appearing tissue that is contiguous to endometrioid endometrial carcinoma as well as in hyperplasia with and without atypia.[34] *RASSF1A* hypermethylation is associated with an advanced stage of disease. In addition, it is identified more commonly in microsatellite unstable tumours. It has therefore been suggested that a methylator phenotype affects the *MLH1* gene as well as others, such as *RASSFIA*. *RASSF1A* hypermethylation is more commonly noted in tumours without KRAS mutations.[34]

2.6.4. *BRAF*

BRAF, as discussed in section 2.5.2.4, is a member of the RAS-RAF-MEK-ERK pathway. Feng et al[62] identified *BRAF* mutations in cases that were negative for *MLH1* by immunohistochemistry which suggests that they may be associated with microsatellite instability.[62] *BRAF* mutations are mutually exclusive with hypermethylation of *RASSF1A* and *KRAS* mutations.[34] The role and incidence of *BRAF* mutations in endometrial carcinomas is not entirely clear at present with differing results having been obtained by different authors.[43,61,63] It has been suggested that the high incidence of *BRAF* mutations noted by Feng *et al* may be attributed to different ethnic populations studied. This however has not been fully established.[34]

2.6.5. THE WNT PATHWAY

CTNNB1 - β-CATENIN

The *CTNNB1* gene, located on chromosome 3p21, encodes the protein, β-Catenin.[16,30,34,77] The Wingless-type or WNT family, encodes glycoproteins which promote cellular growth and differentiation.[80] Frizzled or FZD receptors in the WNT pathway allow for signal transduction to the transcriptional regulator, *CTNNB1* by means of intracellular proteins.[16] β-Catenin is an adherens junction protein allowing for adhesion between cells and is a member of the E-Cadherin-Catenin complex.[30,34,77] By interacting with E-Cadherin at the cell membrane, β-Catenin ensures cell polarity.[16,77] Free β-Catenin within the cytoplasm of cells can interact with the Adenomatous Polyposis Coli (APC) protein and may facilitate transcription. Glycogen Synthase Kinase 3β (GSK3β) along with APC proteins cause serine-threonine residues coded in exon 3 of *CTNNB1*, to undergo phosphorylation. This causes degradation of β-Catenin via the ubiquitin-proteosome pathway. APC therefore, down-regulates β-Catenin. [16,27,77] Binding of WNT to FZD and lipoprotein receptor-related protein 5

or 6 (LRP5/6) results in a complex that, together with Dishevelled and Axin, inhibit β–Catenin phosphorylation and therefore stabilise β–Catenin.[81] This results in cellular proliferation. Mutations in exon 3 of the *CTNNB1* gene facilitates signal transduction, transcription of genes involved in tumourigenesis, causes protein stabilisation and accumulation in the cytoplasm and nucleus.[16,27,77] As such, it may be seen that mutations in *CTNNB1* and other genes results in abnormal build-up of β-Catenin, which then, via the T-cell factor/lymphoid enhancer factor (LEF/Tcf) pathway,[80] culminates in transcription of genes such as cyclin D1 (*CCND1*).[16]

Upregulation of the APC/β-Catenin/Tcf pathway has been identified in a myriad of tumours including endometrioid endometrial carcinomas.[16,34,77]Between 14 to 44% of endometrial carcinomas have documented mutations in *CTNNB1*. [16,27,30,34,80] Mutations occurring within APC are not common in endometrial carcinomas in contrast to colorectal carcinomas.[16,80] Studies by Eskander *et al*[80] have suggested a role for Dickkopf (Dkk) which inhibits WNT.[80] It was shown that there were lowered levels of Dkk protein expression in endometrial carcinomas in contrast to control tissue. Furthermore, these authors demonstrated decreased Dkk mRNA levels which correlated with increased obesity, higher histological FIGO grade and greater infiltration of myometrium.[80]

β-Catenin mutations have been noted in endometrial hyperplasia with squamous morule formation[16,30,34,77] This then implies that *CTNNB1* mutations occur early on in carcinogenesis.[16,34,77] β-Catenin mutations exist separately from microsatellite instability, *PTEN* or *KRAS* mutations.[27] Whilst fair correlation between mutational status of *CTNNB1* and nuclear positivity of β-Catenin exists, there are occasions in which carcinomas exhibit nuclear expression of β-Catenin in the absence of *CTNNB1* mutations. This advocates that there may be variations in other WNT genes that activate transcription.[16,35]

2.6.6. FIBROBLAST GROWTH FACTOR (FGF) PATHWAY

Fibroblast growth factor is identified in many tissue types and is important in the regulation of angiogenesis and wound repair in normal tissues and tumours.[82] FGF receptor activation causes signal transduction via different pathways such as PI3K and RAS-RAF-MEK-ERK.[82] The FGF pathway is regulated by different proteins, of which "Sprouty mammalian genes" (*SPRY*) has a vital role. SPRY proteins control receptor tyrosine kinases as well as the RAS pathway. SPRY proteins are therefore able to regulate angiogenesis in addition to cellular differentiation and proliferation. As a result of FGF pathways being affected in various manners, FGF may have tumour suppressor functionality whilst in other cases it may function as an oncogene culminating in cell proliferation and migration.[82]

It has been demonstrated that *SPRY2* is inactivated in endometrial carcinomas. The inhibitory effect on the FGF pathway is therefore removed. Studies by Velasco *et al*[83] identified decreased immuno-reactivity in approximately one fifth of endometrial carcinomas and inversely correlated with increased cell proliferation. Their study suggests that modifications to SPRY proteins may have a role to play in the development of endometrial carcinoma by virtue of increased cellular proliferation.[83]

Between 6-12% of endometrioid endometrial carcinomas have shown mutations in the fibroblast growth factor receptor 2 (*FGFR2*) tyrosine kinase.[30,34] *FGFR2* mutations are noted to be mutually exclusive with mutations in *KRAS*. However, up to 77% of *FGFR2* mutations occur simultaneously with *PTEN* mutations.[30,34,83] Most mutations in *FGFR2* are missense mutations that occur in the transmembrane and extracellular and kinase domains of proteins. A mutational hotspot exists on codon 252 in the extracellular domain which then facilitates binding of ligands. It is the S252W that is oncogenic and is responsible for approximately

40% of all mutations in endometrial carcinomas.[34] There is much interest in FGFR2 at present in pursuit for possible therapeutic modalities.[35]

2.6.7. *ARID1A*

The AT-rich interaction domain (*ARID*) family is a tumour suppressor gene, encoding BAF250a, which forms part of the SWI/SNF (SWItch/sucrose non-fermenting) chromatin remodelling complex.[34,84,85] Many endometrial carcinomas have shown abnormalities in *ARID1A* and BAF250a. Up to 23% of microsatellite unstable endometrial carcinomas have shown *ARID1a* mutations.[17,84] *ARID1A* mutations are associated with PTEN pathway inhibition whilst they result in activation of the PI3K and Akt-mTor pathway, thus increasing cell proliferation.[85] Undifferentiated carcinomas have also been shown to harbour *ARID1A* mutations.[86]

2.6.8. UNDIFFERENTIATED CARCINOMAS AND EPITHELIAL TO MESENCHYMAL TRANSITIONS (EMT)

During the process of epithelial to mesenchymal transitions or EMT, epithelial cells develop mechanisms and a phenotype that allows for decreased cellular adhesion with subsequent detachment of cells. These cells also gain properties which permit cells to migrate with ease. This is accomplished by upregulation of ZEB-1, SNAIL and TWIST which are EMT associated proteins as a consequence of upregulation of microRNA 200 series. Simultaneously, there is down regulation of E-cadherin.[86] It has been shown that undifferentiated carcinomas have mutations from all four molecular subgroups designated by TCGA.[17,86]

The pathogenetic pathways in endometrial carcinomas are summarised and represented in figure 14.[10]

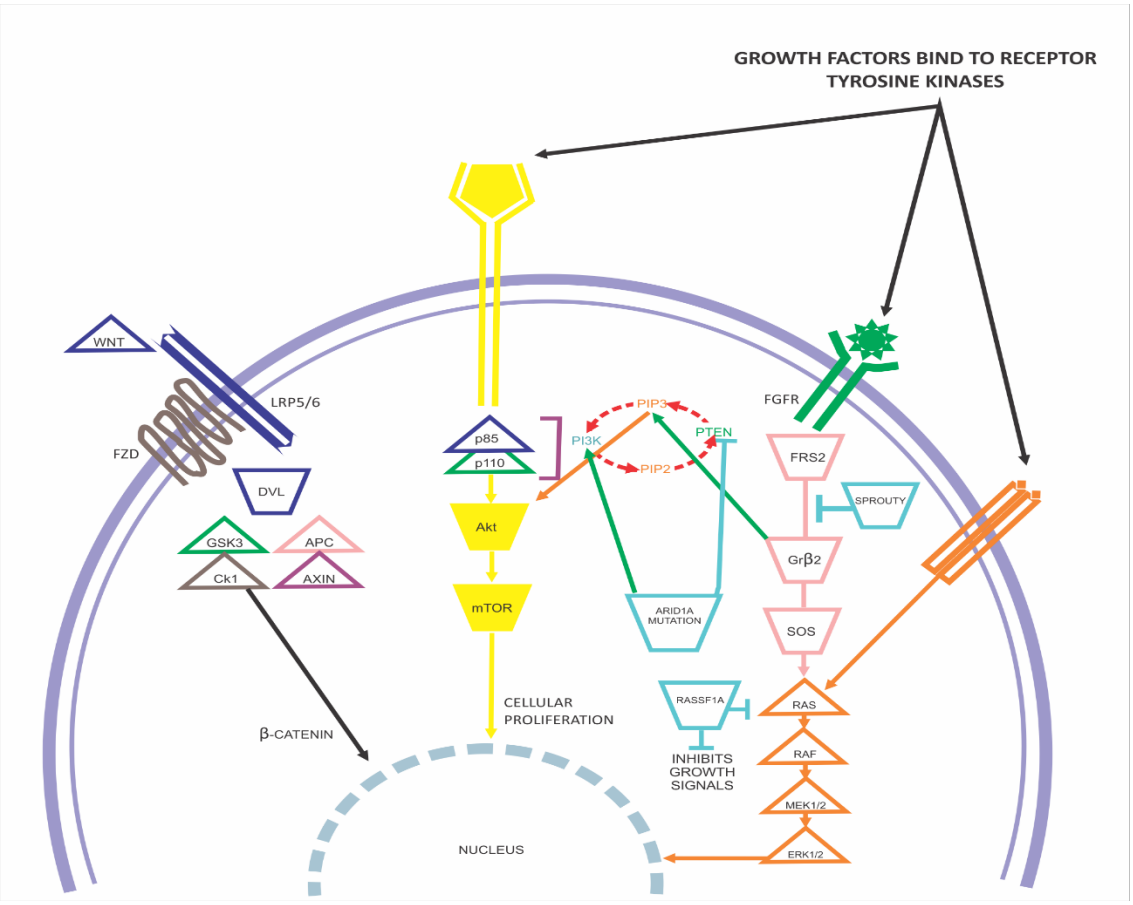

Figure 14. A line diagram illustrating molecular pathways involved in endometrioid endometrial carcinomas (adapted from Wadee and Grayson[10]).

2.7. MOLECULAR PATHWAYS IN NON-ENDOMETRIOID CARCINOMAS OF THE ENDOMETRIUM

2.7.1. *TP53* – p53 MUTATIONS

TP53, found on chromosome 17p13, is a tumour suppressor gene, encoding for the nuclear p53 protein which is a transcription factor.[16,77] When this protein encounters DNA damage it triggers expression of genes causing arrest of the cell cycle and brings about apoptosis.[16,77] Loss of p53 function implies an inhibition of apoptosis, thereby causing loss of heterozygosity and aneuploidy with widespread genetic abnormalities.[16,77]Normally, *p53* is broken down quickly and therefore, there is no immunohistochemical evidence of p53.

However, mutations of *TP53* cause non-functional p53 protein production which is not broken down and is thus detected immunohistochemically.[77] *TP53* is a tumour suppressor, and as such inactivation of both alleles either by means of mutation or via loss of heterozygosity, is required. Consequently, loss of heterozygosity of p53 may not automatically culminate in protein over-expression. There have been instances where some *p53* mutations have caused loss of function with only one allele mutated, which has been described as a consequence of the p53 mutation having a negative effect on wild-type p53.[77] Complete negativity or strong intensity, diffuse nuclear p53 positivity may be seen on an immunohistochemical level in serous carcinomas.[38]

Mutations of p53 are identified in more than half of all neoplasms, including endometrial carcinomas and are the most common molecular aberration in non-endometrioid endometrial carcinomas.[16,30,34,77]It has been shown that up to 90% of serous carcinomas have *TP53* mutations. Mutations of *p53* are early occurrences in carcinogenesis and are identified by "p53 signatures" in which cytologically bland, benign appearing glands demonstrate strong nuclear p53 staining. P53 signatures have been identified in benign epithelium that neighbours serous carcinoma.[34] Mutations of p53 have also been observed in endometrial intraepithelial carcinoma (EIC) where atypical cells are present in the surface epithelial lining. Despite EIC being confined to the endometrial surface epithelial lining or to the surface of an endometrial polyp, it has correlated with a higher tumour stage and heralds a poor prognosis.[77]

It has been shown that *p53* mutations are not a common occurrence in endometrioid endometrial carcinomas, with an incidence between 10-20%, the majority of which are FIGO grade 3 endometrioid endometrial carcinomas.[35,77]There have been no FIGO grade 1 EECs that have had p53 mutations identified.[34] As such, the identification of p53 mutations in EECs lends credence to the belief that *p53* drives evolution of EEC to NEEC with retention of

typical molecular features of EECs.[77] It has been shown that p53 overexpression correlates [17,21,23]with a higher tumour FIGO grade and stage; and ultimately with a worse overall prognosis.

2.7.2. *HER2/neu* – ERBB2

Epidermal growth factor type II receptor or *HER2/neu* is an oncogene that encodes a tyrosine kinase which acts as a receptor for growth factors. It is involved in directing the ERBB pathway. [16,34,77] As *HER2/neu* does not have its own ligand binding it cannot bind growth factors, but it heterodimerizes with *HER1/erbB1*, *HER3/erbB3* and *HER4/erbB4*. These are part of the family of epidermal growth factor receptors (EGFR).[77] *HER2/neu* activation causes an increase in cell proliferation due to increased PI3K and Mitogen-activated protein kinase pathways.[16,77]It has been shown that 20-40% of endometrial carcinomas demonstrate *HER2/neu* overexpression. Additionally, *HER2/neu* amplification has been associated with a higher tumour FIGO grade and stage with a poor overall outcome.[16]

Immunohistochemically, HER2/neu overexpression has been demonstrated in between 17-80% of serous carcinomas of the endometrium; whilst HER/neu amplification as assessed by fluorescence in-situ hybridisation (FISH), has been observed in up to 68% of serous endometrial carcinomas that overexpress the protein as well as in up to 42% of serous carcinomas overall.[34] HER2/neu overexpression and amplification have proven to be far more common in serous endometrial carcinomas than FIGO grade 3 endometrioid endometrial carcinomas. Moreover, FIGO grades 1 and 2 EEC's have illustrated that HER2/neu overexpression is a highly unlikely occurrence.[34]

2.7.3. *CDKN2A (p16)*

CDKN2A (cyclin-dependent kinase Inhibitor 2A)/p16 is a tumour suppressor which is responsible for the inhibition of G1/S phase of the cell cycle. It has been shown in serous

carcinomas that strong intensity diffuse staining of p16 may be seen whereas EECs have shown weak intensity, focal p16 staining.[34]

Positivity of p16 in serous carcinomas is related to loss of p53 function with consequent p16 overexpression.[87] Studies have shown that p16 may be inactivated by mutations, promoter hypermethylation and homozygous deletions.[35,88]

2.7.4. E-CADHERIN

CDH1 is a tumour suppressor gene encoding for E-cadherin; an adhesion molecule. Negativity or low levels of expression of E-cadherin have been documented to occur more commonly in endometrial serous and clear cell carcinomas in contrast to endometrioid endometrial carcinomas. To date, it is not entirely clear as regards what molecular processes occur to culminate in decreased expression of E-cadherin. It has been shown that loss of heterozygosity of the *CDH1* gene occurs more commonly in serous and clear cell carcinomas than EECs.[19] Up to 60% of NEECs have loss of heterozygosity at chromosome 16q22.1.[20] Despite *CDH1* promoter hypermethylation occurring commonly a in endometrial carcinomas, it does not necessarily correlate with lowered expression of the E-cadherin protein.[19] It has been shown that there may be further processes occurring that contribute to lowered expression of E-cadherin such as abnormalities in individual transcriptional repressors of E-cadherin; and dysregulation of these inhibitors causes their overexpression with consequent lowered levels of E-cadherin. E-cadherin loss has been identified in epithelial to mesenchymal transition, whereby cells lose their epithelial appearances and assume a mesenchymal phenotype.[19] High levels of E-cadherin have been linked to a decrease in disease progression and associated mortality.[19, 65]

2.7.5. *PPP2R1A*

PP2A (protein phosphatase 2A), coded for by the gene *PPP2R1A* (Protein Phosphatase 2 Scaffold Subunit Alpha), is a serine-threonine phosphatase, which normally inactivates AKT, similar to PTEN.[34,89] It has been shown that up to 41% of serous carcinomas have somatic mutations.[34] Studies have demonstrated that mutations in *PPP2R1A* occur both early and late in the pathogenesis of serous carcinomas.[89]

2.7.6. CYCLIN E *(CCNE)*

Cyclin E has been shown to be overexpressed immunohistochemically far more commonly in non-endometrioid endometrial carcinomas than in EECs. The increased cyclin E may be a consequence of amplification of the *CCNE* gene. There may also be loss of function mutations in the tumour suppressor gene *FBXW7* which contributes to increased cyclin E expression.[34] *FBXW7* is dependent on p53 and encodes for a substrate recognition component of a ubiquitin-ligase composite which directs the ubiquitin-mediated degradation of cyclin E. Mutations in *FBWX7* and *CCNE* amplification have been shown to increase cyclin E protein levels which facilitates cell cycle to proceed with eventual cell proliferation and carcinogenesis.[90]

2.7.7. CHROMOSOMAL INSTABILITY

Chromosomal instability is a common occurrence in non-endometrioid endometrial carcinomas. Such tumours show widespread chromosomal losses and gains; which are indicative of aneuploidy. Upregulation of genes controlling the mitotic spindle checkpoint; such as STK15, have been identified. STK15 is a serine-threonine kinase which is vital for equal segregation of chromosomes.[16] Studies have shown that overexpression of STK15 overexpression culminates in increased numbers of centrosomes as well as aneuploidy.[16] NEECs often demonstrate STK15 amplification.[35,77]

2.7.8. PIK3CA PATHWAY

This pathway, discussed in section 2.6.2, also plays a role in serous carcinomas. Amplification of PIK3CA or activating mutations result in greater PI3K signalling which ultimately results in cellular proliferation and tumourigenesis.[90]

2.7.9. MOLECULAR TRAITS OF CLEAR CELL CARCINOMAS

This group of tumours were not included in studies by TCGA and as such, there is currently less information on these tumours in comparison to other tumours of the endometrium.[91] Clear cell carcinomas have *PTEN* and PI3K mutations. It has recently been shown that up to 40% of clear cell carcinomas have ARID1 mutations. Le Gallow and colleagues[92] have proposed that clear cell carcinomas have a molecular make-up of serous carcinomas or EECs as p53 and microsatellite instability has been identified. These authors also detected mutations in TATA-box binding associated factor-1 (TAF-1). It is currently believed that further investigations into TAF-1 mutations are necessary to gain more knowledge of their role in this tumour group.[91]

2.7.10. UNDIFFERENTIATED CARCINOMAS AND CARCINOSARCOMAS

As stated in section 2.6.8, undifferentiated carcinomas show mutations from all four subgroups described by TCGA as are therefore molecularly diverse. The majority of carcinosarcomas have shown molecular features of serous carcinomas in which *p53* and *PPP2RIA* have been identified. In contrast, a minority of carcinosarcomas have been shown to have *ARID1A*, *PTEN*, *POLE* and *KRAS* mutations as well as microsatellite instability which insinuates derivation from endometrioid carcinomas.[86]

2.7.11. SUMMARY OF MOLECULAR ALTERATIONS IN ENDOMETRIAL CARCINOMAS

Investigations undertaken by The Cancer Genome Atlas Research Network[17] showed that the majority of Endometrioid carcinomas commonly harboured mutations in *PTEN*, *PIK3CA*, *CTNNB1*, *ARID1A* and *KRAS*. Serous carcinomas on the other hand, demonstrated numerous copy number changes as well as *p53* mutations.

Studies by this group of authors[17] has brought about a molecular re-classification of endometrial carcinomas into four groups, namely: MSI hypermutated, POLE ultramutated, copy-number low and copy-number high. [17,19] Interestingly, the POLE ultramutated group demonstrated good outcomes despite some tumours being of high histological FIGO grade.[17,19,93]

The molecular classification of endometrial tumours is undoubtedly exciting as this now allows for further targeted therapeutic options for patients with endometrial carcinomas.

To the best of the investigator's knowledge, studies on microsatellite instability on endometrioid endometrial carcinomas in the South African context have not been reported in scientific journals and there is currently a paucity of such data in our patient population. This study will examine MSI by both IHC as well as PCR which will allow a comparison of the sensitivity and specificity of the two. In addition, the study intends to ascertain the number of sporadic endometrial carcinomas and will also have a cohort of patients in whom screening modalities suggest LS.

2.8. AIMS

To identify cases of microsatellite instability and cases of suspected Lynch syndrome in cases previously diagnosed as Endometrioid endometrial carcinoma in the period 2009-2015.

2.8.1. OBJECTIVES

1a. To evaluate the percentage of mismatch repair (MMR) deficiency by immunohistochemistry (IHC) and;

1b. To compare microsatellite instability (MSI) to that found by PCR in endometrioid endometrial carcinomas in the greater Johannesburg area.

2. To identify the percentage of MSI-Low versus MSI-High cases of Endometrioid endometrial carcinoma.

3. To determine the methylation status of cases that are deficient for MLH-1 by IHC.

4. To ascertain the *BRAF V600E* status of cases by IHC and BRAF sequencing that are deficient for MLH-1 by IHC.
5. Compare MSI and BRAF status of the tumours to FIGO grade.
6. To identify the number of possible patients with Lynch syndrome by screening modalities.

CHAPTER 3

3.0. MATERIALS AND METHODS

Following acquisition of ethical clearance (Clearance certificate number M151051, Appendix 2), archived blocks of cases previously diagnosed as endometrioid endometrial adenocarcinoma were retrieved from the division of Anatomical Pathology, University of the Witwatersrand/National Health Laboratory Service (NHLS). This study was confined to cases of endometrioid endometrial carcinoma as it has been demonstrated that microsatellite instability is essentially limited to this subtype of endometrial tumour.[44] The cases were identified using the SNOMED search on the NHLS computer database for the period 2009-2013. In addition, a SNOMED search on the LabTrack system for the period 2013-2015 was performed. The H&E stained sections from each case were examined and the diagnoses confirmed. As many cases in the current cohort were curettings and not surgical excision specimens, it was not possible to assess for tumour within the lower uterine segment, peri-tumoural lymphocytes, more than 42 tumour infiltrating lymphocytes per 10 high power fields, tumour heterogeneity, de-differentiation of tumour; all of which have been proposed as histological features suggestive of Lynch syndrome.[94]

Based on international data of approximately 6% of females developing endometrial carcinoma, between 15-30% of these patients may have microsatellite unstable tumours [95]. To observe a significant difference ($p<0.05$) at 80% power between the two groups would require a sample size of 145. Based on the literature and from preliminary data, a difference of 15% is expected between the groups showing microsatellite instability and those that are microsatellite stable. Using the formula for a percentage estimation in the population:

$N= 15.5xpxq/d^2$ for alpha =0.05, 15.5 is a constant, q=1-p, p= expected percentage (according to previous data), d=difference between % expected and observed.

p=30%, q= 70%, d=15%

N= (15.5x0.3x0.7)/0.0225

N= 3.255/0.0225

N=144.6

N=145 cases

Cases of serous or clear cell carcinoma have been excluded. In cases where both a curettage specimen and a hysterectomy specimen for a single patient was found, only the hysterectomy specimen had been used. In cases where the tissue blocks could not be found due to filing errors, the case was excluded. Furthermore, in cases where only a curettage specimen had been submitted and there was too little tissue, the case was excluded.

3.1. IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on 4μm deparaffinised sections using the MMR antibodies; MLH1 (Novocastra, UK), PMS2 (Novocastra, UK), MSH2 (Novocastra, UK) and MSH6 (Novocastra, UK). The tissue sections were cut using a microtome from paraffin embedded blocks. The cut sections were subsequently floated onto slides. The slides then underwent overnight drying at a temperature of 60˚ C. The immunohistochemical stains utilized had previously been optimized according to departmental standard operating procedure. Staining of tissue sections includes the treatment of primary antibody serum. Each of the tumour tissue sections was then subjected to staining with the following antibodies: MLH1 (Clone ES05, 1:50), PMS2 (Clone MOR4G, 1:50), MSH2 (Clone 25D12, 1:50) and MSH6 (PU29, 1:50). Usage of a mouse linker resulted in increased expression of antigens. The sections were subsequently washed with Tris buffered saline (TBS) at pH 7.6. Immunohistochemistry was undertaken using an automated staining machine (DAKO Autostainer Link 48, Denmark). In addition, a ready-made solution which includes EDTA, known as the EnVision™ FLEX target Retrieval Solution, High pH, was used for antigen

retrieval. The chromogen utilized was 3,3' Diaminobenzidine hydrochloride solution (DAB, Sigma); which resulted in the production of a brown pigment. Meyer's haematoxylin was the counterstain applied to tissue sections.

Negative control sections underwent processing and staining in a similar manner as for the investigational material, except that peroxidase labelled secondary anti-sera was utilized in the absence of cell specific antibody. Positive tissue controls were utilized for all the immunohistochemical stains. These control tissue blocks were drawn from the departmental stock of normal colon.

3.1.1 IMMUNOHISTOCHEMICAL EVALUATION

The tissue sections have been assessed in a binary manner whereby there was either retained staining of tumour nuclei (regardless of intensity or volume) or loss of staining of tumour cells, with retention of staining of endothelial cells, lymphocytes and stromal cells which had served as an internal positive control. A retained pattern of staining may be reported as intact/normal/proficient, whereas loss of staining may be reported as deficient or abnormal staining. As the possibility existed of only a few foci of positive staining nuclei identified in certain cases, implying that the mismatch repair genes remained intact, tissue microarrays had not been performed.

3.2. POLYMERASE CHAIN REACTION

Areas of tumour in which a high volume of neoplastic cells were identified, were selected for microdissection.

3.2.1. DNA EXTRACTION

According to departmental standard operating procedure (SOP) and based on the work of Haghighi *et al*[57] genomic DNA was retrieved from formalin-fixed, paraffin-embedded tumour tissue from each case in the following manner:

10µm sections had been prepared from each formalin-fixed paraffin-embedded tissue section that contained both tumour tissue as well as normal, non-neoplastic patient DNA. Areas of tumour in which a high volume of neoplastic cells were identified, had been selected for microdissection. The sections then underwent deparaffinisation utilising 1ml of xylene. Thereafter, the specimens were subsequently treated with 1ml ethanol. Following centrifugation, DNA was extracted from the samples using a DNA Micro QIA amp kit (Qiagen, Whitehead Scientific) according to the manufacturer's instructions.

3.2.2. CONTROL OF CONTAMINATION

The tissue blocks had been sectioned utilising new blades for each sample which prevented cross contamination. The work benches together with work tools were disinfected using 3% Virkon between each tissue block that had been handled. In addition, the work benches were subjected to decontamination using ultraviolet light between each procedure. The extraction procedure was assessed by PCR amplification of the internal ß-globin control and DNA had been quantified using the Nanodrop 1000 Spectrophotometer.

The primers used for amplification of microsatellite sequences in this panel (as indicated in Table 3) are those previously utilised by Haghighi *et al.*[57]

Table 3: Primer sequences utilised for MSI testing[57] (with permission from the corresponding author)

Marker Name:	**Gene:**	**Genbank Number:**	**Repeat:**	**Primer Sequences:**	**Amplicon Size (bp):**
NR-27	Inhibitor of apoptosis protein-1	AF070674	27 A 5'UTR	F: AACCATGCTTGCAAACCACT R:CGATAATACTAGCAATGACC	87
NR-21	SLC7A8	XM 033393	21 T 5'UTR	F: GAGTCGCTGGCACAGTTCTA R: CTGGTCACTCGCGTTTACAA	109
NR-24	Zinc finger 2	X60152	24 T 3'UTR	F: GCTGAATTTTACCTCCTGAC R: ATTGTGCCATTGCATTCCAA	131
BAT-25	c-kit	X06182	25 T intron 16	F: TACCAGGTGGCAAAGGGCA	153

				R: TCTGCATTTTAACTATGGCTC	
BAT-26	hMSH2	U04045	26 A intron 5	F: CTGCGGTAATCAAGTTTTTAG R:AACCATTCAACATTTTTAACCC	183

3.2.3. MULTIPLEX PCR

According to studies undertaken by Haghighi et al, the 5' anti-sense primer was tagged with a fluorescent dye using NED for BAT-25, FAM for BAT-26 and NR-21, and NR-27 and VIC for NR-24. PCR was performed in a total volume of 15µl using 0.5 µM each of sense and antisense primers (Life Technologies, USA) in a True Allele® PCR premix (Life Technologies, USA). The process of denaturation took place at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds. Thereafter, annealing occurred at 55°C for 30 seconds and extension took place at 72°C for 5 minutes.

PCR products were assessed utilising an Applied Biosystems 3730-48 DNA Analyser together with Genemapper v5.0. The sizes of amplicons detected on capillary electrophoresis instruments may differ between one to four nucleotides. The acceptable and expected size ranges of control and test tissue specimens are documented in the table below:

Table 4. Specific markers and expected size of amplicons. Adapted from Haghighi *et al*[45]

Specific marker	Size of Amplicon (bp)	Colour
NR27	87	Green
NR21	109	Blue
NR24	131	Black
BAT25	153	Green
BAT26	183	Blue

Cases that showed a difference in size of alleles compared to the patient's normal DNA control in the representative tissue section, in only one out of the five markers were regarded as MSI-Low (MSI-L). These cases demonstrated a repeat in any one of the Gaussian distribution of peaks within the valid size range of the five markers. Cases showing differences in allele sizes in two or more of the five markers were deemed MSI-High (MSI-

H). Such cases revealed two or more repeats in the Gaussian distribution of peaks within the valid size range. Cases that showed no difference in size were considered Microsatellite Stable (MSS). These cases showed a Gaussian distribution within the valid size ranges.[39–41]

3.2.4. REAL-TIME AMPLIFICATION OF THE ß-GLOBIN GENE:

This assay was performed using the Corbett Research RotorGene 6000 (Whitehead Scientific) RT-PCR machine using the Bioline SensiMix™SYBR No-Rox kit (Celtic Diagnostics). According to the departmental SOP, a final volume of 20µl of a reaction mix was made using 0.2 mM of each primer, 10 µl 2x SensiMix™SYBR No-Rox Master Mix (with $MgCl_2$, 50mM) and 2µl of template DNA. The thermal cycling program for this test consisted of a denaturation step at 95ºC for 10 minutes, followed thereafter by 50 cycles at 95ºC for 10 seconds for the denaturation process. Subsequently, annealing occurred at 55ºC for 10 seconds and elongation ensued at 72 ºC for 15 seconds. Succeeding amplification, a melt curve analysis was performed at 95ºC with a ramp rate 1ºC/5seconds. The average melting temperature ($T_{m)}$ of the β-globin amplicon is 85.5º +/-1.0°C. Gel electrophoresis was subsequently undertaken on samples with doubtful T_m values to confirm the presence of the 268 bp PCR fragment.

Cases that were deemed negative on an MLH-1 immunohistochemistry stain then underwent evaluation using the BRAF V600E immunohistochemical stain as well as real-time PCR for DNA methylation.

3.3. BRAF V600E IMMUNOHISTOCHEMISTRY

BRAF V600E (Clone VE1), immunohistochemistry was performed on 4µm deparaffinised tissue sections. The tissue sections underwent the same technical process for immunohistochemistry as stated in section 3.1.1. The immunohistochemically stained tissue sections were examined for cytoplasmic positivity or negativity in the tumour cells.

3.4. BRAF POLYMERASE CHAIN REACTION

BRAF mutational analysis by sequencing has been performed on DNA extracted from archived tissue blocks. Tissue sections were cut and had undergone deparaffinisation and ethanol washing, drying and placement in Protein K buffer solutions. PCR with primers targeting the *BRAF V600E* mutation site were performed according to the manufacturer's instructions.[96] The kit allowed for four mutation assays to be performed for *V600E, V600Ec, V600D, V600K, V600R*. The *V600E/Ec* assay detects both *V600E* and *V600Ec* mutations but does not distinguish between them.[96] The PCR reactions were performed on a Rotor-Gene Q 5plex HRM instrument with fluorescence channels for Cycling Green and Cycling Yellow and analysis was performed using Rotor-Gene Q Software version 2.1.[96] The handbook states that a cycle threshold value (Ct) between 20.95 and 33 was required in order for DNA of sufficient quantity to be present for assessment. A Ct value between 35-45 implied that only a few amplifiable copies are identified and that a mutation will only be detected if there are mutations on most copies. A Ct value of >33 suggests that mutations are present in low quantities. Mutations were identified by calculating the difference in Ct values between the test sample and the control sample:

ΔCt = (Ct value of mutation) – (Ct value of control tissue)

A value ≤ 7 was indicative of a BRAF mutation being present.[96]

3.5. BRAF SANGER SEQUENCING

Direct sequencing of *BRAF* may identify mutations other than the *V600E*. This allows for comparison of the percentage of cases with BRAF mutations as detected by IHC and direct sequencing.

BRAF mutational analysis by sequencing was performed on DNA extracted from archived tissue blocks. Tissue sections were cut and had undergone de-paraffinisation and ethanol washing, drying and placement in Protein K buffer solutions. PCR with primers targeting the

BRAF V600E mutation site was performed. PCR assessment had taken place utilising polyacrylamide gel electrophoresis and was visualised using ethidium bromide under ultraviolet light. The Biospin PCR purification kit was utilised to purify PCR amplicons (Bioer Technology Co. Ltd Hangzhou, China) according to the manufacturer's instructions. Sequencing of PCR amplicon fragments was performed utilising the BrilliantDye™ Terminator v3.1 Cycle Sequencing Kit (NimaGen BV, Nijmegen, The Netherlands)[97] The sequencing products were subjected to a purification process using the Zymo kit.[98] The sequenced products had thereafter undergone electrophoresis on an Applied Biosystems 3500XL automated genetic analyser. This was performed utilising 50cm capillary arrays together with POP 7 polymer. The sequenced data was then analysed on Geneious software. [99] Sanger sequencing was conducted by Inqaba Biotec utilising BrilliantDye™ Terminator V3.1 cycle sequencing kit (Nimagen, Netherlands) and an ABI 3500XL Genetics analyser (Thermo Fisher Scientific®, UK) according to manufacturer's instructions.

3.6. MLH-1 HYPERMETHYLATION USING EPITYPER

MLH1 Promoter hypermethylation analysis was performed using MassARRAY EpiTyper analysis, by Agena Bioscience at Inqaba Biotec.

EpiTYPER is a system using DNA methylation analysis technology which has demonstrated that it is a quantitative method of DNA methylation analysis that provides reliable results. It has several components including:

1. Software that allows for identification of a target sequence and for designing PCR primers (EpiDesigner). reagents and consumables for all downstream processes, following bisulfite treatment of DNA.
2. Reagent sets that include all reagents needed for reactions after bisulphite treatment (EpiTYPER reagent sets).

3. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer for signal identification and quantification (MassARRAY Analyzer 4).
4. Data analysis and diagrammatic representation of the degree of methylation occurring at each CpG site (EpiTYPER reporting software)

Initially, the target sequence of the MLH1 promoter region, (-248 to -178) according to work undertaken by Pérez-Carbonell,[100] were entered into the software package which then identified primers allowing for the best possible DNA coverage. The forward primers were: AGGAAGAGCGGATAGCGATTT and the reverse primers were: TCTTCGTCCCTCCCTAAAACG. This yielded a product size of 187 base pairs and allowed for evaluation of 11 CpG islands. There were 3 CpG targets that could not be assessed due to lower mass cleavage products, which is a known disadvantage of EpiTYPER.[101] The remaining 8 CpG sites could be assessed.

One (1) μg of genomic DNA was required for bisulphite treatment and a ratio of between 1.7 to 2.0 was recorded for each sample using a Nanodrop spectrophotometer. DNA tubes were stored at 4ºC. The forward and reverse primers were reconstituted with Nuclease-free water at equimolar concentrations of 100 μM. These were subsequently diluted to a working solution of 1 μM.[102 6]

3.6.1. BISULPHITE CONVERSION

Conversion of unmethylated Cytosine to Uracil was achieved by the addition of bisulphite to DNA. This process resulted in the methylation dependent sequence change of Cytosine (C) to Thymidine (T). On the reverse strand, the C/T variations are identified as Guanine (G) to Adenine (A). This resulted in a mass difference of 16 Daltons (Da) per CpG site. The difference in mass was detected by the MassARRAY system.

3.6.2. CT CONVERSION REAGENT PREPARATION

As the reagents are light sensitive, they had little light exposure. Briefly, 750 µl of water was added to 210 µl of M-Dilution Buffer to a tube of CT Conversion Reagent and the substances underwent vortexing for 1-2 minutes for a total of 10 minutes. The prepared CT conversion reagent was stored at room temperature, away from light. Each tube of conversion reagent treated 10 DNA samples.

An M-Wash buffer was prepared using 24ml of 100% ethanol to the M-Wash buffer concentrate.

For the bisulphite conversion, 5µl of M-Dilution Buffer was added to the 1µg DNA sample to reach a total volume to 50 µl with water. The sample was mixed, incubated at 37°C for 15 minutes and then had 100 µl of the prepared CT conversion reagent added to each sample, which was then mixed.

The samples were then incubated in a Mastercycler Nexus (Eppendorff) thermocycler at 95ºC for 20 cycles for 30 seconds; 50ºC for 15 minutes and then at 4ºC (for the holding step). Thereafter, 400µl of M-Binding Buffer was added to a Zymo-Spin™ IC Column. The column was placed into a collection Tube. The sample from the previous step was placed into the Zymo-Spin™ IC Column with M-Binding Buffer. The closed tube was mixed by inverting the column several times. Thereafter, the samples underwent centrifugation for 30 seconds and any flow-through was discarded. This was followed by the addition of 200 µl of M-Wash Buffer to the column, followed by centrifugation for 30 seconds. The column had 200 µl of M-Desulphonation Buffer added and was left to stand at room temperature for 15 minutes. Following incubation, the mixture was centrifuged for 30 seconds and 200 µl of M-Wash Buffer was added to the column. This was then followed by centrifugation at full speed for 30

seconds; an additional 200 µl of M-Wash Buffer was added and then centrifuged again for 30 seconds. The column was placed into a 1.5 ml microcentrifuge tube. Thereafter, 20 µl of pre-warmed M-Elution Buffer was added to the column matrix and incubated for a minute at room temperature; followed by centrifuging for 30 seconds.

3.6.3. POLYMERASE CHAIN REACTION (PCR)

The DNA then underwent PCR using a pre-mixed cocktail that was prepared according to the manufacturer's protocol. In each PCR reaction, a no-template control (NTC) was run to identify any false positive reactions. Eight (8)µL of the cocktail was placed into a 96-well microtiter plate and 2 µL of 10ng of Bisulfite added to each well. The plate was centrifuged at 1000 rotations per minute. PCR was undertaken with the following cycling program: 94°C for 4 minutes, 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 60 seconds, repeat 94ºC for 20 seconds, 45 more times, 72°C for 3 minutes and lastly 4°C (hold).

Following PCR, the samples were treated with shrimp alkaline phosphatase which was prepared according to the manufacturer's instructions. The shrimp alkaline phosphatase (SAP) was used to cleave phosphate groups from the 5' terminus. Treatment with SAP was performed in order to remove remaining, un-incorporated deoxyribonucleotide triphosphate from the amplification products. Two (2)µl of the shrimp alkaline phosphatase solution was added to each PCR product containing well and incubated at 37°C for 20 minutes, 85°C for 5 minutes and 4°C (hold).

3.6.4. *IN-VITRO* TRANSCRIPTION AND CLEAVAGE

The in-vitro RNA transcription and cleavage master mix were prepared according to the manufacture's instruction. Two (2) ul from the shrimp alkaline phosphatase treated plate (total vol. 9 ul) was transferred to a 96 well plate containing 5ul of T Cleavage

Transcription/RNase A cocktail. The mixture then underwent vortexing and centrifugation and incubation of the plate at 37°C for 3 hours. The process of in-vitro transcription and cleavage were performed in one step using the PCR product following shrimp alkaline phosphatase addition. Using the PCR product as a template, single stranded DNA molecules were made by polymerases which targeted the promoter tags attached to PCR primers. In the same reaction RNaseA was used to cleave the reverse transcripts where uracil (U) was translated to thymidine (T) on DNA.

Thereafter, a clean-up step was performed by the addition of resin to primer extension reaction products. The resin allowed for the removal of sodium, potassium and magnesium salt, so that these ions would not result in background noise in the mass spectra and their removal thus improved mass spectrometry assessment.

Analyte products from microtiter plates on were placed on SpectroCHIPs. Twelve (12) nl of the cleaved products were added to A 3-hydroxypicolinic acid matrix for MALDI-TOF and arrayed onto existing matrix spots on the silica chip. This process used the Nanodispenser according to the manufacturer's instructions.

3.6.5. MASS SPECTROMETRY CLEAVED PRODUCT DETECTION

In this final step, the cleaved products were detected using the MassARRAY compact mass spectrometer and real-time software by Agena. Specific patterns, assessed by MALDI-TOF mass spectrometry, were created from different cleavage products of methylated or unmethylated target regions. The relative amount of methylation was calculated by a comparison of signal intensity between mass signals from unmethylated and methylated template DNA. A percentage of methylation for each case was calculated and depicted in an Epigram (Appendix 3).

3.7. THE *ONESTEP* qMETHYL™ KIT FOR PCR

This kit and the protocol by which the tests were performed, were developed by the Zymo Research corporation, Irvine, California.[103] The kit allowed for the detection of a single region of the MLH1 promoter region. As the C-region of MLH1 is short, and based on the chemistry of the kit, the C-region could not be assessed. However, the D-region could be analysed.

This kit was used for the identification of specific regions of DNA methylation using amplification of methylated cytosines in CpG dinucleotides. During this process, the DNA test samples were divided into two parts; namely a test reaction and a reference reaction. For the test reactions, DNA was digested with Methylation Sensitive Restriction Enzymes (MSRE), whereas DNA in the reference reaction was not digested with these enzymes. DNA from both samples then underwent real-time PCR amplification using a fluorescent dye and were then quantitated. Cycle threshold (Ct) values for both the test and reference DNA were noted and varied depending on methylation status. Large Ct differences were seen with non-methylated DNA.

Each kit allowed for 22 samples of DNA to be assessed in duplicate, using a 96-well plate. In order to validate the MSRE digestion/real-time PCR step, the provided samples of methylated and non-methylated DNA standards were used.

Test and reference reaction master mix were made according to the manufacturer's instructions, to accommodate the DNA samples and the methylated and non-methylated DNA standards. Thereafter, 15 µl of the reference reaction and test master mix were added to a 96-well real-time PCR plate, followed by the addition of 5 µl of the appropriate DNA sample to those selected wells in duplicate and then transferred to a Mastercycler Nexus real-time PCR instrument. Real-time PCR was performed using the following temperatures and times:

MSRE digestion at 37ºC for 2 hours, initial denaturation at 95ºC for 10 minutes, denaturation at 95 ºC for 30 seconds, annealing at 54ºC for 60 seconds, extension at 72 º for 60 seconds and final extension at 72ºC for 7 minutes followed by a hold step at 4ºC for more than 5 minutes. Denaturation, extension and annealing were performed for 40 cycles (Appendix 4).

Thereafter, the methylation levels were calculated using the following equation:

Percent Methylation = 100 x $2^{-\Delta Ct}$ where ΔCt = the average Ct value from the test reaction minus the average Ct values from the reference reaction.

3.8. STATISTICAL ANALYSIS

Data from various observations had been entered into a Microsoft Excel 2013 programme. Statistical analyses were undertaken utilising STATA version 15 (StataCorp LP, College Station TX).

Categorical variables were presented as frequencies, proportions, percentages and charts. Normally distributed continuous variables were presented as means and standard deviations whilst non-normally distributed continuous variables were presented as medians and interquartile range. According to the central limit theorem, a sample size of >30, the sampling distribution tends to be normal.[104]

Age was categorised as <60 and ≥60 years. In general, 50 years of age is generally accepted as the age of menopause[105], however, an age cut-off of 60 was used as a low percentage of patients (2/145) were under the age of 50.

Pearson's Chi-squared or Fischer's exact tests were used to compare categorical variables.

The Mann-Whitney U test was utilised to compare median values among categorical variables while the Student's t-test was used to compare mean values of categorical variables with 2 categories. Similarly, Kruskal-Wallis test and Analysis of Variance (ANOVA) were

used for non-normally and normally distributed continuous variables among categorical variables with more than 2 categories.

Sensitivity and specificity analyses (based on the confusion matrix) was used to assess the degree of sensitivity of each of the procedures in comparison to tests that are regarded as the gold standard.[106] The degree of agreement is calculated using: k = (Observed agreement - Chance agreement) / (Maximum agreement - Chance agreement),[106] or k= (total accuracy-random accuracy)/(1-random accuracy).[107] A diagnostic test is expected to have a high degree of sensitivity and specificity. This type of test should have both low false positive and negative results.

Cohen's Kappa was used to assess agreement/concordance between and among the different test procedures such as IHC and PCR for microsatellite instability as well as BRAF IHC, PCR and Sequencing. Cohen's kappa test is used for assessing inter-rater agreement where the rating is categorical such as yes/no for the same measured item. In this study, the measured item is the tumour and the "raters" are the diagnostic procedures such as IHC, PCR and Sequencing. A high value indicates agreement (consistency) between the procedures in terms of detecting the presence of mutations. In a perfect setting, each diagnostic test should identify the same mutated measured items (tumour) and should be negative for cases where there is no mutation). A lower value, however, indicates discord results in the testing procedures, thus suggesting poor consistency.

Table 5. Interpretation of Cohen's kappa values.[108] (Reproduced and adapted with permission from Dr. M. McHugh)

Kappa value	Level of agreement	% of data that are reliable
0-0.20	None	0-4
0.21-0.39	Minimal	4-15
0.40-0.59	Weak	14-25
0.60-0.79	Moderate	35-63
0.80-0.90	Strong	64-81
>0.90	Almost perfect	82-100

p-Values <0.05 were deemed significant.

3.9. STUDY LIMITATIONS

1. Slides and/or tissue blocks of some cases were not found due to filing errors.
2. Due to the weak Rand, the cost of consumables had unfortunately increased thus adding to the preliminary costs. Additional funding had to be sought.
3. It is not appropriate to generalise results from this localised study to the general South African female population as the patient samples were only from the greater Johannesburg area.

CHAPTER 4

4.0. RESULTS

Table 6. Age and pathological distribution of the study samples.

Parameter/Characteristic	Number (%) (n=145)	
Age (years) Mean ± SD <60 years (reproductive age group) ≥ 60 years (post-menopausal age group)	65.24 (±9.2) years 48 (33.1) 97 (66.9)	
Tumour FIGO grade		Mean Age +/- SD *
1	44 (30.34)	64.4+/- 7.8
2	63 (43.45)	65.5 +/- 10.2
3	38 (26.21)	65.8+/- 9.2
Deficient staining: IHC total number of cases MLH1 MSH2 MSH6 PMS2 Proficient staining	41 (28.28) 36 (24.83) 2 (1.38) 5 (3.45) 20 (13.79) 104 (71.72)	
Mutation: PCR MSI-Low MSI-High Microsatellite stable No result	46 (31.7) 35 (24.1) 11 (7.6) 94 (64.8) 5 (3.5)	
Abnormality by IHC or PCR No abnormality by IHC or PCR	66 (45.51) 79 (54.48)	
BRAF (n=37) Mutation by IHC Mutation by PCR Mutation by Sanger Sequencing	5 (16.21) 1 (2.7) 4 (10.81) 1 (2.7)	
Methylation (n=52) Methylation by EpiTYPER Methylation by OneStep qMethyl™ Kit	 40 (76.92) 36 (69.23)	

*Analysis of variance p-value = 0.76

4.1. CLINICAL PARAMETERS

4.1.1. AGE AND PATHOLOGICAL DISTRIBUTION OF THE STUDY SAMPLES.

Table 6 shows that the mean age of the 145 patients with endometrial tumours over the study period, was 65.24 (±9.2) years. About two-thirds (66.9%) of patients were menopausal and were 60 years or older. Furthermore, 30.34% were FIGO grade 1 tumours, 43.45% were

FIGO grade 2 tumours and 26.21% were FIGO grade 3 tumours. Although not reaching statistical significance, the results indicate an increasing trend in age with tumour FIGO grade ($p = 0.76$)

Forty-one (28.28%) cases showed deficient staining by immunohistochemical analysis and were thus regarded as demonstrating mismatch repair deficiency. In contradistinction, 71.72% of cases showed retained staining. Of the 41-mismatch repair deficient cases, 25.52% showed MLH1 loss, 1.38% had MSH2 loss, 3.45% had MSH6 loss and 13.79% had PMS2 loss.

Forty-six (37.1%) specimens from patients demonstrated mutation/microsatellite instability by PCR. Of the 46 cases, 24.1% were microsatellite low and 7.6% were microsatellite high. Microsatellite instability was detected in 64.8% of cases and 3.5% of cases did not generate a result.

Abnormalities were identified by either IHC or PCR (or were abnormal overall), in 45.51% of cases. The remaining 54.48% of cases demonstrated no abnormality by either test. Only 6 (16.22%) cases showed a BRAF mutation by IHC, PCR or Sanger sequencing.

Out of 52 comparable cases, methylation was identified in 40 (76.92%) of cases by EpiTYPER analysis and 36 (69.23%) using the OneStep qMethyl^{TM} kit.

4.2. MISMATCH REPAIR IMMUNOHISTOCHEMISTRY

Collectively, each of the 145 cases were subjected to four immunohistochemical stains rendering a total of 580 tissue sections. Of the 580 immunohistochemically stained sections, 64 (11.03%) tissue sections showed loss of nuclear staining and were thus deemed mismatch repair deficient (Table 7A). This table demonstrates that the most commonly identified MMR deficient IHC marker was MLH1 (n=37/145, 25.52%)

Table 7. The number of deficient immunohistochemical stains per marker (A) and the number of mismatch repair deficiencies per immunohistochemical marker per patient sample (B).

A. Number of deficient stains per immunohistochemical marker	
IHCs	**MMR deficiency, n=145, (%)**
MLH1	37 (25.52)
MSH2	2 (1.38)
MSH6	5 (3.45)
PMS2	20 (13.79)
Total	64 of 580 (11.03) tissue sections
B. Mismatch repair deficiencies per immunohistochemical marker per patient sample.	
IHCs	**MMR Deficient n=145, (%)**
MLH1 only **MLH1 and PMS2**	16 (11.03) 20 (13.79)
MSH6 only **MSH6 and MSH2** **MSH6, MLH1 and PMS2**	2 (1.38) 2 (1.38) 1 (0.69)
Total	41 (28.28)

Table 7B shows the number of deficient staining for each of the immunohistochemical stains. Twenty (13.79%) cases of the 145, showed deficiency for both MLH1 and PMS2. Two cases (1.38%) showed deficiency for both MSH2 and MSH6. Sixteen cases (11.03%) showed loss of only MLH1. Two cases (1.34%) were deficient for MSH6 alone and one case (0.69%) was deficient for three immunohistochemical markers, namely MLH1, PMS2 and MSH6. Thus, out of 145 cases, 41 (28.28%) demonstrated loss of staining of one or more markers by immunohistochemistry and were mismatch repair deficient (table 6). Conversely, 104 (71.72%) cases showed retention of mismatch repair stains.

4.2.1. MLH1

Of the 145 cases of endometrioid endometrial carcinoma, 108 (74.5%) had retained nuclear staining with MLH1. Thirty-seven (25.52%) showed loss of nuclear staining of tumour cells (figures 15, 16 and 17).

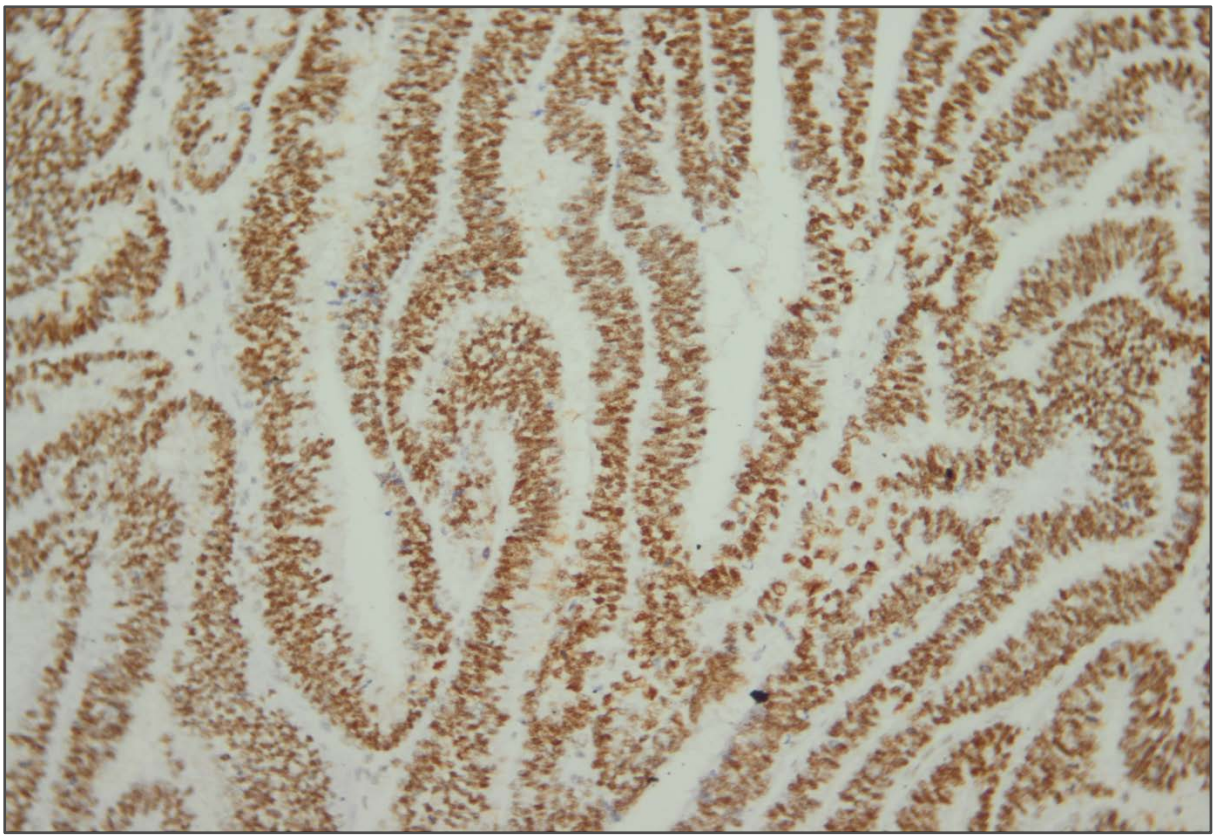

Figure 15. Retained nuclear staining in tumour cells on an MLH1 stain. 4µm MLH1 stained section. Original magnification: 200x

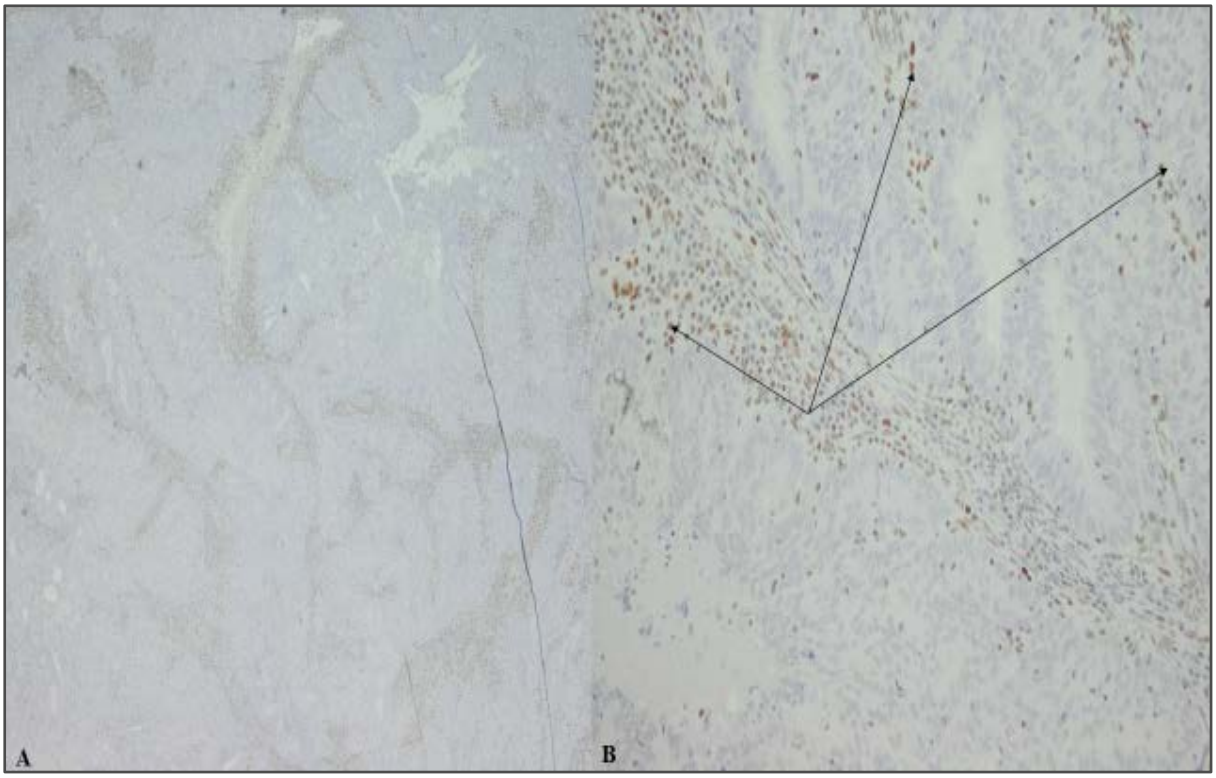

Figure 16. Loss of MLH1 staining in tumour nuclei. The lymphocytes and stromal cells serve as an internal control and are positive (arrows). 4µm MLH1 stained sections. Original magnification 40x (A) and 400x (B).

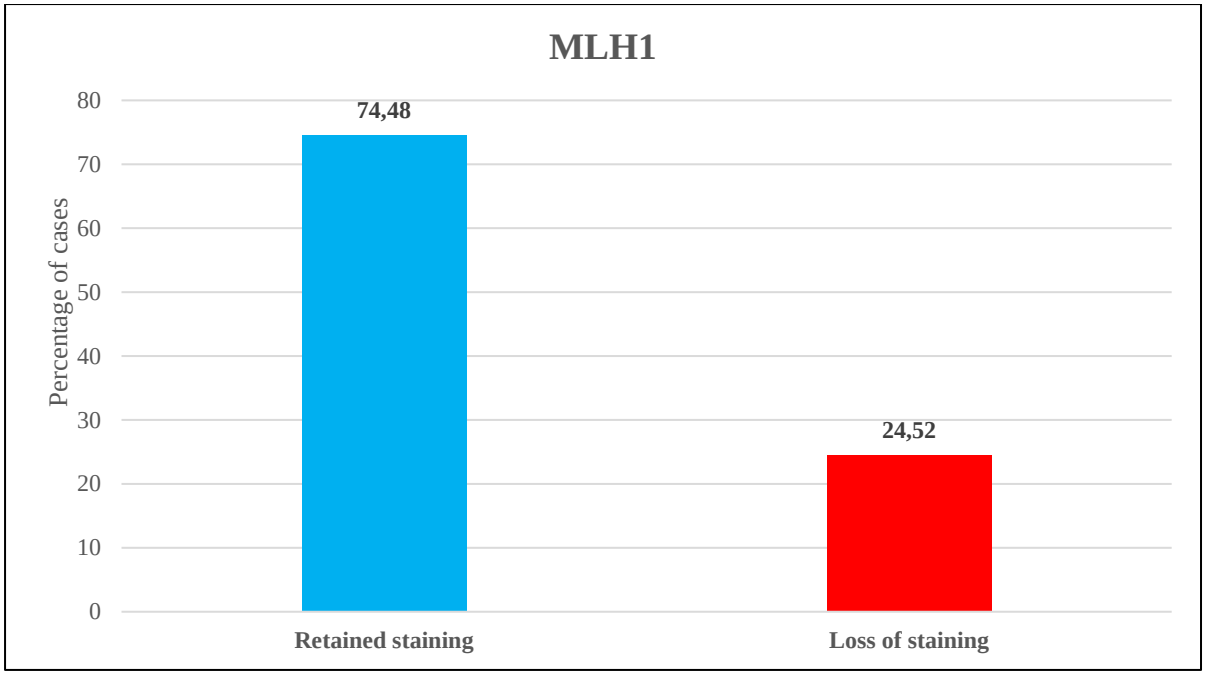

Figure 17. MLH1 staining of endometrial carcinomas.

4.2.2. MSH2

There were 2 (1.38%) out of 145 cases that showed loss of MSH2 staining. One hundred and forty-three cases (98.62%) demonstrated retained nuclear positivity (figures 18, 19 and 20).

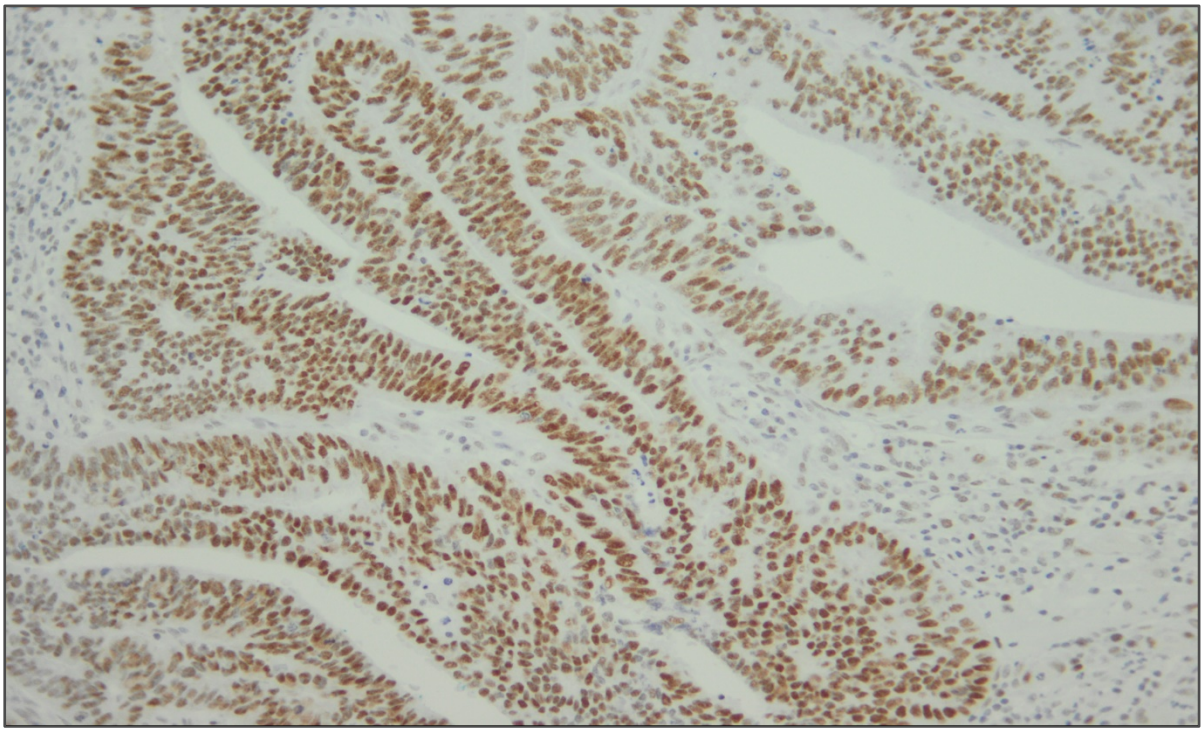

Figure 18. Retained nuclear staining of tumour cells is noted on an MSH2 stain. 4µm MSH2 stained section. Original magnification: 200x

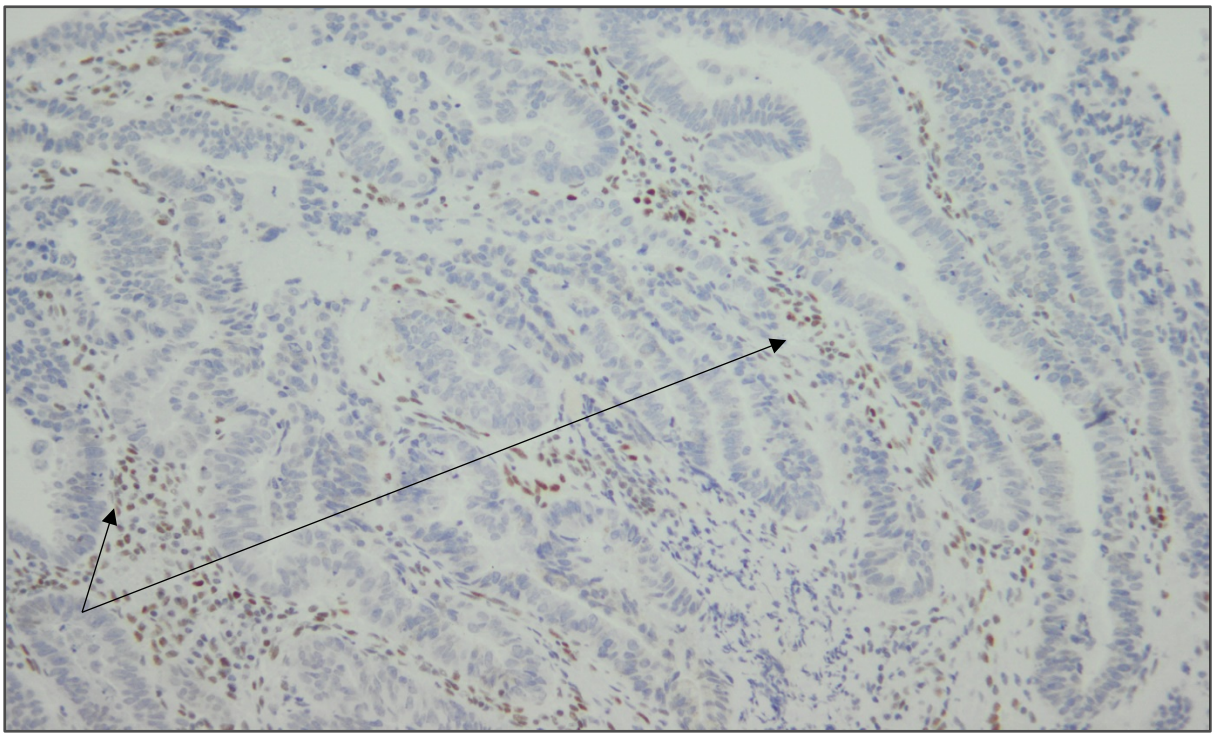

Figure 19. Lymphocytes and stromal cell nuclei show retention of staining (arrows) whilst tumour nuclei show loss of staining. 4µm MSH2 stained section. Original magnification: 200x

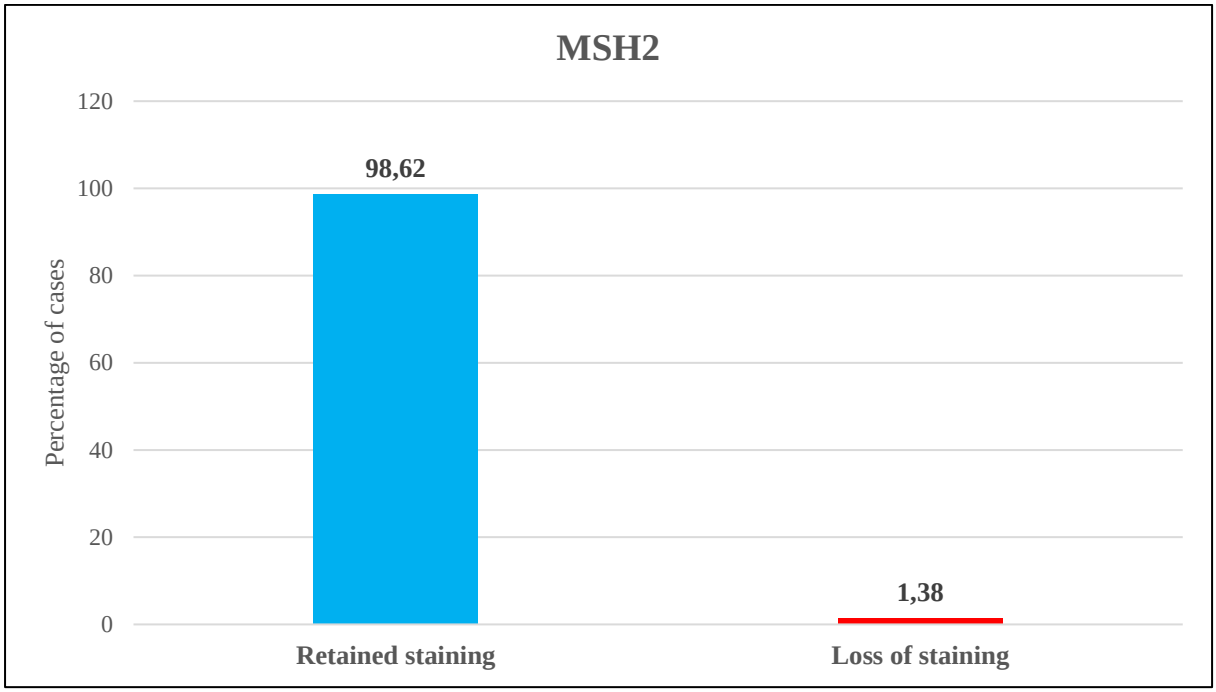

Figure 20. MSH2 Staining of endometrial carcinomas.

4.2.3. MSH6

Five (3.45%) of the 145 cases (3.45%) showed loss of MSH6, whilst 140 (96.55%) cases showed retention of staining (figures 21, 22 and 23).

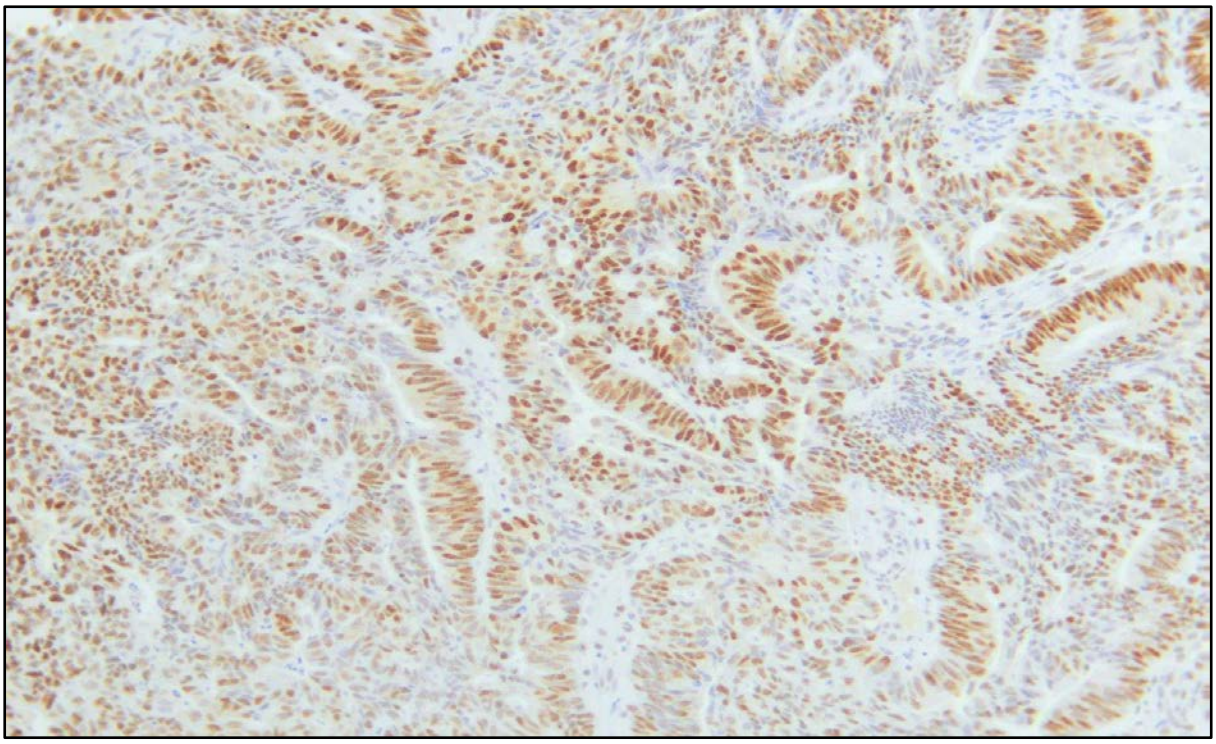

Figure 21. A retained nuclear signal is noted in the tumour cells. 4µm MSH6 stained section. Original magnification: 200x

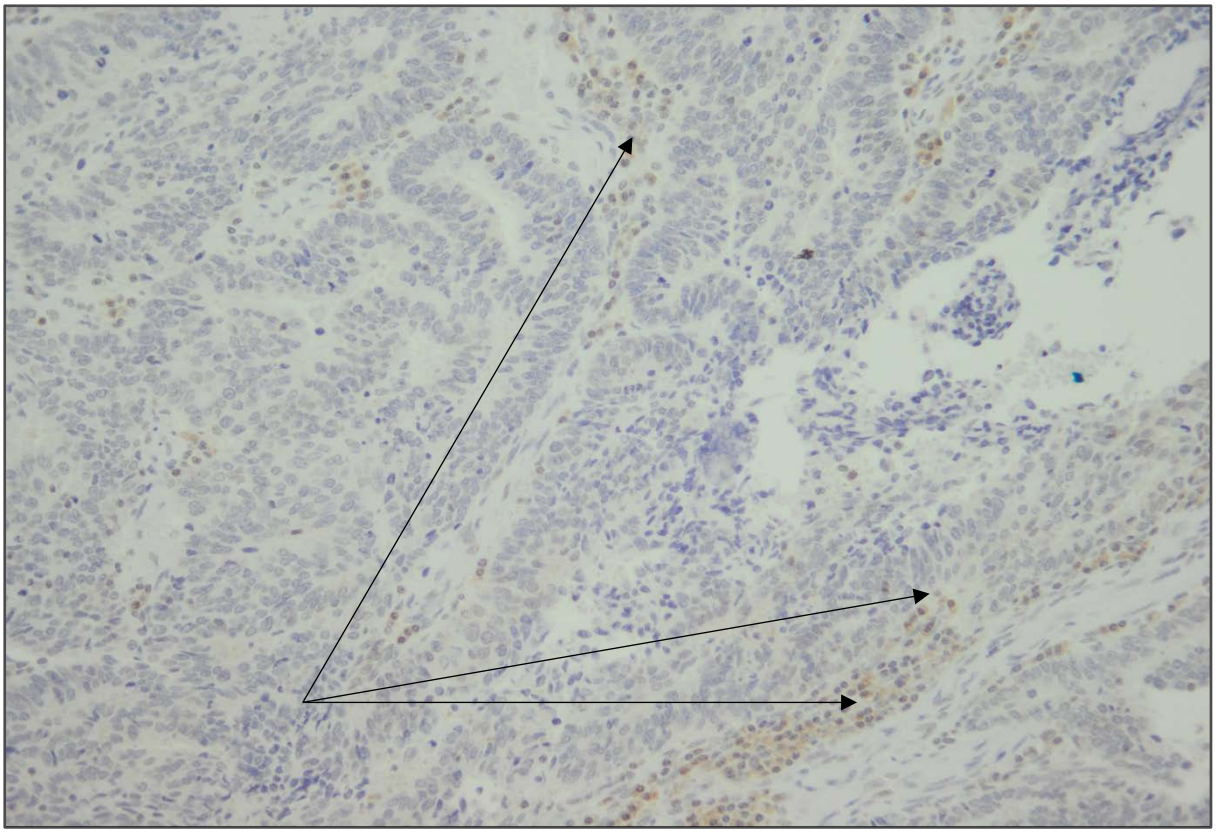

Figure 22. Lymphocytes and stromal cell nuclei show retained staining (arrows). The tumour nuclei show loss of staining. 4µm MSH6 stained section. Original magnification: 200x

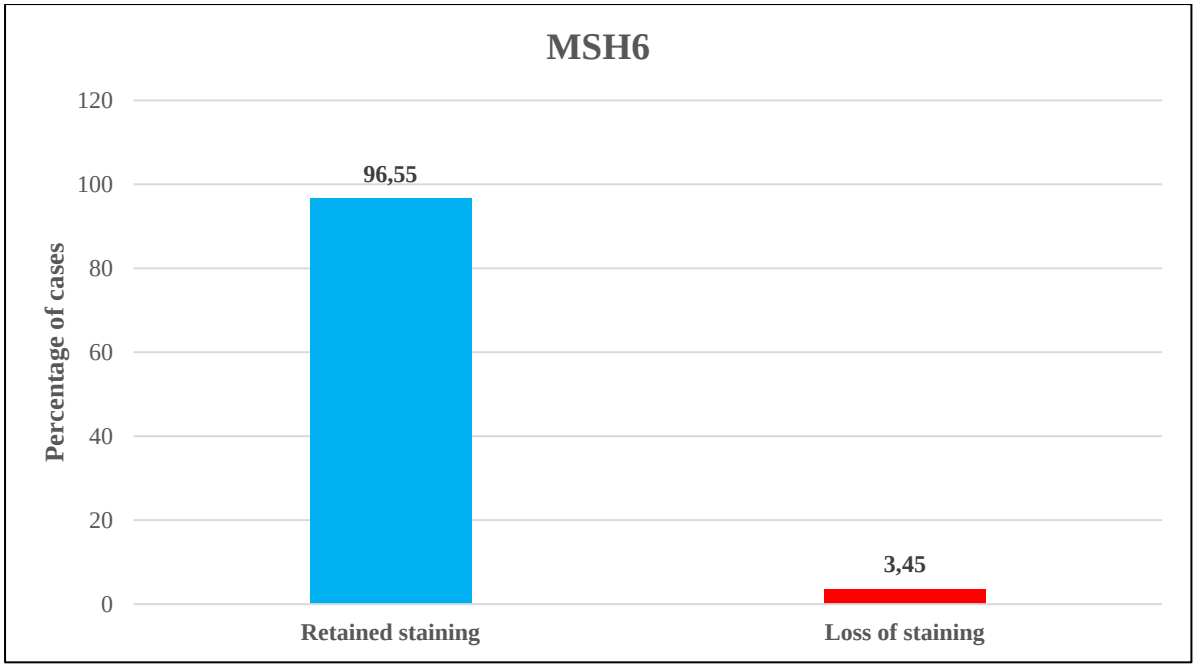

Figure 23. MSH6 Staining of endometrial carcinomas.

4.2.4. PMS2

Twenty-one cases (14.48%) demonstrated deficiency of PMS2, whilst one hundred and twenty-four cases (85.5%) showed retention of staining (figures 24, 25 and 26).

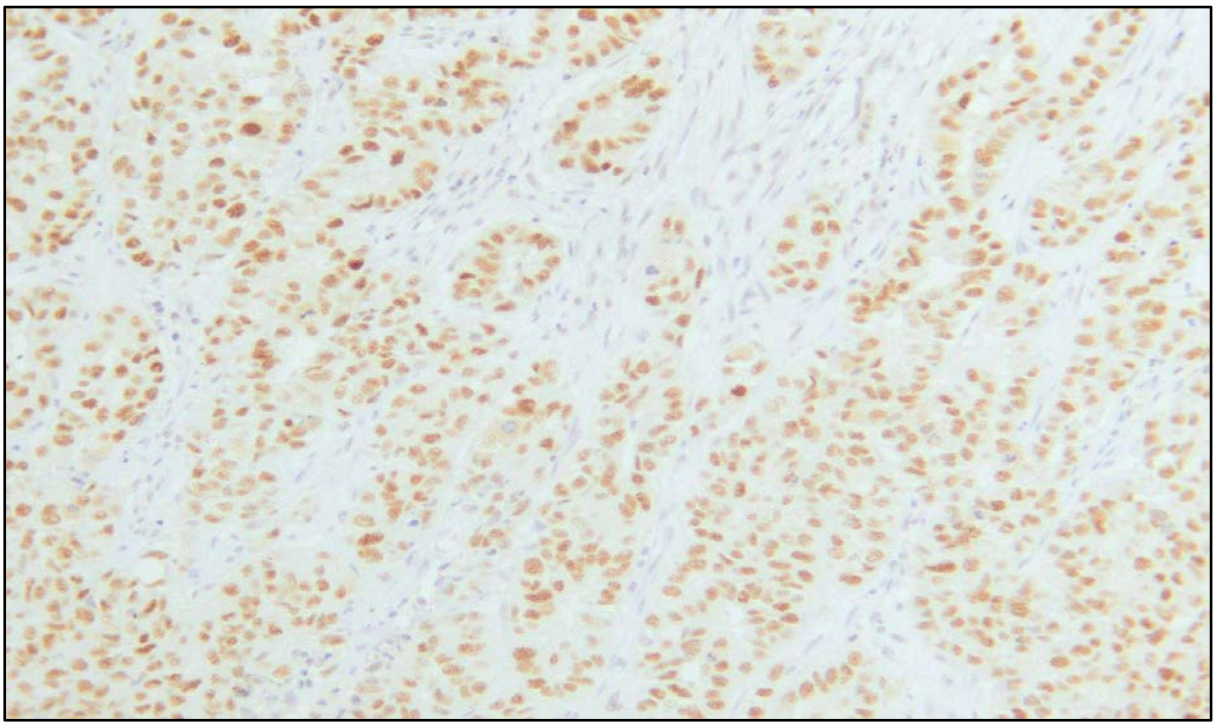

Figure 24. Retained staining of tumour nuclei is noted. 4μm PMS2 stained section. Original magnification: 200x

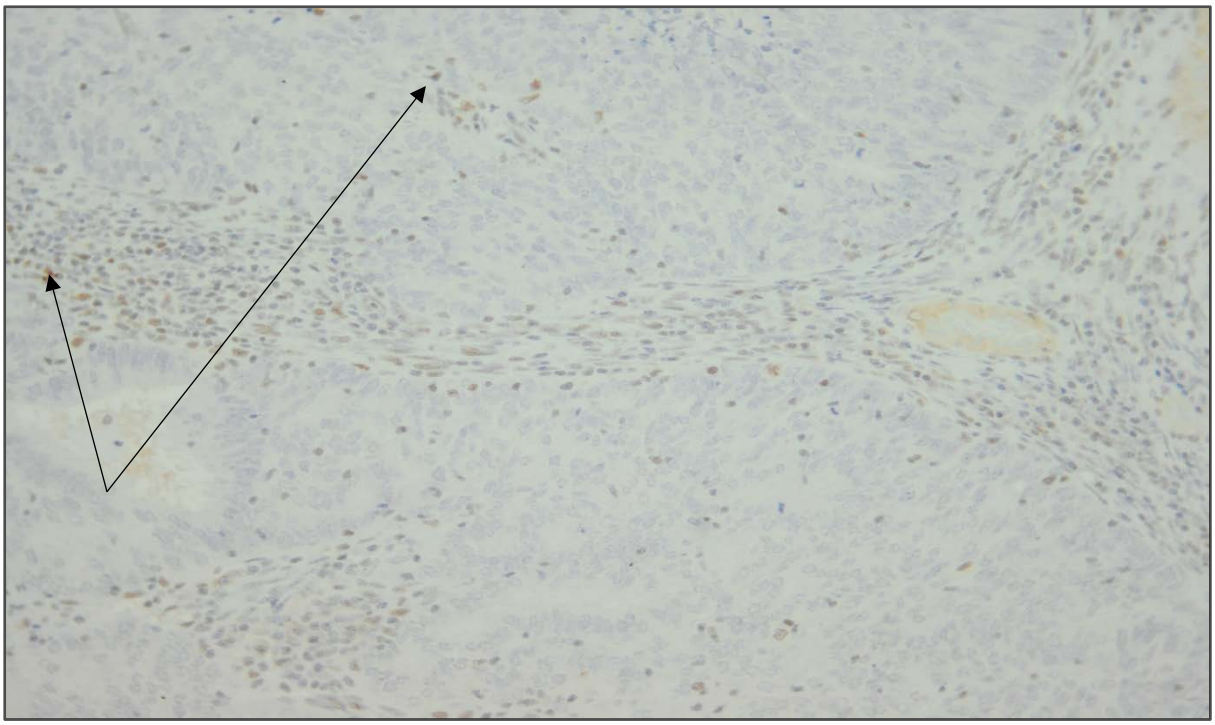

Figure 25. Lymphocytes and stromal cell nuclei show retained staining (arrows). The tumour nuclei show loss of PMS2 staining. 4μm PMS2 stained section. Original magnification: 200x

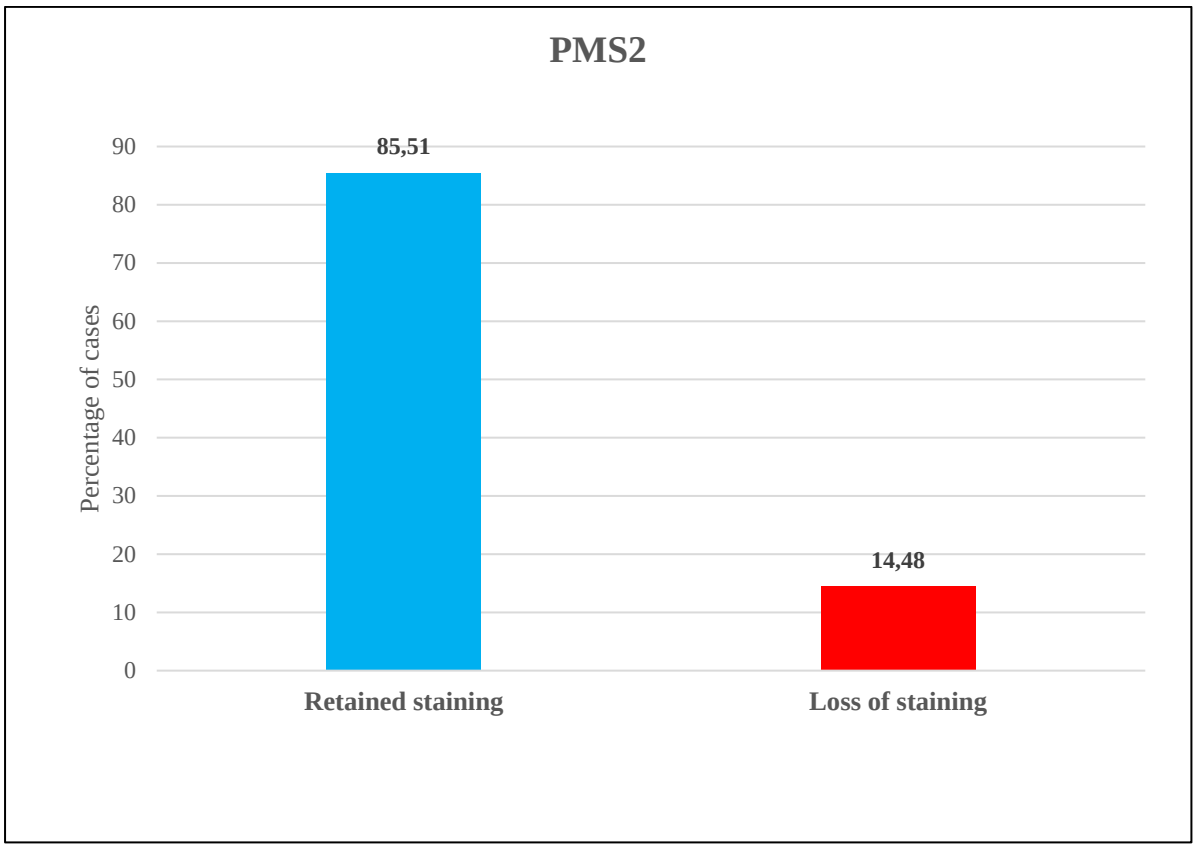

Figure 26. PMS2 staining of endometrial carcinomas.

4.2.5. FOCAL/WEAK STAINING OF IMMUNOHISTOCHEMICAL STAINS

There were 22 cases out of 145(15.17%) which showed focal or weak nuclear staining and were thus considered MMR proficient. Of these 22 cases, 4 (18.18%) showed variable staining for all 4 markers, 5 cases (22.73%) showed variable staining for both MLH1 and PMS2, 5 cases (22.73%) showed isolated variable staining for PMS2, 1 case (4.56%) showed variable staining for both MSH2/MSH6, 3 cases (13.64%) showed variable MSH6 staining, 1 case (4.56%) had isolated variable MSH2 staining, 2 cases (9.09%) had variable staining for both MSH6/PMS2 and 1 case (4.56%) showed variable staining for three stains namely MLH1/MSH6/PMS2 (Figure 27).

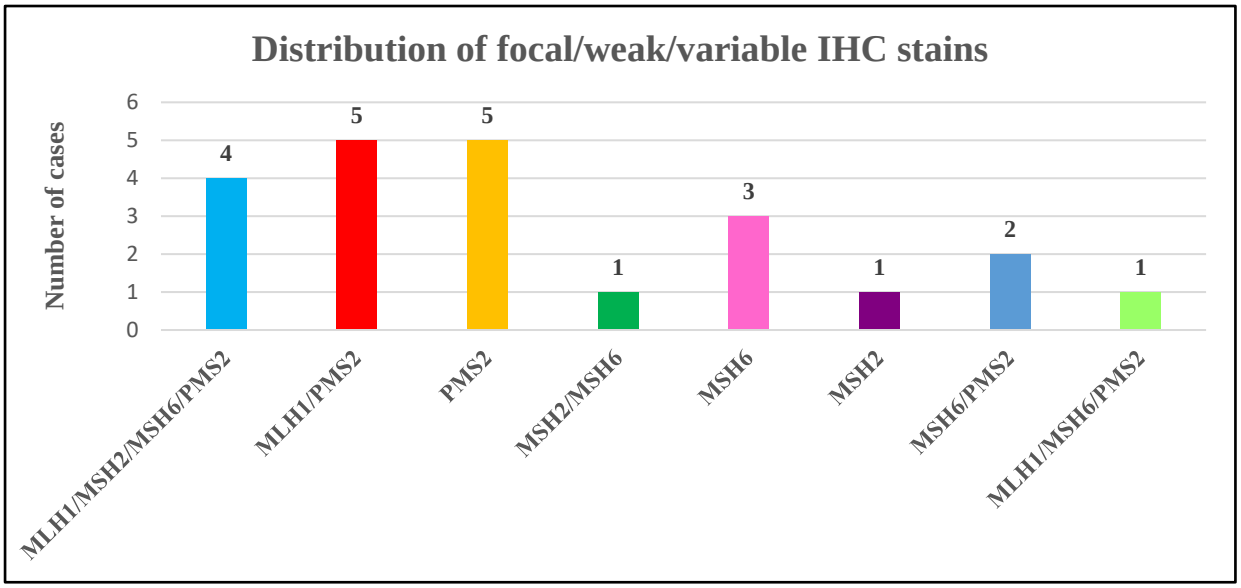

Figure 27. Distribution of immunohistochemical stains that demonstrated focal, weak or variable staining.

Figure 28 below highlights the cases that showed focal/weak staining immunohistochemically and were thus interpreted as MMR proficient, in relation to their MSI status by PCR and their methylation status. These cases did not undergo BRAF mutational analysis as they were positive for MLH1 irrespective of the variability of staining.

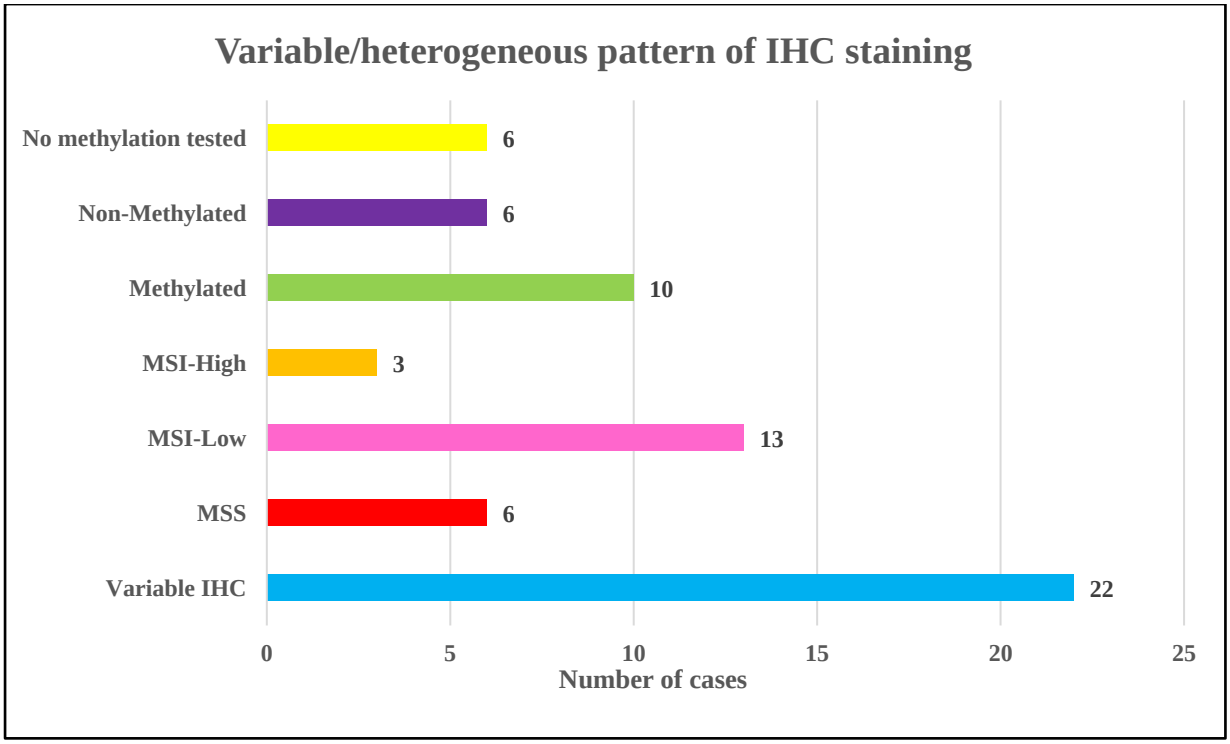

Figure 28. Heterogenous pattern of immunohistochemical staining.

4.3. COMPARISON OF AGE AND TUMOUR FIGO GRADE IN MUTATIONS IDENTIFIED BY IHC, PCR AND OVERALL MUTATIONAL DIAGNOSIS.

Table 8. Comparison of age and tumour FIGO grades of cases detected by IHC (A), PCR (B) and overall abnormality/mutational status (C).

8A. Comparison of age and tumour FIGO grades of cases with loss of nuclear staining and those with retained nuclear staining based on IHC.

Factor	**Retained staining**		**Loss of staining**		Total		Test statistics	P value
	N=104	**%**	**N= 41**	**%**	**N=145**	**%**		
Age (years) (Mean ± SD)	65.2± 9.1		65.3± 9.7		65.0 ± 9.2		-0.0619^	0.95
<60	33	31.7	15	36.6	48	33.1	0.313#	0.58
≥60	71	68.3	26	63.4	97	66.9		
Tumour FIGO grades								
FIGO grade 1	35	33.7	9	22.0	44	30.3	12.2#	0.002*
FIGO grade 2	36	34.6	27	65.9	63	43.5		
FIGO grade 3	33	31.7	5	12.2	38	26.2		

SD: Standard deviation #Chi-square test; ^ Student's t-test;

8B. A comparison of age and tumour FIGO grades of those with mutation and those without mutation based on PCR

Factor	No Mutation		Mutation		Total		Test statistics	P value
	N= 94	**%**	**N= 46**	**%**	**N=140**	**%**		
Age (years) (Mean ± SD)	64.8 ± 8.9		65.3 ± 9.9		65.0 ± 9.2		-0.2917^	0.77
<60	32	34.0	16	34.8	48	34.3	0.0075#	0.93

≥60	62	66.0	30	65.2	92	65.7		
Tumour FIGO grades								
FIGO grade 1	32	34.0	10	21.7	42	30.0	2.41[#]	0.30
FIGO grade 2	37	39.4	23	50.0	60	42.9		
FIGO grade 3	25	26.6	13	28.3	38	27.1		
SD: Standard deviation [#] Chi-square test; ^ student t-test;								

8C. Comparison of the age and tumour FIGO grades of those with abnormalities/mutation and those without abnormalities/mutation based on overall abnormality/mutational status

Factor	No abnormality		Abnormality		Total		Test statistics	P value
	N=75	**%**	**N= 65**	**%**	**N=140**	**%**		
Age (years) (Mean ± SD)	65.4± 9.1		64.5 ± 9.4		65.0 ± 9.2		-0.525^	0.6005
<60	23	30.7	25	38.5	48	34.3	0.9391	0.3003
≥60	52	69.3	40	61.5	92	65.7		
Tumour FIGO grades								
FIGO grade 1	27	36.00	15	23.08	42	30.00	7.90[#]	0.030*
FIGO grade 2	24	32.00	36	55.38	42	42.82		
FIGO grade 3	24	32.00	14	21.54	38	27.14		
SD: Standard deviation [#] Chi-square test; ^ Student's t-test;								

Two-sample t test with equal variances

For comparative purposes Table 8B and Table 8C have been placed here but will be discussed under their respective subsections.

Table 8A demonstrates that there were no statistically significant differences in the age of those who had deficient staining versus those who had retained staining (p=0.58). Thus, 68.3% of the 104 patients with retained staining were over 60 years of age, while of the 41 patients who showed loss of staining were detected by IHC, 63.4% of patients were over the age of 60 years.

There was a statistically significant association between the FIGO grade of the tumour and abnormality (p=0.002). Out of the 104 patients who had retention of staining by IHC, 33.7% had FIGO grade 1 tumours whereas 22% of 41 patients with a loss of staining had a FIGO grade 1 tumour. Of the 104 cases, 34.6% showed intact IHC staining but were identified as FIGO grade 2 tumours; whilst 65.9% of the 41 cases with deficient staining, showed FIGO grade 2 histological features. Therefore, there was approximately double the proportion of cases showing deficient staining with FIGO grade 2 features than those showing retained

immunohistochemical staining. Furthermore, 31.7% of 104 patients with retained staining were FIGO grade 3 tumours while 12.2% of the 41 cases showing loss of IHC staining were identified as FIGO grade 3 tumours. Thus, the proportion of FIGO grade 3 IHC proficient tumours is approximately three times that of cases showing loss of staining.

Among 44 cases of FIGO grade 1 tumours; the proportion of those retained staining (n=35/44, 79.55%) were four times more than the proportion of those with loss of staining (n=9/44, 20.45%). Likewise, FIGO grade 3 tumours showed that the proportion of those with retained staining (n=33/38, 86.84%) were four times more than the proportion of those with loss of staining (n=5/38 13.16%) However, in FIGO grade 2 tumours, there was a slightly higher proportion of tumours with retained staining (n=36/63, 57.14%) as compared to those with loss of staining (n=27/63, 42.86%). (Table not shown.)

4.4. MICROSATELLITE INSTABILITY BY PCR

Out of the 145 cases, 94 (64.8%) were microsatellite stable and 34 (23.45%) had 1 mutation identified out of the five markers tested. Approximately 8.28% (12 cases) were deemed microsatellite high as they had 2 or more mutations out of the 5 markers examined. As such a total of 46 cases were microsatellite unstable (31.72%) (Figure 29). Microsatellite instability was determined by the identification of a shift in the length of the microsatellite due to deletion or insertion of a repetitive unit which subsequently resulted in a new length allele in the tumour tissue relative to the patient's normal DNA. (Figures 30-32). There were 5 cases (3.5%) that yielded no PCR reaction despite PCR having been performed on three occasions for all 5 cases. (Figure 33)

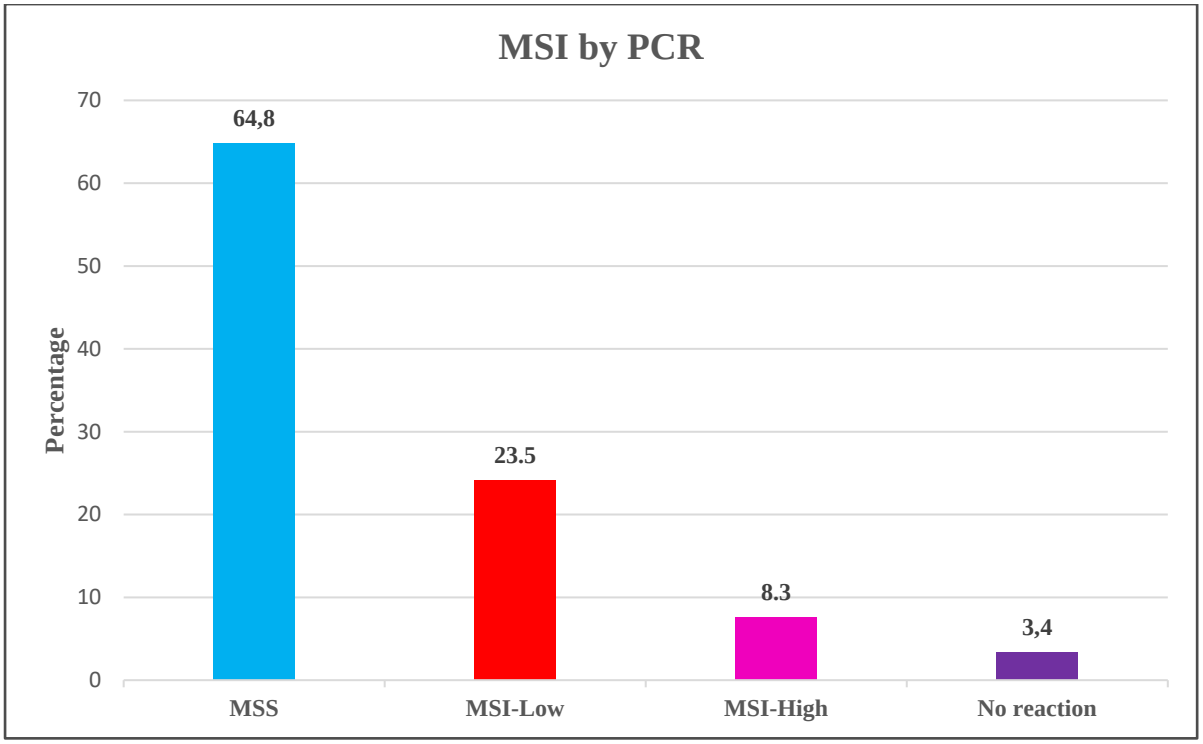

Figure 29. Microsatellite instability as demonstrated by PCR

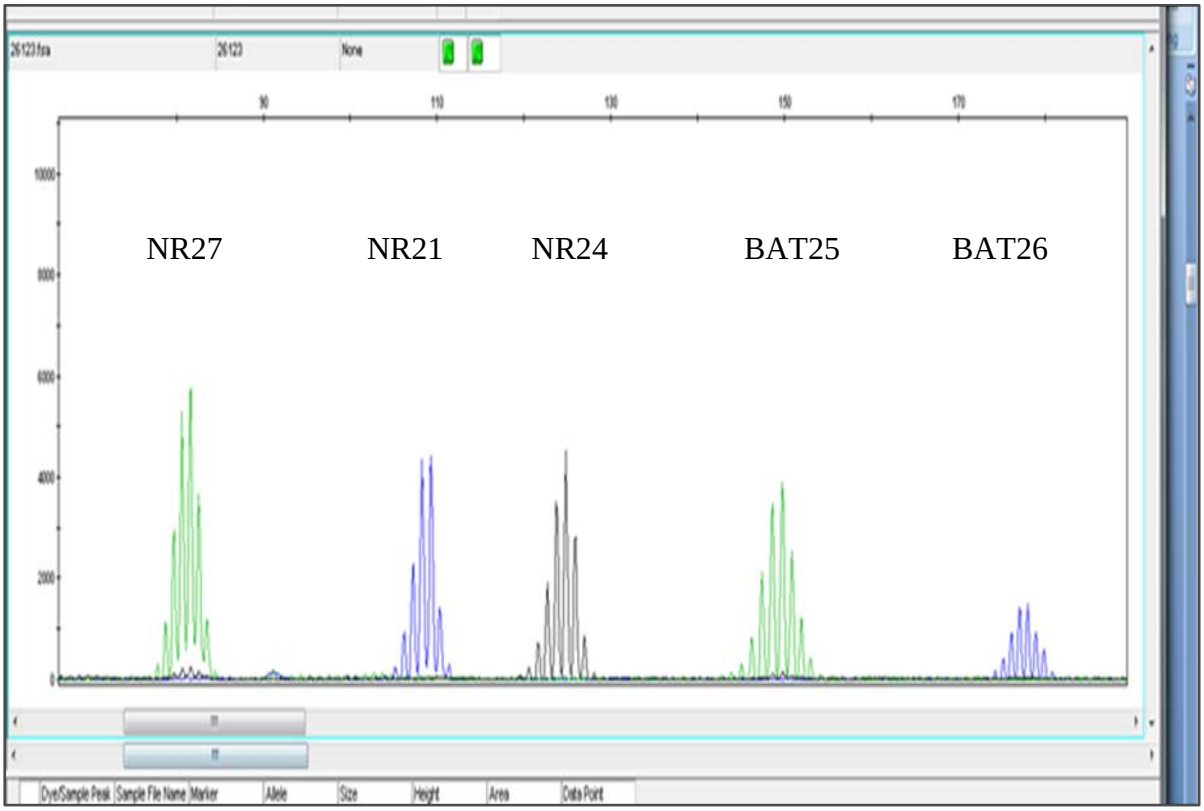

Figure 30. An electropherogram illustrating microsatellite stability in all five markers in an endometrial carcinoma.

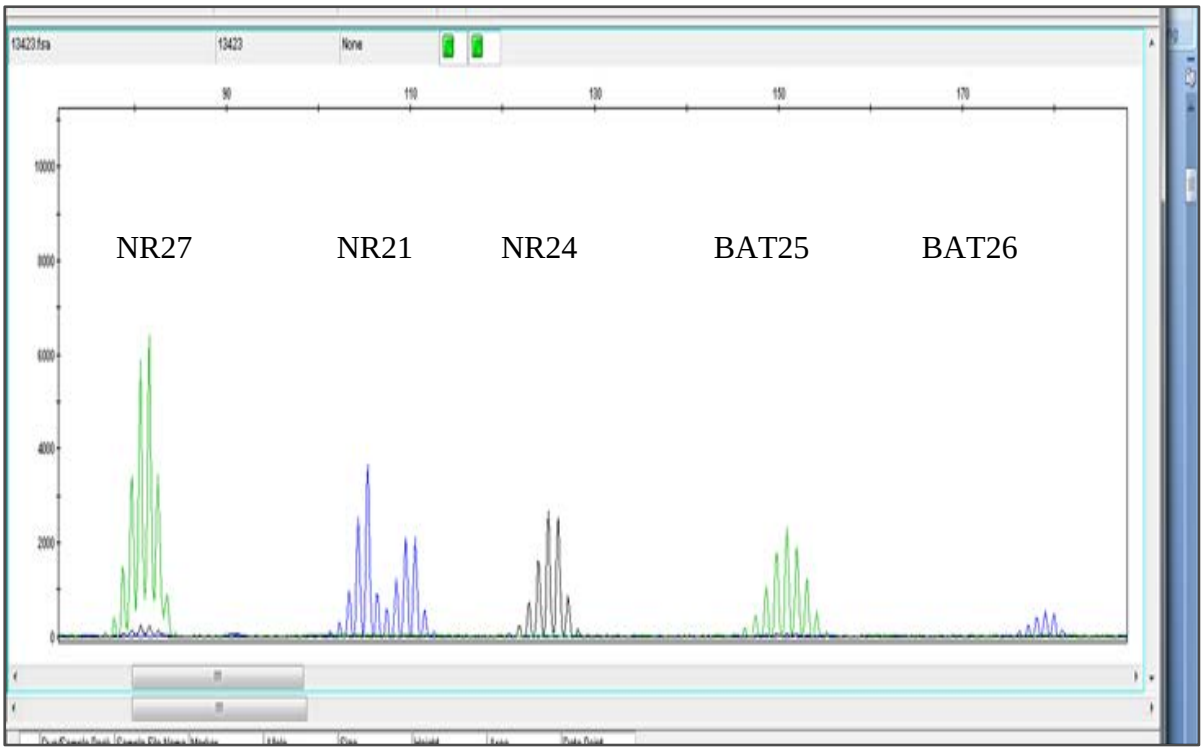

Figure 31. An electropherogram demonstrating a microsatellite-Low tumour. There is a mutation in NR21.

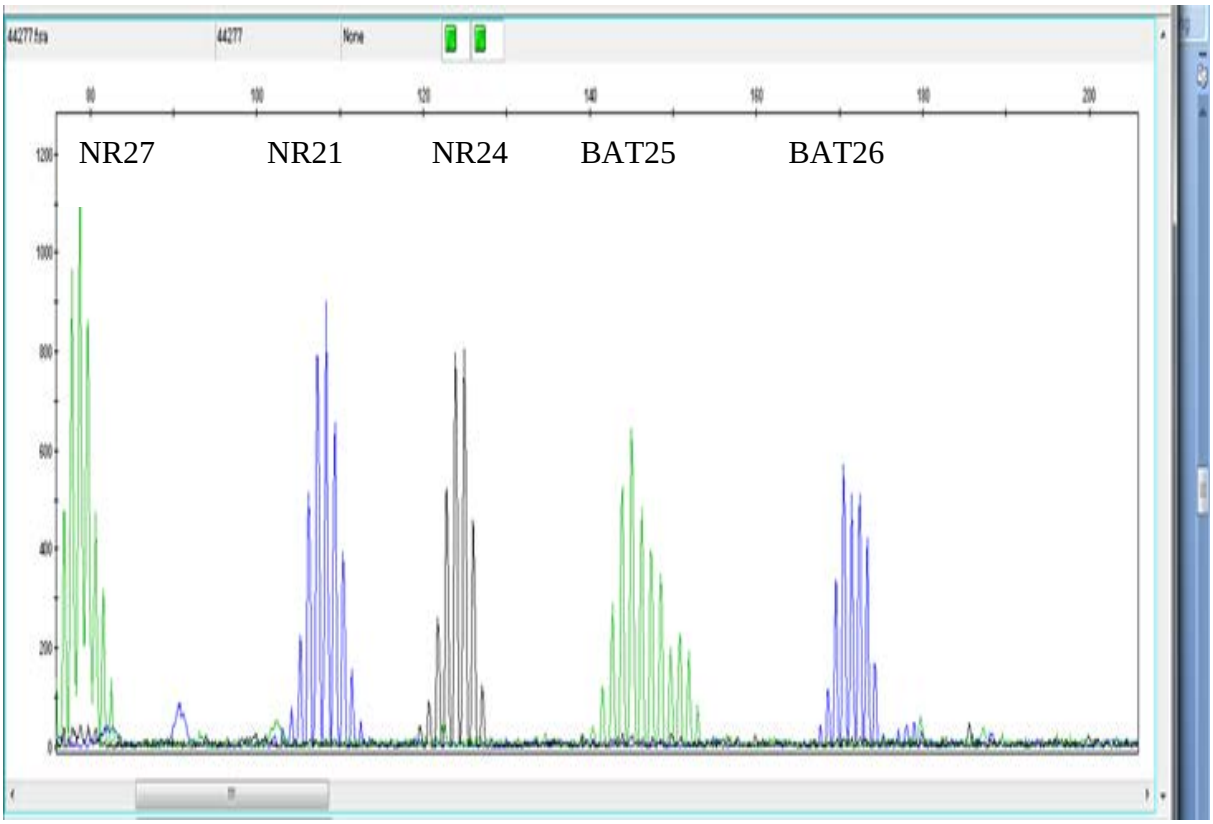

Figure 32. A microsatellite-high tumour is depicted in this electropherogram. Mutations are present in BAT25 and BAT26.

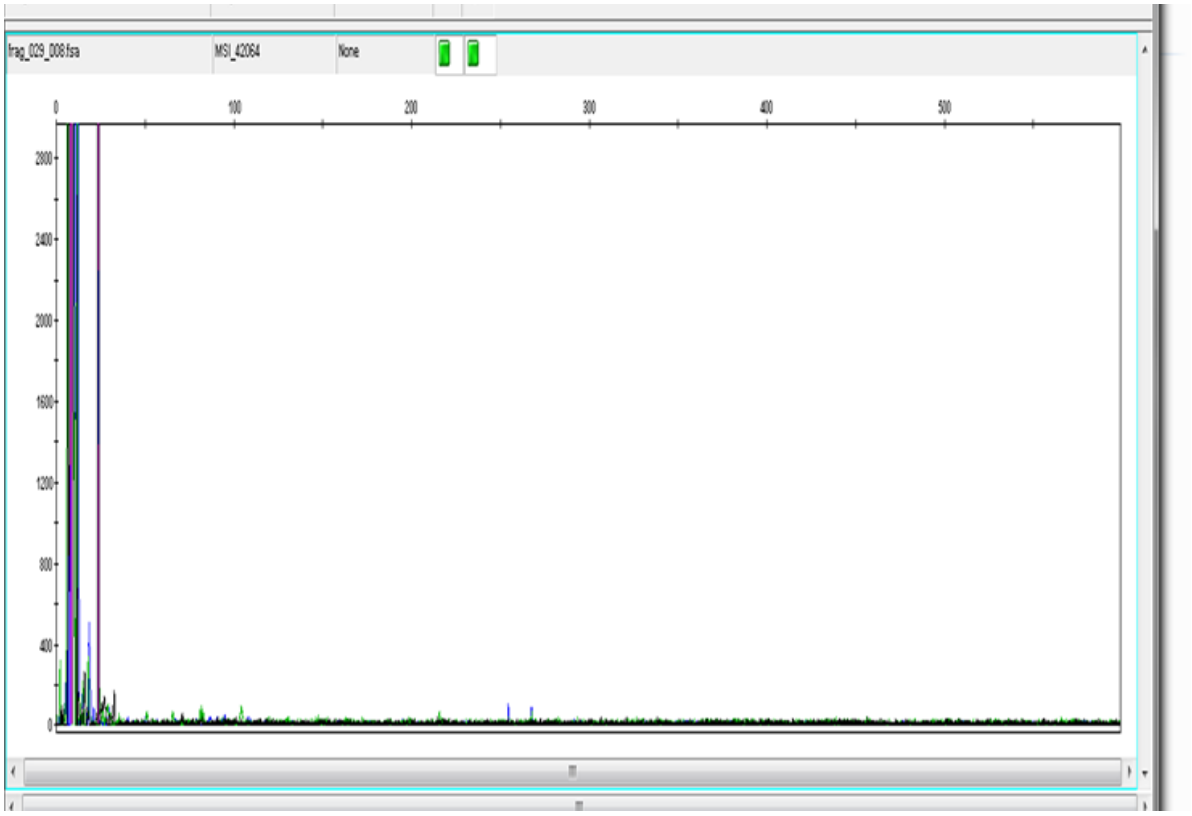

Figure 33. This electropherogram depicts one of the cases in which no result could be obtained.

Table 8B shows that of the 94 cases where no mutation was identified by PCR, 34% of patients were under the age of 60. Similarly, from the 46 cases that demonstrated a mutation, 34.8% were below the age of 60. There was no statistically significant difference in the mean age of cases diagnosed as having a PCR detected mutation as compared to those without a mutation. (p=0.77). Of the 94 cases without a detectable mutation, 34% showed FIGO grade 1 histological features whereas 21.7% of the 46 cases with a mutation were designated FIGO grade 1. Of the 94 microsatellite stable cases, 39.4% were FIGO grade 2 tumours whilst 50% of the 46 microsatellite unstable tumours were FIGO grade 2. A further 26.6% of the 94 non-mutated tumours showed FIGO grade 3 features whereas 28.3% of the 46 tumours with mutation were FIGO grade 3 tumours histologically. Proportionately, there were 1.5 times as many FIGO grade 1 tumours without mutation than those with a mutation. Similarly, there were 1.3 times as many FIGO grade 2 tumours with a PCR detected mutation than those

without mutation. However, the association between tumour FIGO grade and PCR detection of mutation did not reach statistical significance (p value = 0.30).

4.4.1. COMPARISON OF NUMBER OF MUTATIONS IN THE MICROSATELLITE HIGH GROUP OF TUMOURS

Out of the 12 cases (8.23%) that were identified as being microsatellite high, 9 (75%) harboured two mutations and 3 cases (25%) had 3 mutations (figure 34).

None of the cases contained 4 or 5 mutations.

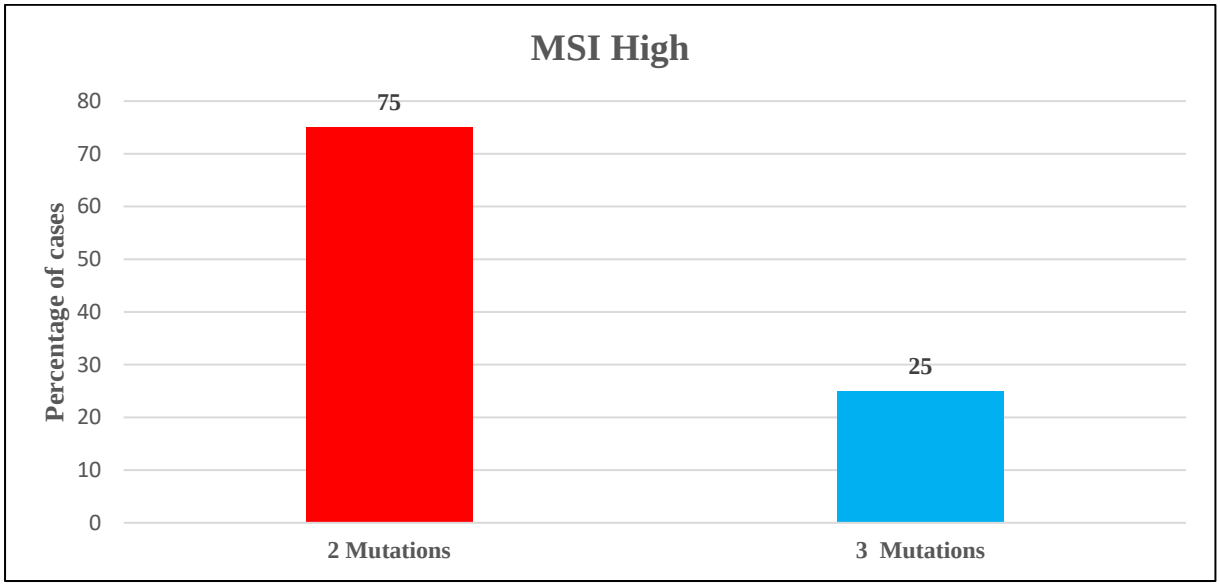

Figure 34. MSI-High tumours.

4.4.2. SUBTYPES OF VARIOUS MICROSATELLITE UNSTABLE MUTATIONS

Of the 46 microsatellite unstable cases, each with a maximum of 5 mutations, a total of 60 mutations was noted. Table 9 below summarises the numbers of the various mutations seen in each MSI marker. This is illustrated in figure 35.

Table 9. Numbers of mutations per molecular marker.

Molecular Marker	NR27	NR21	NR24	BAT25	BAT26
Frequency of mutations	12	10	3	30	5

The most frequently mutated marker was BAT25, followed by NR27, NR21, BAT26 and NR24 respectively.

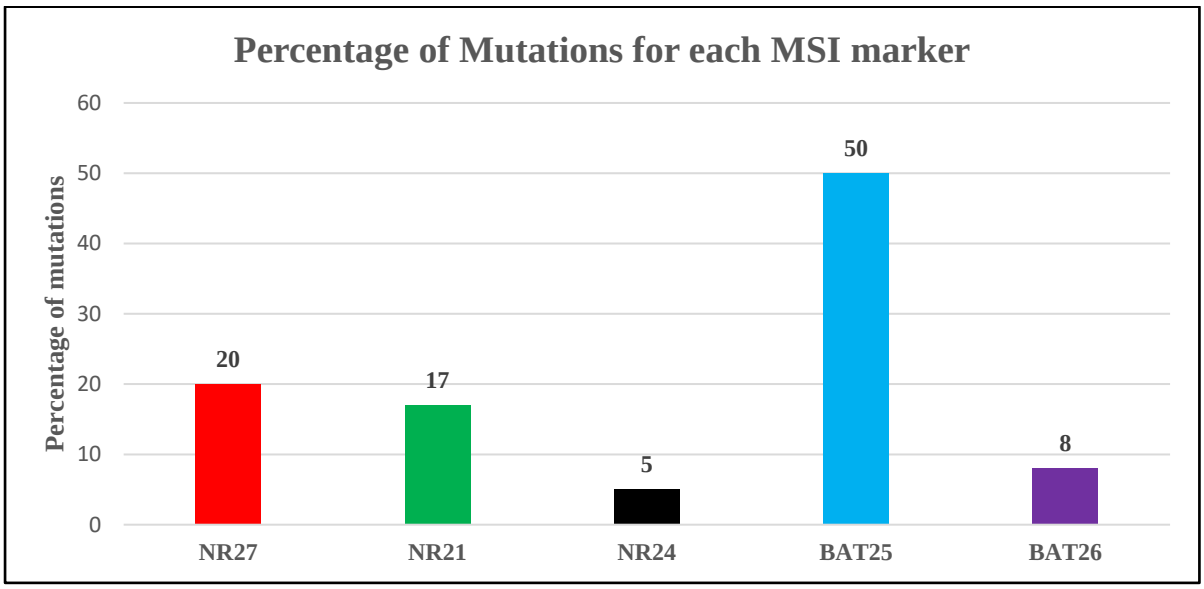

Figure 35. Percentage of mutations in each of the PCR markers

4.5. DETECTION OF MISMATCH REPAIR DEFICIENCY/MICRSATELLITE INSTABLITY BY IMMUNOHISTOCHEMISTRY VERSUS PCR

A total of 41 (28.28%) immunohistochemical stains out of 145 cases showed loss of staining; thus, demonstrating mismatch repair deficiency. In contrast, 46 (31.72%) out of 145 cases demonstrated microsatellite instability by PCR (table 6).

Five cases yielded no result by PCR despite having been performed on three separate occasions. Of these 5 cases, 3 demonstrated retention of staining of all four immunohistochemical markers, whilst 2 cases had MMR deficiency by immunohistochemistry. As such, in order to compare IHC results against PCR, these 5 cases have been excluded from statistical analysis, thus bringing the total number of cases available for comparison to 140.

Table 8C shows that of the 75 patients without a detected abnormality, 30.7% were under 60 years of age. Similarly, 38.5% of the 65 patients who had an abnormality were below 60 years. There was no statistically significant difference in the mean age of patients whose tissue samples had an abnormality versus those without mutations (p=0.76). There was an almost equal proportion of tumours showing no abnormality with FIGO grades 1 to 3; whereas more than half the number of cases with abnormalities showed FIGO grade 2 features (60%). A statistically significant association is identified between tumour FIGO grade and abnormality status (p-value of 0.020).

4.5.1. AGREEMENT OF MUTATION/ABNORMAL DIAGNOSIS OF IHC AND PCR

Table 10 below, showed MMR deficiency by IHC in eighteen cases, but these cases were stable by PCR (false positive). Twenty-one cases were unstable for both PCR and IHC (true positive). Twenty-five cases were proficient by IHC staining, but unstable by PCR (false negative). Seventy-six cases were stable for both test methods (true negative). Of the 140 cases, 101 cases showed retention of staining of all four markers by immunohistochemistry. The total number of cases that showed IHC loss of staining, was 39. The total number of cases that were deemed microsatellite unstable by PCR, was 46. The total number of cases that were microsatellite stable (MSS) was 94. The 25 discordant IHC/PCR cases subsequently underwent methylation assessment by EpiTYPER and the OneStep qMethyl™ kit (figure 36).

Table 10. Abnormalities/mutations detected by IHC versus PCR.

		PCR		
		Mutation	**No Mutation**	**Total**
	Abnormal/Deficient staining	True Positive (TP) 21	False Positive (FP) 18	**39**

IHC	**Normal/Retained staining**	False Negative (FN) 25	True Negative (TN) 76	**101**
	Total	**46**	**94**	**140**

If PCR is regarded as the gold standard,[109,110] utilising these figures:

Sensitivity = $\frac{\text{True Positive}}{\text{(True Positive+False Negative)}} = \frac{21}{(21+25)} = 45.65\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=45.65 \pm 1.96\sqrt{\frac{45.65(100-45.65)}{140}}$$

$$=45.65 \pm 1.96\sqrt{\frac{17.7219}{140}}$$

$$=45.65 \pm 8.251106992$$

=37.40 53.90

Specificity = $\frac{\text{True Negative}}{\text{(True Negative+False Positive)}} = \frac{76}{(76+18)} = 80.85\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=80.85 \pm 1.96\sqrt{\frac{80.85(100-80.85)}{140}}$$

$$=80.85 \pm 1.96\sqrt{\frac{11.059125}{140}}$$

$$=80.85 \pm 6.518031497$$

=74.33 87.37

Positive Predictive Value = $\frac{\text{True Positive}}{\text{(True Positive+False Positive)}} = \frac{21}{(21+18)} = 53.85\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=53.85 \pm 1.96\sqrt{\frac{53.85(100-53.85)}{140}}$$

$$=53.85 \pm 1.96\sqrt{\frac{17.75126786}{140}}$$

$$=53.85 \pm 8.257921688$$

=45.59 62.11

Negative Predictive Value = $\frac{\text{True Negative}}{\text{(True Negative+False Negative)}} = \frac{76}{(76+25)} = 75.25\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=75.25 \pm 1.96\sqrt{\frac{75.25(100-75.25)}{140}}$$

$$=75.25 \pm 1.96\sqrt{\frac{13.303125}{140}}$$

$$=75.25 \pm 7.148796052$$

=68.10 82.40

Thus, the sensitivity of IHC is 45.65%; 95% CI (37.40-53.90), the specificity is 80.85%, 95% CI (74.33-87.37. The positive predictive value (PPV) is 53.85%, 95% CI (45.59-62.11) and the negative predictive value (NPV) is 75.25%, 95% CI (68.10-82.40).

The overall accuracy = $\frac{\text{(True Positive + True Negative)}}{\text{(True Positive + True Negative+ False Positive + False Negative)}}$

$$= \frac{(21+76)}{(21+76+18+25)}$$

$$= \frac{97}{140}$$

= 69.29%

The average between the specificity and sensitivity or balanced accuracy is:

$$\frac{(80.85+45.65)}{2}$$

= 63.25%.

Thus, a total of 39 (27.86%) cases demonstrated microsatellite instability out of 140. One hundred and one (72.14%) cases showed no abnormality and were microsatellite stable. These results demonstrate a concordance of 69.29%.

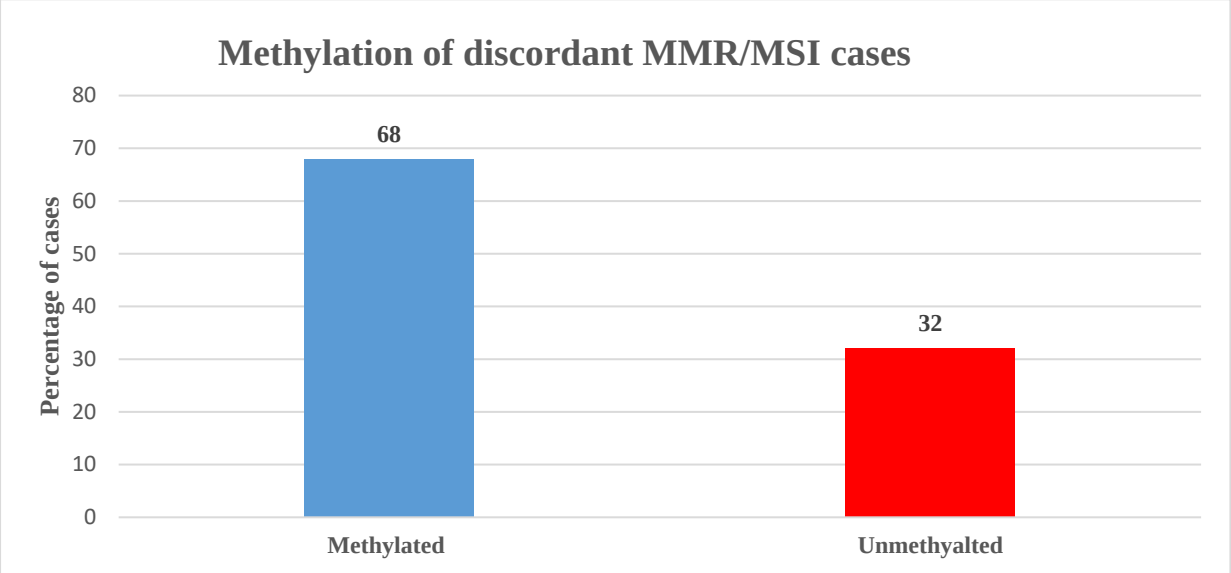

Figure 36. Methylation of cases that demonstrated MMR/MSI discordance using both EpiTYPER and the OneStep qMethyl™ kit.

4.6. STATISTICS OF MICROSATELLITE INSTABILITY MUTATIONAL STATUS

4.6.1. VALIDITY OF IMMUNOHISTOCHEMICAL DIAGNOSIS IN ENDOMETRIAL CARCINOMA

IHC VERSUS PCR

Table 11. This table illustrates the concordance between IHC and PCR.

Agreement	Kappa	Std. Error	p-Value
69.29%	0.2757	0.0839	0.0005

The degree of agreement or concordance between IHC and PCR from the confusion matrix in Table 10 is = (TN + TP)/Total

= (76+21)/140

= 97/140

= 69.29%.

From Table 11, there was a statistically significant, minimal level of agreement between IHC and PCR in the identification of MSI in endometrial carcinomas (kappa score = 0.2757, p=0.0005 According to McHugh[108] a kappa value of 0.21-0.39 shows a minimal level of agreement. The p-value, being statistically significant indicates that the kappa score is valid.

Table 12. Odds ratio of abnormalities/mutations by IHC in comparison to PCR

		PCR			
		Odds ratio	**Std error**	**p-value**	**95%Conf interval**
IHC	**Abnormality/ mutation**	3.546667	1.402313	0.001	1.634047 7.697969
	Normal/No mutation	0.3289474	0.0758421	0.000	0.2093502 0.5168677

4.6.2. LOGISTIC REGRESSION: IHC ABNORMALITIES/MUTATIONS IN COMPARISON TO PCR

Table 12 shows that the odds of a mutation being identified by PCR is 3.5 times more likely amongst those who have a mutation by IHC compared to those who do not have a mutation by IHC (p=0.001, CI 1.63-7.70)

4.6.3. IHC VERSUS PCR BY AGE

Table 13. This table shows agreement between IHC and PCR according to age (A) and by tumour FIGO grade (B).

A. Agreement between IHC and PCR according to age.				
Agreement	**Age**	**Kappa**	**Std. Error**	**p-Value**
64.58%	<60	0.1905	0.1442	0.0932
71.74%	>60	0.3220	0.1030	0.0009
B. Agreement between IHC and PCR stratified by tumour FIGO grade.				
Agreement	**FIGO grade**	**Kappa**	**Std. Error**	**p-Value**
76.19%	1	0.2953	0.1528	0.0266
61.67%	2	0.2087	0.1284	0.0520
73.68%	3	0.3141	0.1356	0.0103

Assessing the degree of agreement between IHC and PCR in patients under the age of 60 shows concordance of 64.58%. The kappa score is poor (0.1905), showing that there is no level of agreement[108] and there is no statistically significant association between IHC and PCR results in patients under 60 years.

Assessing the concordance between IHC and PCR in patients over the age of 60, however, shows agreement of 71.74% and a p-value of 0.0009 which is statistically significant.

Evaluation of the association between IHC and PCR with regards to FIGO grade 1 tumours (table 13B) shows an agreement of 76.19%. The kappa score (0.2953) shows minimal agreement[108] whilst the p-value is not statistically significant.

Assessing the relationship between IHC and PCR for FIGO grade 2 tumours shows a 61.67 % agreement. The kappa score suggests only minimal agreement.[108]The p-value is however tending toward significance (p-value = 0.0520).

Examination of IHC and PCR in relation to FIGO grade 3 tumours shows the highest percentage of agreement amongst the three tumour FIGO grades (73.68%). The kappa score is indicative of only minimal agreement whilst the p-value reveals statistical significance between the two test methods and high FIGO grade of a tumour (p=0.0103).

4.6.4. LOGISTIC REGRESSION: IHC IN COMPARISON TO PCR BY AGE

Table 14. Odds ratio of abnormalities/mutations by IHC in comparison to PCR stratified by age.

PCR						
IHC		**Odds ratio**	**Std error**	**p-value**	**95%Conf interval**	
	Age	1.005767	0.019693	0.769	0.9679001	1.045114
	Cons	0.3366349	0.4336389	0.398	0.0269573	4.203792

From table 14, the odds calculation: (1- 1.005767) x 100 = -0.005767 x 100

= 0.6%

The likelihood of having a PCR mutation increases by 0.6% on an annual increase in age. However, this relationship does not reach statistical significance (p-value = 0.769; odds ratio 1.005767).

4.6.5. LOGISTIC REGRESSION: PCR MUTATIONS AND TUMOUR FIGO GRADES

Table 15. The odds of PCR mutations and tumour FIGO grades.

PCR				
A. Tumour FIGO grades 2 and 3 compared to tumour FIGO grade 1				
	Odds ratio	**Std error**	**p-value**	**95% CI**
Tumour FIGO grade 2	1.989189	0.8934876	0.126	0.8247843 4.797465
Tumour FIGO grade 3	1.664	0.8289544	0.307	0.6267733 4.417699
Cons	0.3125	0.1132139	0.001	0.1536289 0.6356632
B. Tumour FIGO grades 1 and 2 compared to tumour FIGO grade 3				
	Odds ratio	**Std error**	**p-value**	**95% CI**
Tumour FIGO grade 1	0.6009616	0.2993809	0.307	.2263622 1.595473
Tumour FIGO grade 2	1.195426	0.5175358	0.680	.5116961 2.792759
Cons	0.52	0.1778089	0.056	.2660378 1.016397

Table 15A shows that there is no statistical significance between tumour FIGO grade and the likelihood of a PCR detected mutation. However, the pattern suggests that the odds decreased from approximately 2 times to 1.7 times in moving from FIGO grade 2 to FIGO grade 3.

Table 15B: Odds ratio calculation for FIGO grade 1 tumours: (1 – 0.6) x 100 = 0.4 x 100

= 40%

Odds ratio calculation for FIGO grade 2 tumours: (1-1.195426) x 100 = 0.195426 x100

= 19.5%

There is a 40% reduced likelihood of having a PCR mutation amongst tumours with FIGO grade 1 histological features compared to those with FIGO grade 3 features; whilst there is almost a 20% increased likelihood of PCR mutation amongst FIGO grade 2 carcinomas compared to FIGO grade 3 carcinomas.

4.6.6. IHC VERSUS PCR IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 16. The agreement between IHC and PCR in relation to tumour FIGO grade, stratified by age.

FIGO grade	Age	Agreement	Kappa	Std. Error	p-Value
1	<60	80.00%	0.2857	0.2508	0.1273
2	<60	47.62%	-0.0359	0.2093	0.5680
3	<60	75.00	0.4375	0.2387	0.0334
1	>60	74.07%	0.2921	0.1915	0.0635
2	>60	69.23%	0.3500	0.1601	0.0144
3	>60	73.08%	0.2353	0.1643	0.0760

Evaluation of the association between IHC and PCR in FIGO grade 1 tumours in patients under 60 years of age, shows an 80% agreement with a poor kappa score[108] (0.2857) and a p-value that is not statistically significant.

Table 16 above demonstrates that there is only a 47.62% degree of association between IHC and PCR in FIGO grade 2 tumours in patients below 60. The kappa score shows no association[108] (-0.0359). The p-value is however, tending toward statistical significance at 0.5680.

There is a 75% level of agreement between the two test methods and FIGO grade 3 tumours in patients under 60. The kappa score shows a weak[108] level of association whilst the p-value is statistically significant.

Table 16 demonstrates an agreement of 74.07% between IHC and PCR in FIGO grade 1 tumours in patients over 60. Only a minimal degree of association is seen on the kappa score (0.2921). The p-value is not statistically significant (p=0.0635).

FIGO grade 2 tumours show a concordance of 69.23% between IHC and PCR in patients over the age of 60. A minimal degree of association is noted with a kappa score of 0.3500. The p-value is statistically significant.

Evaluating the relationship between IHC and PCR with FIGO grade 3 tumours in patients over 60 shows an agreement of 73.08%. The kappa score shows minimal association[108] between these two test methods in this tumour FIGO grade. The p-value is not statistically significant (p=0.0760).

Table 17. The odds ratio for PCR detected mutations and tumour FIGO grades 2 and 3 compared to FIGO grade 1 tumours.

		PCR			
		Odds ratio	**Std error**	**p-value**	**95%Conf interval**
IHC	**Abnormality/ Mutation**	3.598606	1.511608	0.002	1.579733 8.197564
	Tumour FIGO grade 2	1.474278	0.7017547	0.415	0.5799736 3.747575
	Tumour FIGO grade 3	1.857822	0.9590188	0.230	0.6754742 5.109747
	Age	1.005185	0.0205765	0.801	0.9656541 1.046334
	Cons	0.1654822	0.2276031	0.191	0.011169 2.45182

From table 17 above, the likelihood of a PCR detected mutation is 3.6-fold amongst those with IHC abnormality compared to those without an IHC abnormality (p-value =0.002). There is, however, no statistical significance between tumour FIGO grade and age and the likelihood of a PCR mutation amongst those with an IHC abnormality compared to those without an IHC mutation.

4.7. IHC VERSUS OVERALL ABNORMALITY/MUTATIONAL STATUS

The degree of agreement or concordance of IHC to overall abnormality/mutational status is calculated using the confusion matrix (table 10): (76 + 39)/140

= 115/140

= 82.14%

Table 18. Agreement of IHC to overall abnormality/mutational status.

Agreement	Kappa	Std. Error	p-Value
82.14%	0.6288	0.0785	0.0001

There was a moderate degree of concordance between IHC and overall abnormality/mutational status (kappa score =0,6288, p-value=0.0001).

4.7.1. IHC VERSUS OVERALL ABNORMALITY/MUTATIONAL STATUS STRATIFIED BY AGE

Table 19. Agreement between IHC and overall abnormality/mutational status. Comparison of abnormality/mutational status stratified by age (A), in relation to each tumour FIGO grade (B) and consolidated status in relation to age stratified by tumour FIGO grade (C).

A. Concordance between IHC and overall abnormality/mutational status, stratified by age.				
Agreement	**Age**	**Kappa**	**Std. Error**	**p-Value**
81.25%	<60	0.6250	0.1338	0.0001
82.61%	>60	0.6290	0.0968	0.0001
B. Agreement between IHC and overall abnormality/mutational status in the three tumour FIGO grades				
Agreement	**FIGO grade**	**Kappa**	**Std. Error**	**p-Value**
85.71%	1	0.6400	0.1440	0.0001
83.33%	2	0.6753	0.1221	0.0001
76.32%	3	0.4124	0.1313	0.0008

C. Agreement between IHC and overall abnormality/mutational status stratified by age against each tumour FIGO grade					
Agreement	**Age**	**FIGO grade**	**Kappa**	**Std. Error**	**p-Value**
86.67%	<60	1	0.5946	0.2360	0.0059
80.95%	<60	2	0.6111	0.2010	0.0012
75.00%	<60	3	0.4375	0.2387	0.0334
85.19%	>60	1	0.6538	0.1806	0.0001
84.62%	>60	2	0.6977	0.1526	0.0001
76.92%	>60	3	0.3953	0.1562	0.0057

Table 19A shows that the degree of agreement between IHC and overall abnormality/mutational status in patients under 60 years of age shows concordance of 81.25%, a moderate kappa score[108] (0.6250). The p-value is 0.0001 which is statistically significant agreement and indicates that IHC is a good test to use in patients under 60 years of age. A concordance level of 82.61% is seen when comparing IHC to overall abnormality/mutational status in patients over 60 years of age. A moderate kappa score of 0.6290[108] is noted. A p-value of 0.0001 is achieved which signifies good agreement between IHC and overall abnormality/mutational status in patients 60 years and over.

Table 19B shows that there is an 85.71% concordance level between IHC and overall abnormality/mutational status in FIGO grade 1 tumours; with moderate agreement[108] and statistical significance (kappa =0.6400; p-value= 0.0001). FIGO grade 2 tumours demonstrate agreement of 83.33%; with a moderate kappa score and statistical significance (kappa = 0.6753, p-value = 0.0001). FIGO grade 3 tumours demonstrate the lowest level of agreement between IHC and overall abnormality/mutational status; with a weak[108] kappa score and statistical significance (kappa= 0.4124, p-value =0.0008).

Table 19C shows an 86.67% level of agreement between IHC and overall abnormality/mutational status for FIGO grade 1 tumours in cases from patients under the age of 60. A weak association[108] with statistical significance is noted (kappa score = 0.5946; p-value =0.0059). With regard to FIGO grade 2 tumours in patients below 60 years of age, there is an 80.95% agreement level between IHC and overall abnormality/mutational status, with a moderate degree of association[108] (0.6111) and statistical significance (kappa=0.6111; p-value=0.0012) which indicates that IHC is a good test to use in patients under the age of 60 years. There is a 75.00% level of agreement between IHC and overall abnormality/mutational status for FIGO grade 3 tumour in cases from patients younger than 60; with a weak kappa score, but statistically significant (kappa=0.4375; p-value=0.0334).

FIGO grade 1 tumours in cases from patients over 60 years of age shows agreement of 85.19% between IHC and overall abnormality/mutational status with a moderate kappa score[108] and a statistically significant p-value (p=0.001). There is an agreement of 84.62% between IHC and overall abnormality/mutational status in FIGO grade 2 tumours from patients over the age of 60. A moderate kappa score of 0.6977 is noted with a statistically significant p-value (p=0.0001). With regard to FIGO grade 3 tumours in patients older than 60 years, an agreement level of 76.92% is seen. There is minimal association [108] as indicated by a kappa score of 0.3953. The p-value is tending toward significance (p=0.0057).

4.8. VALIDITY OF PCR DIAGNOSIS IN ENDOMETRIAL CARCINOMA

4.8.1. PCR VERSUS OVERALL ABNORMALITY/MUTATIONAL STATUS

The level of agreement between PCR MSI and overall abnormality/mutational status was assessed.

Table 20. This table show concordance between PCR and overall abnormality/mutational status.

Agreement	Kappa	Std. Error	p-Value
87.14%	0.7351	0.0815	0.0001

Assessment of agreement between PCR and overall abnormality/mutational status is calculated using the confusion matrix: (21+ 101)/140

= 122/140

= 87.14%

There was a moderate degree of concordance between PCR and overall abnormality/mutational status (kappa score =0.7351, p-value=0.0001).

4.8.2. PCR VERSUS OVERALL ABNORMALITY/MUTATIONAL STATUS STRATIFIED BY AGE

Table 21. Concordance between PCR and overall abnormality/mutational status according to age.

A. Concordance between PCR and overall abnormality/mutational status according to age.				
Agreement	Age	Kappa	Std. Error	p-Value
83.33%	<60	0.6667	0.1361	0.0001
89.13%	>60	0.7723	0.1015	0.0001

B. Concordance between PCR and overall abnormality/mutational status in relation to tumour FIGO grade.				
Agreement	FIGO grade	Kappa	Std. Error	p-Value
90.48%	1	0.7692	0.1501	0.0001
78.33%	2	0.5860	0.1175	0.0001
97.37%	3	0.9426	0.1620	0.0001

C. Concordance between PCR and overall abnormality/mutational status stratified by age for each tumour FIGO grade.					
Agreement	Age	FIGO grade	Kappa	Std. Error	p-Value
93.33%	<60	1	0.8148	0.2537	0.0007
66.67%	<60	2	0.3951	0.1738	0.0115
100%	<60	3	1.0000	0.2887	0.0003
88.89%	>60	1	0.7461	0.1861	0.0001
84.62%	>60	2	0.6977	0.1526	0.0001
96.15%	>60	3	0.9128	0.1954	0.0001

Table 21A shows 83.33% degree of agreement between PCR and overall abnormality/mutation in patients under 60 years of age; with a moderate kappa score[108] and statistical significance (kappa= 0.6667; p-value =0.0001). This indicates that PCR is a good test to use in patients under 60 years of age. A concordance level of 89.13% with a moderate kappa score of 0.7723 was noted when comparing PCR to overall abnormality/mutational status in patients over 60 years of age. The p-value is 0.0001 which signifies good agreement between PCR and overall abnormality/mutational status in patients 60 years and over.

Table 21B shows that there is 90.48% concordance between PCR and overall abnormality/mutational status in FIGO grade 1 tumours; with moderate agreement, kappa score of 0.7692, and p–value of 0.0001. FIGO grade 2 tumours demonstrate the lowest degree of agreement of 78.33%. There is weak agreement[108] on kappa score (0.5860) but similar to that seen in FIGO grade 1 tumours, with statistical significance (p=0.0001). FIGO grade 3 tumours demonstrate the highest level of agreement between PCR and overall abnormality/mutational status. The kappa score shows strong association between abnormality/mutational status and PCR mutations (kappa score 0.9426; p-value =0.0001).

Table 21C shows that there is a 93.33% level of agreement between PCR and overall abnormality/mutational status for FIGO grade 1 tumours in cases from patients under the age of 60, with a strong kappa score of 0.8148 and p-value of 0.0007 which indicates that PCR is a good test to use in patients under the age of 60 years. FIGO grade 2 tumours in patients below 60 years of age showed A 66.67% level of agreement between PCR and overall abnormality/mutational status, with a weak degree of association[108] (0.3951) whilst the p-value is statistically significant (p-value =0.0115). There was a 100% level of agreement between PCR and overall abnormality/mutational status for FIGO grade 3 tumours in cases from patients younger than 60. The kappa score shows a perfect score and the p-value is statistically significant (p=0.0003).

In patients over the age of 60 years; FIGO grade 1 tumours showed agreement of 88.89% between PCR and overall abnormality/mutational status with a moderate kappa score of 0.7461 and a statistically significant p-value (p=0.001). This suggests that PCR is a good test to use in patients over the age of 60 years. There is agreement of 84.62% between PCR and overall abnormality/mutational status in FIGO grade 2 tumours; with a moderate kappa score and statistical significance (kappa= 0.6977; p=0.0001). With regard to FIGO grade 3 tumours, there is agreement of 96.15% with good association (kappa score = 0.9128), and statistical significance (p-value =0.0001). This suggests that PCR is a good test to use in patients over the age of 60 years.

4.9. BRAF ASSESSMENT OF ENDOMETRIAL CARCINOMAS

4.9.1. VALIDITY OF BRAF V600E IHC DIAGNOSIS IN ENDOMETRIAL CARCINOMA

Only one (2.7%) of the thirty-seven cases that showed loss of MLH-1 immunohistochemically, showed very focal cytoplasmic positivity with the BRAF V600E

immunohistochemical stain (figure 37). Five cases demonstrated aberrant nuclear positivity, which according to the package insert, should be interpreted as negative (figure 38).[111] Figure 39 shows a comparison of negative and positive BRAF IHC stains.

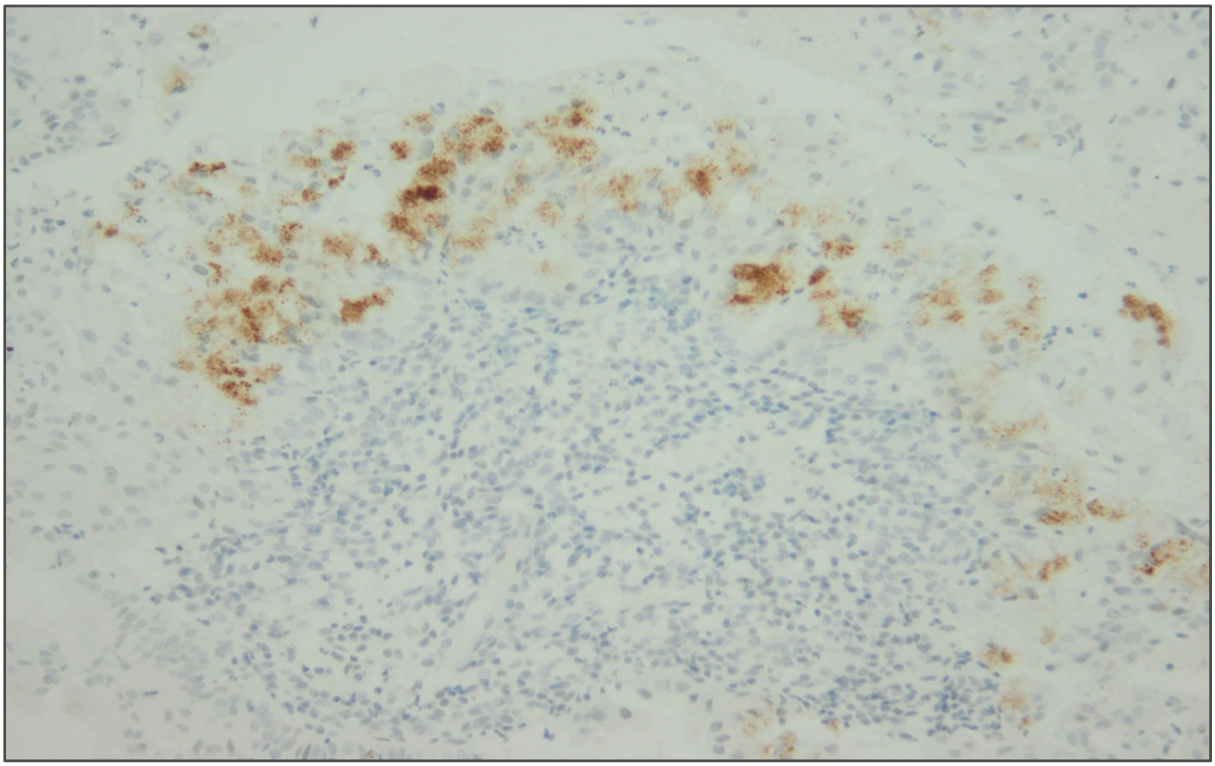

Figure 37. Focal cytoplasmic staining of tumour cells is identified. 4µm BRAF V600E stained section. Original magnification: 200x

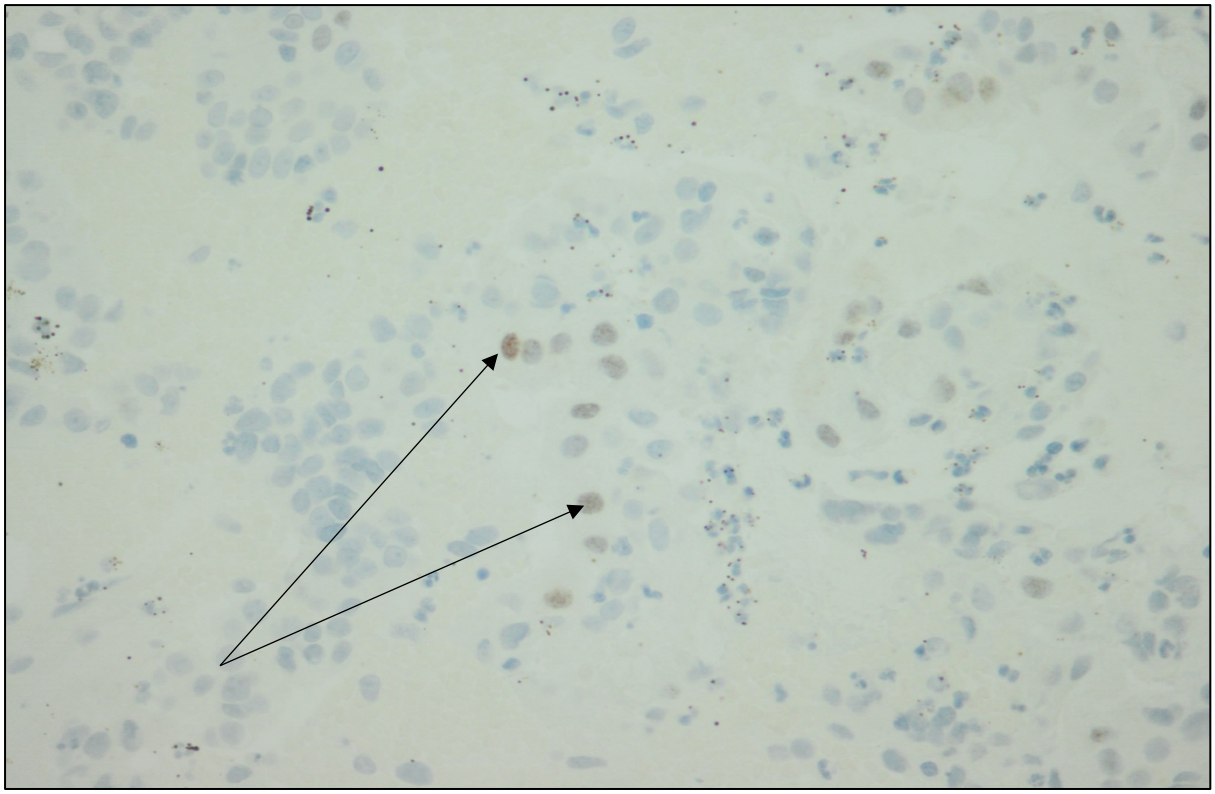

Figure 38. Aberrant nuclear staining of tumour cells is noted (arrows). 4µm BRAF V600E stained section. Original magnification: 400x

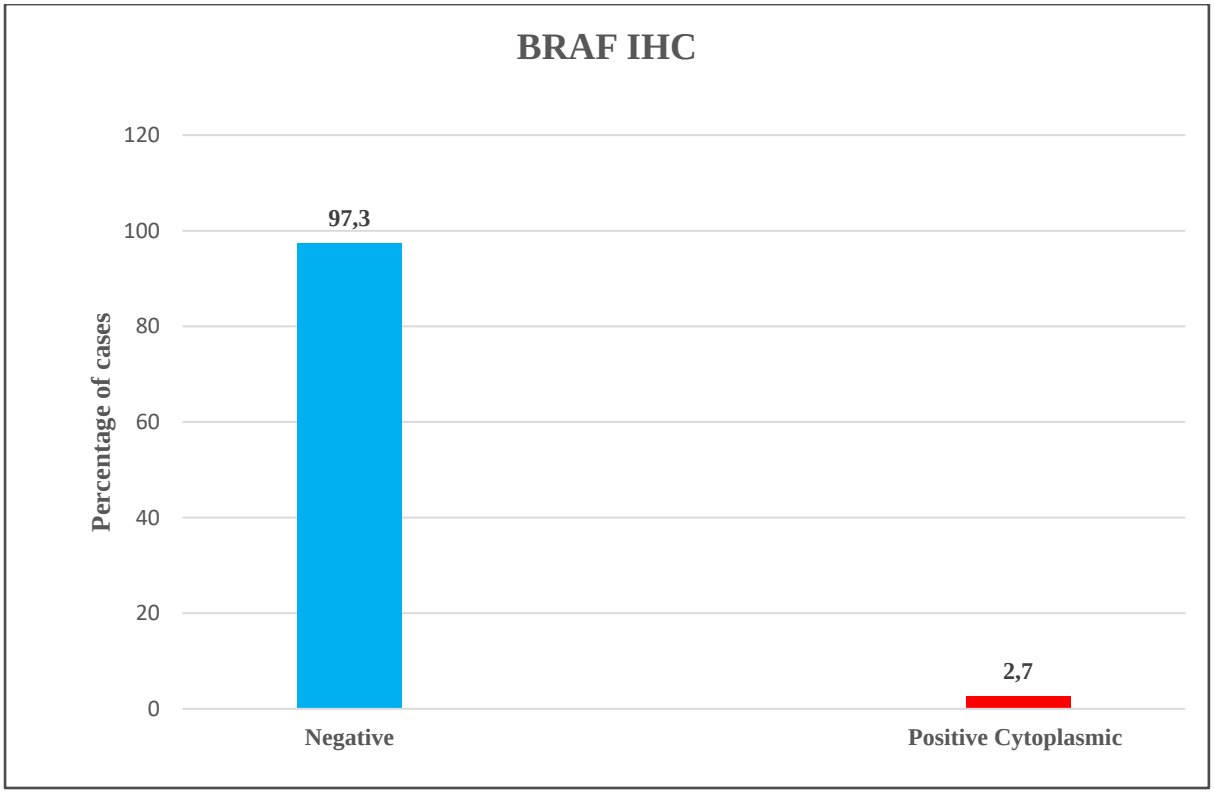

Figure 39. BRAF V600E staining by immunohistochemistry.

4.9.2. BRAF PCR

Four (10.81%) of the thirty-seven cases that showed loss of MLH1 staining demonstrated a BRAF mutation by PCR (figure 40). Of the 4 mutations detected, 3 (75%) harboured BRAF V600E mutations whilst only a single (25%) case showed a mutation in V600D (figure 41).

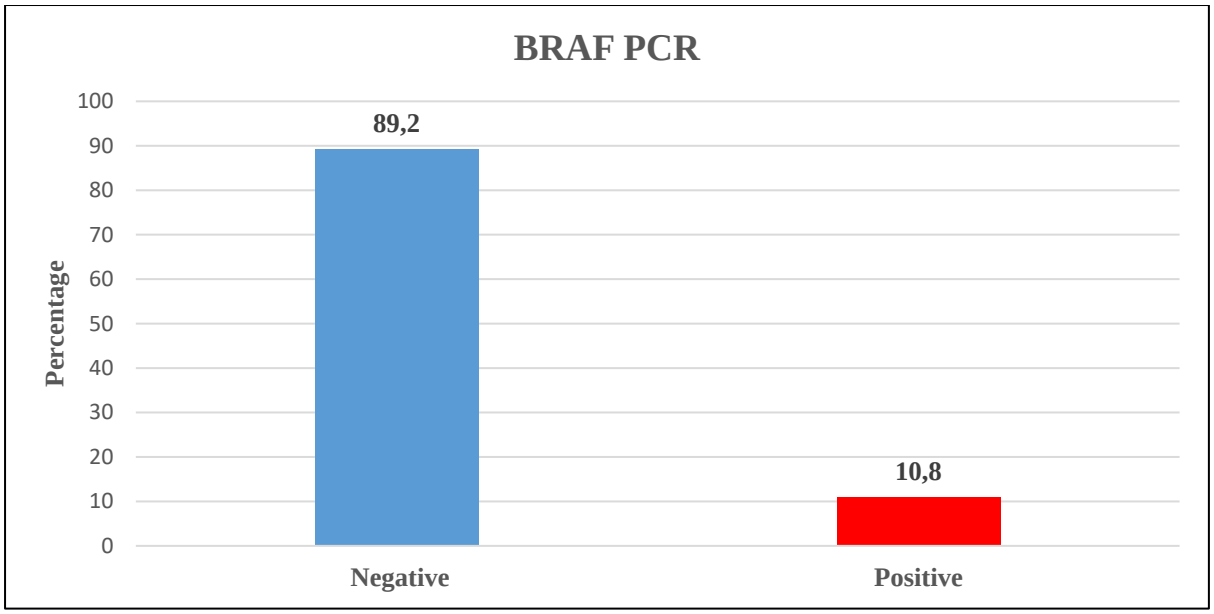

Figure 40. BRAF results by PCR.

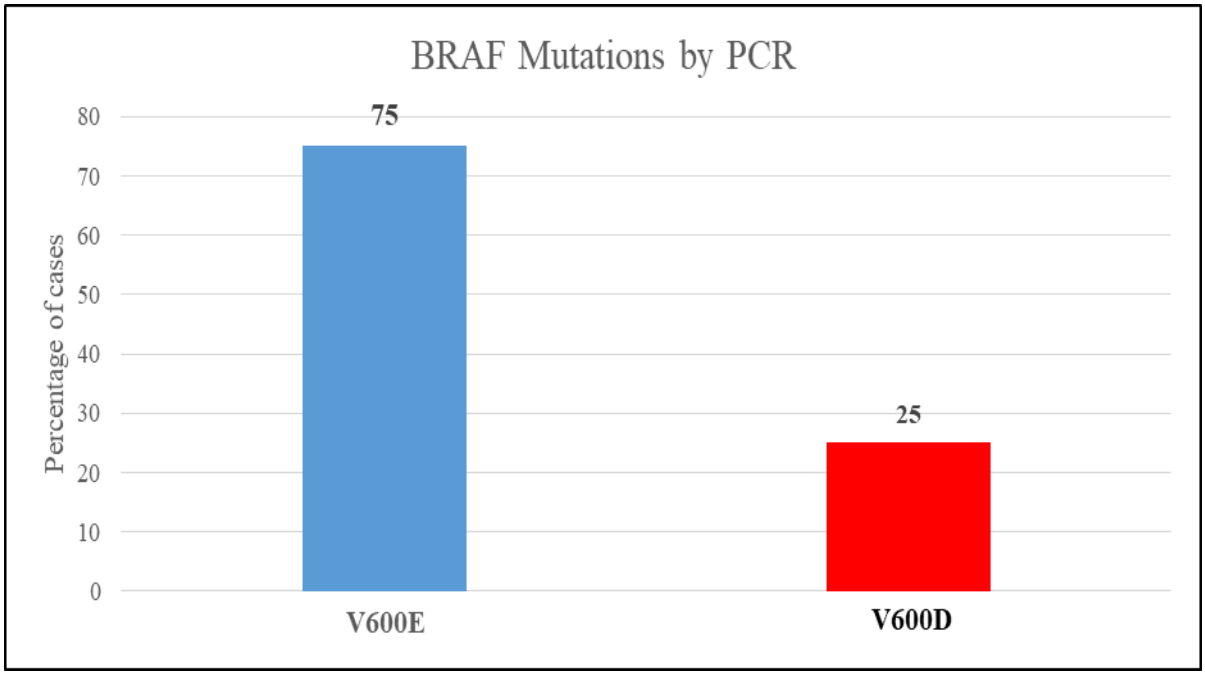

Figure 41. Specific BRAF mutations identified by PCR.

4.9.3. BRAF SEQUENCING

Only one (2.7%) of the thirty-seven cases that showed loss of MLH-1 immunohistochemically, showed a *BRAF V600E* mutation as detected by Sanger sequencing (figure 42). Figure 43 demonstrates wild-type, non-mutational *BRAF*.

Figure 42. A BRAF V600E mutation is indicated by the arrows.

Figure 43. Wild-type *BRAF* is depicted above.

4.9.4. AGREEMENT OF MUTATIONAL DIAGNOSIS OF IHC AND SANGER SEQUENCING

Results obtained from BRAF IHC and Sanger sequencing were compared to one another (table 22).

Table 22. Mutations detected by IHC versus Sanger sequencing.

		Sanger sequencing		
		Mutation	**No Mutation**	**Total**
IHC	**Mutation**	True Positive (TP) 0	False Positive (FP) 1	**1**
	No mutation	False Negative (FN) 1	True Negative (TN) 35	**36**
	Total	**1**	**36**	**37**

Considering Sanger sequencing as the gold standard[112,113] utilising these figures:

$$\text{Sensitivity} = \frac{\text{True Positive}}{(\text{True Positive}+\text{False Negative})} = \frac{0}{(0+1)} = 0\%$$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=0 \pm 1.96\sqrt{\frac{0(100-0)}{37}}$$

$$=0 \pm 1.96\sqrt{\frac{0}{37}}$$

$$=0$$

$$\text{Specificity} = \frac{\text{True Negative}}{(\text{True Negative}+\text{False Positive})} = \frac{35}{(35+1)} = 97.22\%$$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=97.22 \pm 1.96\sqrt{\frac{97.22(100-97.22)}{37}}$$

$$=97.22 \pm 1.96\sqrt{\frac{270.06}{37}}$$

$$= 97.22 \pm 5.2952$$

$$=91.92 \quad 102.52$$

Positive Predictive Value = $\frac{\text{True Positive}}{\text{(True Positive+False Positive)}} = \frac{0}{(0+1)} = 0\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=0 \pm 1.96\sqrt{\frac{0(100-0)}{37}}$$

$$=0 \pm 1.96\sqrt{\frac{0}{37}}$$

$$=0$$

Negative Predictive Value = $\frac{\text{True Negative}}{\text{(True Negative+False Negative)}} = \frac{35}{(35+1)} = 97.22\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=97.22 \pm 1.96\sqrt{\frac{97.22(100-97.22)}{37}}$$

$$=97.22 \pm 1.96\sqrt{7.3046}$$

$$=97.22 \pm 5.2952$$

$$=91.92 \quad 102.52$$

Thus, the sensitivity of IHC is 0%; 95% CI (0), the specificity is 97.22%, 95% CI (91.92-102.52). The positive predictive value (PPV) is 0%, 95% CI (0) and the negative predictive value (NPV) is 97.22%, 95% CI (91.92-102.52).

The overall accuracy = $\frac{\text{(True Positive + True Negative)}}{\text{(True Positive + True Negative+ False Positive + False Negative)}}$

$$= \frac{(0+35)}{(0+35+1+1)}$$

$$= \frac{35}{}$$

37

= 94.59%

The average between the specificity and sensitivity or balanced accuracy is:

(0+97.22)

2

= 48.61%.

4.9.5. AGREEMENT OF MUTATIONAL DIAGNOSIS OF BRAF PCR AND SANGER SEQUENCING

Results of BRAF PCR and BRAF Sanger sequencing were compared (table 23).

Table 23. Mutations detected by PCR versus Sanger sequencing

		Sanger sequencing		
		Mutation	**No Mutation**	**Total**
PCR	**Mutation**	True Positive (TP) 0	False Positive (FP) 4	**4**
	No Mutation	False Negative (FN) 1	True Negative (TN) 32	**33**
	Total	**1**	**36**	**37**

Considering Sanger sequencing as the gold standard[112,113] utilising these figures:

Sensitivity = True Positive / (True Positive+False Negative) = 0 / (0+4) = 0%

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$=0 \pm 1.96\sqrt{\frac{0(100-0)}{37}}$

$=0 \pm 1.96\sqrt{\frac{0}{37}}$

$=0$

Specificity = True Negative / (True Negative+False Positive) = 32 / (32+4) = 88.89%

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=88.89 \pm 1.96\sqrt{\frac{88.89(100-88.89)}{37}}$$

$$=88.88 \pm 1.96\sqrt{26.69102432}$$

$$=88.89 \pm 1.96 \times 5.166335676$$

$$=88.89 \pm 10.12601793$$

$$=78.76 \quad 99.02$$

Positive Predictive Value = $\frac{\text{True Positive}}{\text{(True Positive+False Positive)}} = \frac{0}{(0+4)} = 0\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=0 \pm 1.96\sqrt{\frac{0(100-0)}{37}}$$

$$=0 \pm 1.96\sqrt{\frac{0}{37}}$$

$$=0$$

Negative Predictive Value = $\frac{\text{True Negative}}{\text{(True Negative+False Negative)}} = \frac{32}{(32+1)} = 96.97\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=96.97 \pm 1.96\sqrt{\frac{96.97(100-96.97)}{37}}$$

$$=96.97 \pm 1.96\sqrt{7.941056757}$$

$$=96.97 \pm 5.523256615$$

$$=91.45 \quad 102.49$$

Thus, the sensitivity of PCR is 0%; 95% CI (0), the specificity is 88.89%, 95% CI (78.76-99.02). The positive predictive value (PPV) is 0%, 95% CI (0) and the negative predictive value (NPV) is 96.97%, 95% CI (91.45-102.49).

The overall accuracy = $\frac{\text{(True Positive + True Negative)}}{}$

(True Positive + True Negative+ False Positive + False Negative)

$$= \frac{(0+32)}{(0+32+4+1)}$$

$$= 32/37$$

$$= 86.47\%$$

The average between the specificity and sensitivity or balanced accuracy is:

$$\frac{(88.89+0)}{2}$$

$$= 44.46\%.$$

4.10. STATISTICS OF BRAF MUTATIONAL ANALYSIS

4.10.1. INCIDENCE OF BRAF MUTATIONS USING IHC, PCR AND SEQUENCING

Table 24. The incidence of BRAF mutations using IHC, PCR and Sequencing.

Factor	No Mutation			Mutation			**Total**		**P value**
	N= 31	**83.78%**	**95% CI: 67.99-93.81**	**N= 6**	**16.21 %**	**95% CI: 6.19-32.01**	**N=37**	**100%**	
Age (years) (Mean)	66.87 (63.62 -70.12)			62.67 (52.54-72.92)					0.3019
<60	9	29.03%		3	50%		12	32.43	0.367
≥60	22	70.97%		3	50%		25	67.57	
Tumour FIGO grades									
FIGO grade 1	9	29.03%		1	16.67%		10	27.03	0.794
FIGO grade 2	19	61.29%		5	83.33%		24	64.86	
FIGO grade 3	3	9.68%		0	0		3	8.11	

Table 24 shows that the prevalence of BRAF mutations was 16.21% (95% CI: 6.19 – 32.01). Whilst the median age of BRAF mutation negative women was 67 years and was greater than the median age of mutation positive women (63 years), the difference was not statistically significant (p-value = 0.3019). There was no statistically significant association between tumour FIGO grade and BRAF result. However, 83.33% of cases with a positive BRAF mutation demonstrated FIGO grade 2 histological features.

4.10.2. AGREEMENT OF BRAF ASSESSMENT USING IHC, PCR AND SEQUENCING

Table 25. Agreement between BRAF results obtained by IHC, PCR and Sequencing.

	Agreement	Kappa	Std. Error	p-Value
IHC vs PCR	86.49%	-0.0452	0.1281	0.6379
IHC vs Sequencing	94.59%	-0.0278	0.1644	0.5671
PCR vs Sequencing	86.49%	-0.0452	0.1281	0.6379

There is concordance of 86.49% between both IHC and PCR as well as between PCR and sequencing, but the kappa score indicates no agreement[108] and is not statistically significant (kappa= -0.0452, p=0.6379). Whilst there is agreement of 94.59% between BRAF results by IHC and sequencing, the kappa score shows no concordance and the result is not statistically significant (kappa= -0.0278, p=0.5671) (table 25).

4.10.3. BRAF IHC MUTATIONS IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 26. Comparison of age and tumour FIGO grades of cases with mutation and those without mutation based on BRAF IHC.

Factor	No Mutation			Mutation			**Total**		**P-value**
	N=36	**97.30 %**	**95% CI: 81.74-99.66**	**N= 1**	**2.70 %**	**95% CI: 0.34-18.26**	**N=37**	**%**	
Age (years) (Median)	66.5 (63.48-69.52)			55					0.1592
<60	11	30.56		1	100		12	32.43	
≥60	25	69.44		0	0		25	67.57	
Tumour FIGO grades									
FIGO grade 1	10	27.78		0	0		10		
FIGO grade 2	23	63.89		1	100		24		
FIGO grade 3	3	8.33		0	0		3		

Table 26 above illustrates that just a single case demonstrated a mutation by immunohistochemistry. The age of the patient from whom the single case with the BRAF mutation was derived, was under 60 years and the tumour had FIGO grade 2 histological features. Although the median age of BRAF mutation negative women was greater than the median age of mutation positive women, the difference was not statistically significant (BRAF Negative versus BRAF Positive: 66.5 years (age range: 59 -72.5) versus 55 years; p-value = 0.1592).

4.10.4. BRAF IHC VERSUS OVERALL MUTATIONAL STATUS STRATIFIED BY AGE AND TUMOUR FIGO GRADE

Table 27. Agreement of results obtained by BRAF IHC versus overall mutational status stratified by age, according to the three tumour FIGO grades.

FIGO grade	Age	Agreement	Kappa	Std. Error	p-Value
1	<60	-	-	-	-
2	<60	80.00%	0.2857	0.1355	0.0175
3	<60	-	-	-	-
1	>60	87.50%	0.0000	0.0000	0.0001
2	>60	85.71%	0.000	0.0000	0.5000
3	>60	-	-	-	-

Table 27 above shows a good level of agreement between IHC and overall mutational status for FIGO grade 2 tumours in both patients over and under 60 years of age; and with statistical significance noted in FIGO grade 2 tumours in those under the age of 60. The kappa score however showed no level of agreement. A good level of agreement was noted in FIGO grade 1 tumours in patients over the age of 60 years, with statistical significance (p=0.0001). For the remainder, statistical analysis was not possible due to too few rating categories being present in light of the small sample size (37 cases).

4.10.5. BRAF PCR MUTATIONS IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 28. Comparison of age and tumour FIGO grades of cases with mutation and those without mutation based on BRAF PCR

Factor	No Mutation			Mutation			**Total**		**P value**
	N=33	**89.19%**	**95% CI: 73.53-96.08**	**N= 4**	**10.81%**	**95% CI: 3.92-26.47**	**N=37**	**%**	
Age (years)	66.15 (62.95-69.36)			66.5 (50.56-82.44)					0.9219
<60	11	33.33		1	25		12	32.43	
≥60	22	66.67		3	75		25	67.57	
Tumour FIGO grades									
FIGO grade 1	9	27.27		1	25		10	27.03	
FIGO grade 2	21	63.64		3	75		24	64.86	
FIGO grade 3	3	9.09		0	0		3	8.11	

Table 28 shows that 10.81% of cases demonstrated a BRAF mutation by PCR. 75% of the patients who harboured a PCR detectable BRAF mutation were over 60 years of age. In addition, 75% of tumours with BRAF mutations were assessed histologically as FIGO grade 2 tumours. Although the median age of BRAF mutation positive women was marginally

greater than the median age of BRAF mutation positive women, the difference was not statistically significant (BRAF positive versus BRAF negative: 66.5 (age range: 50-82) years versus 66.15 (age range: 62.95-69.36) years; p-value = 0.9219).

4.10.6. BRAF PCR VERSUS OVERALL MUTATIONAL STATUS STRATIFIED BY AGE AND TUMOUR FIGO GRADE

Table 29. Concordance of results obtained by BRAF PCR versus overall mutational status stratified by age, according to the three tumour FIGO grades.

FIGO grade	Age	Agreement	Kappa	Std. Error	p-Value
1	<60	-	-	-	-
2	<60	80.00%	0.2857	2.11	0.0175
3	<60	-	-	-	-
1	>60	87.50%	0.4667	2.83	0.0023
2	>60	85.71%	0.4615	3.74	0.0001
3	>60	-	-	-	-

From table 29, it may be seen that there is a good level of agreement between results obtained by BRAF PCR and overall mutational status with statistical significance noted for FIGO grades 1 and 2 tumours in patients over the age of 60; as well as for FIGO grade 2 tumours in patients under the age of 60 (p=0.0023, 0.0001 and 0.0175 respectively). Statistical analysis was not possible for the remaining tumour FIGO grades as too few rating categories were present.

4.10.7. BRAF SEQUENCING MUTATIONS IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 30. Comparison of age and tumour FIGO grades of cases with mutation and those without mutation based on BRAF Sequencing.

Factor	No Mutation			Mutation			Total		P-value
	N=36	97.30 %	95% CI: 81.74-99.66	N= 1	2.70 %	95% CI: 0.34-18.26	N=37	%	
Age (years) (Median)	66.5 (63.48-69.52)			55					-
<60	11	30.56		1	100		12	32.43	0.324
≥60	25	69.44		0	0		25	67.57	
Tumour FIGO grades									
FIGO grade 1	10	27.78		0	0		10	27.03	1.00
FIGO grade 2	23	63.89		1	100		24	64.86	
FIGO grade 3	3	8.33		0	0		3	8.11	

Table 30 demonstrates that 2.70% of cases had a mutation detected by BRAF sequencing and that the only patient with a mutation was under the age of 60 and that this patient's tumour showed FIGO grade 2 histological features. Whilst the median age of the BRAF mutation positive patient was lower than the BRAF negative patients, the difference was not statistically significant (BRAF positive versus BRAF negative: 55 versus 66.5 (age range:63.48-69.52) years, p-value = 0.324.

4.10.8. BRAF SEQUENCING VERSUS OVERALL MUTATIONAL STATUS

Table 31. Agreement of results obtained by BRAF Sequencing versus overall mutational status stratified by age, according to the three tumour FIGO grades.

FIGO grade	Age	Agreement	Kappa	Std. Error	p-Value
1	<60	-	-	-	-
2	<60	80.00%	0.4118	0.2557	0.0537
3	<60	-	-	-	-
1	>60	87.50%	0.0000	0.0000	-
2	>60	85.71%	0.000	0.0000	-
3	>60	-	-	-	-

Table 31 above demonstrates good agreement between results obtained by BRAF sequencing versus overall mutational status for FIGO grade 2 tumours in patients below the age of 60 with statistical significance (p=0.0537) as well as for patients with FIGO grade 1 and 2 tumours who were over 60 years of age.

4.10.9. BRAF IHC, PCR AND SEQUENCING VERSUS OVERALL MUTATIONAL STATUS

Table 32. Concordance between IHC, PCR and Sequencing results and overall BRAF mutational status.

	Agreement	Kappa	Std. Error	p-Value
IHC	86.49%	0.1475	0.0575	0.0052
PCR	94.59%	0.7702	0.1600	0.0001
Sequencing	86.49%	0.1475	0.0575	0.0052

From table 32, the agreement between results obtained by IHC versus overall BRAF mutational status as well as Sequencing and overall mutational status, was 86.49%. There was a statistically significantly low, non-random concordance[108] (kappa =0.15, p-

value<0.005). There was agreement of 94.59% between PCR and overall mutational status. There was statistically significant, moderate, non-random concordance[108] (kappa=0.77, p=0.0001).

4.11. EpiTYPER MassARRAY MLH-1 HYPERMETHYLATION ANALYSIS

The 37 cases that were deficient for MLH1 and/or PMS2 by immunohistochemistry underwent methylation analysis of the MLH1 promoter region. Of these 37 cases, 1(2.7%) case had insufficient DNA for further analysis; whilst 5 of the remaining 36 (13.89%) showed methylation levels below 10%; of which 5 cases, 3 showed methylation levels of less than 5%. Thirty-one (86.11%) out of 36 cases showed hypermethylation, with the lowest percentage in the acceptable range being 13% and the greatest degree of methylation being 74% (figure 44).

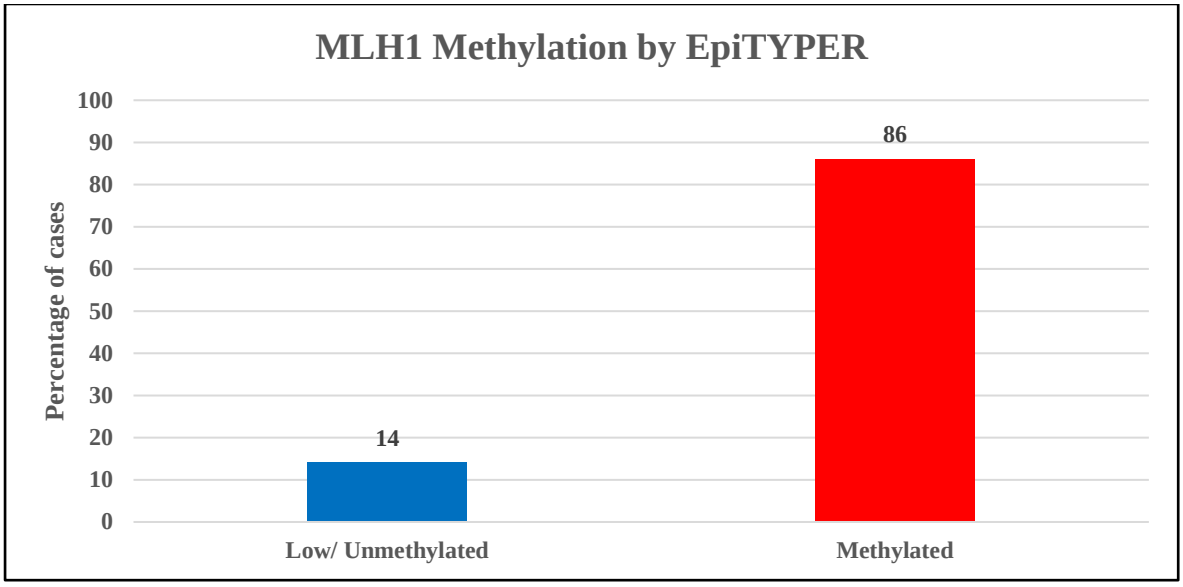

Figure 44. *MLH1* methylation by EpiTYPER.

Enriched methylation positions located up to 151 base pairs upstream of the transcription initiation site were analysed. Eight positions were amenable to analysis whilst 3 positions could not be assessed due to low mass which could not be detected by the MassARRAY equipment.

Assessment of regions C and D of the *MLH1* promoter revealed that in each of the CpG sites in region C, positions 40 to 151, the average methylation was 43%; for positions 39 to 149 on *MLH1* C (reverse), the average methylation was 37% and for MLH1 D positions 36 to 156, the average methylation was 37%. Thus, the average methylation for region C of the *MLH1* promoter was 40% and for region D the average methylation was 37% (figure 45).

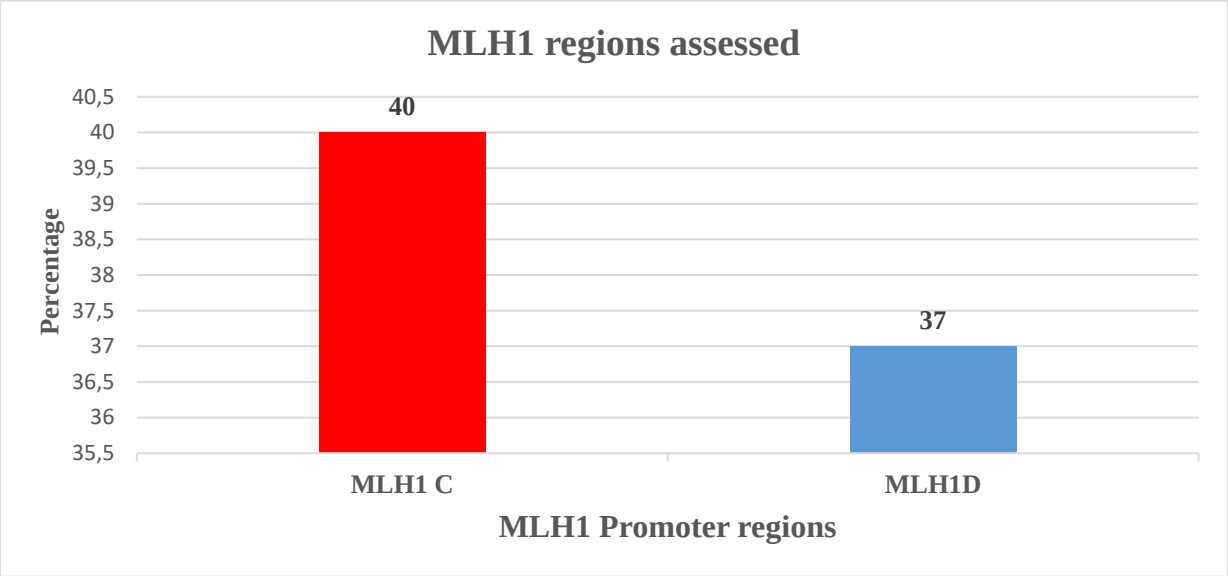

Figure 45. Assessment of methylation of *MLH1* regions C and D.

Table 33. Results of EpiTYPER testing on sixty-one cases.

EiTYPER	Frequency (%)	Total	Std error	95% Conf Interval		P-value
Negative	17 (27.87)	27.87	2.265088	61.90411	71.50765	0.6964*
Positive	44 (72.13)	72.13	1.356642	62.62771	68.09957	
Total	61 (100)	100	1.157041	63.42328	68.05213	

*T-test

A total of 62 cases underwent EpiTYPER analysis. These 62 cases comprised the 37 cases that were deficient for MLH1 immunohistochemistry in addition to 25 cases that showed discrepant IHC MMR and PCR MSI results. Of these 62 cases, only one case did not have enough DNA for analysis and has thus been excluded for comparative purposes (table 33). A value of ≥10% was regarded as showing hypermethylation. Table 33 above shows that 27.87% of cases were not methylated whilst 72.13% of cases were hypermethylated.

Table 34. Methylation of cases detected by EpiTYPER, stratified by age and tumour FIGO grade.

Factor	No Methylation			Methylation			**Total**		**P-value**
	N= 17	**27.9%**	**95% CI: 61.90411 71.50765**	**N=44**	**72.1 %**	**95% CI: 62.62771 68.09957**	**N=61**	**100%**	
Age (years) (Mean)	66.70588			65.36364					0.3036
<60	3	17.65%		17	38.64%		20		0.117*
≥60	14	82.35%		27	61.36%		41		
Tumour FIGO grades									
FIGO grade 1	8	47.06%		7	15.91%		15	24.59%	0.036*
FIGO grade 2	6	35.29%		28	63.64%		34	55.74%	
FIGO grade 3	3	17.65%		9	20.45%		12	19.67%	

*Pearson chi2

Table 34 above demonstrates that there was a 4-fold increase in the number of patients over the age of 60 years relative to those under 60 years of age in those who did not have methylated tumours. In patients who had methylated tumours there was a 1.5-fold increase in the number of patients who were over 60 years of age in contrast to those who were under the age of 60 years. In the unmethylated group of patients, most cases demonstrated FIGO grade 1 histological features (47.06%) with FIGO grade 3 tumours accounting for the minority of cases (17.65%). Contrastingly, most methylated cases (63.64%) had FIGO grade 2 tumours with FIGO grade 1 tumours making up the minority of cases (15.91%). There is a statistically significant relationship between methylation status and tumour FIGO grade (p-value= 0.036).

Table 35. Methylation by EpiTYPER against IHC abnormality

EpiTYPER METHYLATION				
IHC		**No Methylation**	**Methylation**	**Total**
	Retained staining	12 (TN) (70.59%)	13 (FP) (29.55%)	**25 (40.98%)**
	Loss of staining	5 (FP) (29.41)	31 (TP) (70.45%)	**36 (59.02)**
	Total	**17 (100%)**	**44 (100%)**	**61 (100%)**

Pearson chi2(1) = 8.5401 Pr = 0.003

Table 35 shows that 70.59% of cases showed no methylation or loss of IHC staining; whilst nearly 30% of cases that had intact MMR IHC staining were methylated. There were 5 cases with deficient MMR that were not methylated; whereas approximately 70% of MMR deficient cases were hypermethylated.

Table 36. Agreement between EpiTYPER methylation and PCR mutations

EpiTYPER METHYLATION				
PCR		**No Methylation**	**Methylation**	**Total**
	No Mutation	2(TN) (12.50%)	14 (FN) (32.56%)	**16** **(27.12%)**
	Mutation	14 (FP) (87.50%)	29 (TP) (67.44%)	**43** **(72.88%)**
	Total	**16 (100%)**	**43 (100%)**	**59 (100%)**

Pearson chi2(1) = 2.3737 P-value = 0.123

Table 36 shows that 12.50% of cases that had no PCR mutation were not methylated; whereas nearly one third of cases were methylated were microsatellite stable. Of the 16 cases that were not methylated, 87.50% showed MSI by PCR; whereas two-thirds of methylated cases had microsatellite instability.

Table 37. Agreement between EpiTYPER methylation and PCR mutational status

EpiTYPER METHYLATION				
PCR		**No Methylation**	**Methylation**	**Total**
	Microsatellite stable	2 (12.50%)	14 (32.56%)	**16** **(27.12%)**
	MSI-Low	13 (**81.25%**)	20 (**46.51%**)	**33** **(16.95%)**
	MSI-High	1 **(6.25%)**	9 **(20.93%)**	**10** **(16.95%)**
	Total	**16 (100%)**	**43 (100%)**	**59 (100%)**

Pearson chi2(2) = 5.7286 Pr = 0.057

The confusion matrix in table 37 above illustrates that of the unmethylated cases, 12.50% were microsatellite stable; whilst the majority (81.25%) were MSI-Low and only 1 case had

MSI-High mutations. Approximately one third of methylated cases were microsatellite stable. Nearly 47% of methylated cases had MSI-Low mutations whilst one-fifth of methylated cases were MSI-High. The p-value is tending toward statistical significance (p=0.057).

Table 38. Agreement between EpiTYPER methylation and PCR microsatellite instability.

EpiTYPER METHYLATION				
PCR MSI		**No Methylation**	**Methylation**	**Total**
	MSI-Low	13 (**92.86%**)	20 (**68.97%**)	**33 (76.74%)**
	MSI-High	1 **(7.14%)**	9 **(31.03%)**	**10 (23.26%)**
	Total	**14 (100%)**	**29 (100%)**	**43 (100%)**

Pearson chi2(1) = 3.0198 Pr = 0.082 Fisher's exact = 0.128

1-sided Fisher's exact = 0.084

Table 38 shows that in the unmethylated tumours, the vast majority (92.86%) had MSI-Low mutations whilst a single case was MSI-High. Of the methylated tumours, approximately two-thirds were MSI-Low whereas nearly one-third were MSI-High. The p-value is however not statistically significant.

Table 39. Concordance levels between EpiTYPER and IHC loss of staining.

Agreement	**Kappa**	**Std. Error**	**p-Value**
70.49%	0.3586	0.1227	0.0017

Table 39 shows that there was minimal agreement between methylation status by EpiTYPER and IHC deficiency, but the relationship is statistically significant (kappa = 0.3586, p-value = 0.0017).

Table 40. Agreement between EpiTyper and IHC stratified by age.

Agreement	**Age**	**Kappa**	**Std. Error**	**p-Value**
60.00%	<60	0.1398	0.1708	0.2066
75.61%	>60	0.4757	0.1553	0.0011

There is no level of agreement between EpiTYPER methylation and IHC deficient cases in patients under the age of 60 years (kappa =0.1398, p-value = 0.2066) whereas there was a

weak association between EpiTYPER methylation and IHC loss in patients over 60 years of age, which was statistically significant (kappa=0.4757, p-value = 0.0011) (table 40).

Table 41. Agreement between EpiTyper and IHC stratified by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	73.33%	0.4737	0.2491	0.0286
2	76.47%	0.3585	0.1624	0.0137
3	37.50%	0.2000	0.1732	0.1241

Table 41 illustrates a weak degree of agreement between EpiTYPER methylation and IHC deficiency in FIGO grade 1 carcinomas and a minimal degree of agreement in FIGO grade 2 tumours with statistical significance (kappa = 0.4737, p-value = 0.0286 and kappa=0.3585 and p-value = 0.0137 respectively).

Table 42. Concordance between EpiTyper and PCR mutations.

Agreement	Kappa	Std. Error	p-Value
52.54%	-0.2006	0.1302	0.9383

The table above shows no association between EpiTYPER methylation and PCR mutations with no statistical significance (kappa = -0.2006, p-value = 0.9243). These values have been calculated using values in table 47.

Table 43. Agreement between methylation and PCR mutation by age.

Agreement	Age	Kappa	Std. Error	p-Value
60.00%	<60	-0.0127	0.1928	0.5262
48.72%	>60	-0.2500	0.1550	0.9466

In patients over 60 years of age there is no degree of association between methylation and PCR mutations (kappa = -0.2500, p-value = 0.9466) (table 43). Similarly, there is no association between methylation and PCR mutations in patients under the age of 60 years kappa = -0.0127; p-value=0.5262)

Table 44. Agreement between methylation and PCR mutations by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	35.71%	-0.2857	0.2415	0.8816
2	54.55%	-0.1538	0.1607	0.8308
3	66.67%	-0.1429	0.2369	0.7268

Table 44 above shows that there is no degree of agreement between methylation and PCR with respect to the three tumour FIGO grades; with no statistical significance.

Table 45. Agreement between EpiTYPER methylation and overall abnormality/mutational status.

Agreement	Kappa	Std. Error	p-Value
72.13%	0.000	-	-

The table above illustrates that there is no association between methylation and overall abnormality/mutational status.

Table 46. Agreement between methylation and overall abnormality/mutation by age.

Agreement	Age	Kappa	Std. Error	p-Value
85.00%	<60	0.0000	0.0000	0.5000
65.85%	>60	0.0000	-	0.5000

Table 46 illustrates that there is no association between methylation and overall abnormality/mutational status by age.

Table 47. Agreement between EpiTYPER methylation and overall abnormality/mutation by tumour FIGO grade

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	46.67%	0.0000	0.0000	0.5000
2	82.35%	0.0000	0.0000	0.5000
3	75%	0.0000	0.0000	-

The table above illustrates that there is no association between methylation and overall abnormality/mutational status by tumour FIGO grade.

Table 48. Agreement between EpiTYPER methylation and BRAF IHC

Agreement	Kappa	Std. Error	p-Value
17.14%	0.00098	0.0236	0.3394

Table 48 shows that there is no agreement or statistically significant relationship between EpiTYPER methylation and BRAF IHC (kappa=0.00098; p-value=0.3394)

Table 49. Agreement between EpiTYPER methylation and BRAF IHC by age group

Agreement	Age	Kappa	Std. Error	p-Value
18.18%	<60	0.0198	0.0597	0.3701
16.67%	≥60	0.0000	-	-

Table 49 shows that there is no level of agreement or statistically significant relationship between EpiTYPER methylation and BRAF IHC stratified by age.

Table 50. Agreement between EpiTYPER methylation and BRAF IHC by tumour FIGO grade

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	33.33%	0.0000	-	-
2	13.04%	0.0086	0.0273	0.3762
3	0.00%	0.0000	0.0000	-

Table 50 shows that there is no agreement or a statistically significant relationship between EpiTYPER methylation and BRAF IHC when stratified by 3 tumour FIGO grades.

Table 51. Agreement between EpiTYPER methylation and BRAF PCR

Agreement	Kappa	Std. Error	p-Value
19.44%	-0.0316	0.0464	0.7522

Table 51 shows that there is no level of agreement or statistically significant relationship between EpiTYPER methylation and BRAF PCR stratified. Calculations were based on the fact that of 37 test samples, 1 did not have result by EpiTYPER methylation. Therefore, the calculations are only based on 36 cases (tables 51-53).

Table 52. Agreement between methylation and BRAF PCR by age group

Agreement	Age	Kappa	Std. Error	p-Value
18.18%	<60	0.0198	0.0597	0.3701
20.00%	≥60	-0.0549	0.0628	0.8087

Table 52 shows that there is no level of agreement or statistically significant relationship between EpiTYPER methylation and BRAF PCR stratified by age.

Table 53. Agreement between methylation and BRAF PCR by tumour FIGO grade

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	22.22%	-0.2353	0.1569	0.9332
2	20.83%	0.0256	0.0459	0.2883
3	0.00%	0.0000	0.0000	-

Table 53 shows that there is no agreement or statistically significant relationship between EpiTYPER methylation and BRAF PCR when stratified by 3 tumour FIGO grades.

Table 54. Agreement between EpiTYPER methylation and *BRAF* Sequencing

Agreement	Kappa	Std. Error	p-Value
13.89%	0.0000	0.0000	-

Table 54 shows that there is no statistically significant relationship between EpiTYPER methylation and BRAF Sequencing (kappa=0.0000; p-value =0.5000). Calculations were

based on the fact that of 37 test samples, 1 did not have result by EpiTYPER methylation. Therefore, the calculations are only based on 36 cases (tables 54-56).

Table 55. Agreement between EpiTYPER methylation and *BRAF* Sequencing by age.

Agreement	Age	Kappa	Std. Error	p-Value
9.09%	<60	0.0000	0.0000	-
16.00%	≥60	0.0000	-	-

The table above (table 55) demonstrates that there is no relationship between methylation using EpiTYPER and BRAF sequencing regardless of patient age.

Table 56. Agreement between EpiTYPER methylation and *BRAF* sequencing stratified by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	33.33%	0.0000	-	-
2	8.33%	0.0000	0.0000	0.5000
3	0.00%	0.0000	0.0000	-

Table 56 shows that there is no statistically significant relationship between EpiTYPER methylation and BRAF sequencing when stratified by 3 tumour FIGO grades.

Table 57. Agreement between EpiTYPER methylation and overall BRAF mutation.

Agreement	Kappa	Std. Error	p-Value
22.22%	-0.0223	0.0524	0.6649

Table 57 demonstrates that there is no agreement or statistically significant relationship between EpiTYPER methylation and overall BRAF mutations. Of the 37 cases, 36 were used in the analysis as 1 case did not have a result by EpiTYPER analysis (tables 57-59).

Table 58. Agreement between EpiTYPER methylation and overall BRAF mutation by age.

Agreement	Age	Kappa	Std. Error	p-Value
27.27%	<60	0.0435	0.0879	0.3105
20.00%	≥60	-0.0549	0.0628	0.8087

Table 58 shows that there is no agreement or statistically significant relationship between EpiTYPER methylation and overall BRAF mutations in patients below and over the age of 60 years.

Table 59. Agreement between EpiTYPER methylation and overall BRAF mutation stratified by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	22.22%	-0.2353	0.1569	0.9332
2	25.00%	0.0357	0.0541	0.2544
3	0.00%	0.0000	0.0000	-

Table 59 demonstrates that there is no agreement or statistically significant relationship between EpiTYPER methylation and overall BRAF mutations when stratified by 3 tumour FIGO grades.

4.12. *ONESTEP* qMETHYL^{TM} KIT MLH1 HYPERMETHYLATION ANALYSIS

Using EpiTYPER for methylation analysis both regions C and D of the *MLH1* promoter region were assessed. However, region C is small and the chemistry of the OneStep qMethyl^{TM} kit did not permit for optimal reactions. Thus, only region D could be assessed using the OneStep qMethyl^{TM} kit.

The 37 MLH1 deficient cases were analysed for methylation using the OneStep qMethyl^{TM} kit. One case (2.7%) did not have sufficient DNA for assessment whilst 2 (5.4%) cases yielded no result. Five out of 34 cases with sufficient DNA for assessment (14.71%) cases had methylation values below 10%, of which 2 cases were under 5%. These 5 cases are interpreted as not being methylated. The remaining 29 (85.29%) cases demonstrated methylation levels ranging from 13% to 92% (figure 46).

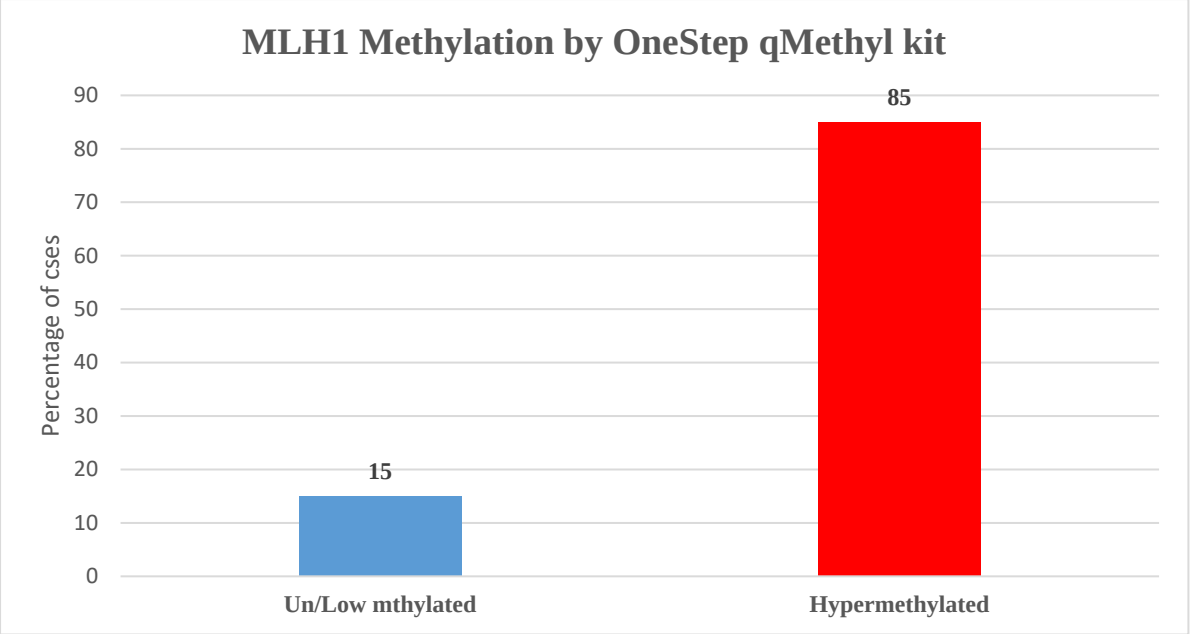

Figure 46. *MLH1* methylation using the OneStep qMethyl™ Kit.

4.13. COMPARISON OF METHYLATION BY EPITYPER AND ONESTEP qMETHYL™ KIT

Table 60. A comparison of methylation percentages in the 37 MLH1 deficient cases.

Case Number	EpiTyper % Methylation	OneStep qMethyl™ Kit % Methylation
1	62	84
2	16	33
3	54	60
4	6	4
5	61	69
6	49	65
7	7	8
8	4	No result
9	34	28
10	56	50
11	61	65
12	5	3
13	29	39
14	30	30
15	49	30
16	20	36
17	48	No result
18	74	69
19	37	60
20	51	68
21	56	83
22	68	72
23	58	83

24	26	27
25	26	13
26	43	56
27	46	37
28	44	44
29	Insufficient DNA	Insufficient DNA
30	28	58
31	57	38
32	**13**	**7**
33	63	66
34	58	92
35	55	37
36	45	43
37	7	4

Table 60 shows only one case (case 32) had a discordant result; with methylation identified by EpiTYPER but not the OneStep qMethyl™ kit. Both test methods had one case which contained insufficient DNA for assessment. Two cases obtained no result with the OneStep qMethyl™ kit. Of these 2 cases, 1 was methylated by EpiTyper whilst 1 demonstrated a value of 4% and is thus deemed not methylated. These three cases have been excluded for comparative purposes. Thus, out of 34 cases, 30 were methylated (88.24%) and four (11.76%) were not methylated by EpiTYPER, whilst 29 out of 34 (85.29%) were methylated and 5 out of 34 (14.70%) were not methylated using the OneStep qMethyl™ kit. These results are graphically represented below (figure 47).

Methylation using EpiTYPER and the OneStep qMethyl™ kit were compared.

Table 61. Methylation detected by EpiTYPER versus the OneStep qMethyl™ kit.

		EpiTYPER		
		Methylation	**No Methylation**	**Total**
OneStep qMethyl™ Zymo Kit	**Methylation**	True Positive (TP) 29	False Positive (FP) 0	**29**
	No Methylation	False Negative (FN) 1	True Negative (TN) 4	**5**
	Total	**30**	**4**	**34**

Sensitivity = $\frac{\text{True Positive}}{\text{(True Positive+False Negative)}} = \frac{29}{(29+1)} = 96.67\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=96.67 \pm 1.96\sqrt{\frac{96.67(100-96.67)}{34}}$$

$$=96.67 \pm 1.96\sqrt{\frac{321.9111}{34}}$$

$$=96.67 \pm 6.030934182$$

$$=90.64 \quad 102.70$$

Specificity = $\frac{\text{True Negative}}{\text{(True Negative+False Positive)}} = \frac{4}{(4+0)} = 100\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=100 \pm 1.96\sqrt{\frac{100(100-100)}{34}}$$

$$=100 \pm 1.96\sqrt{\frac{0}{34}}$$

$$=100 \pm 0$$

$$=100 \quad 100$$

Positive Predictive Value = $\frac{\text{True Positive}}{\text{(True Positive+False Positive)}} = \frac{29}{(29+0)} = 100\%$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=100 \pm 1.96\sqrt{\frac{100(100-100)}{34}}$$

$$=100 \pm 1.96\sqrt{\frac{}{34}}$$

$$=100 \pm 0$$

$$100 \quad 100$$

Negative Predictive Value = $\frac{\text{True Negative}}{} = \frac{4}{} = 80\%$

(True Negative+False Negative) (4+1)

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$=80 \pm 1.96\sqrt{\frac{80(100-80)}{34}}$$

$$=80 \pm 1.96\sqrt{\frac{1600}{34}}$$

$$=80 \pm 13.44548908$$

$$=66.55 \quad 93.45$$

Thus, the sensitivity of the OneStep qMethyl™ Zymo kit is 96.67%; CI (90.64-102.70), the specificity is 100%, 95% CI (100). The positive predictive value (PPV) is 100%, 95% CI (100) and the negative predictive value (NPV) is 80%, 95% CI (66.55-93.45).

The overall accuracy = $\frac{\text{(True Positive + True Negative)}}{\text{(True Positive + True Negative+ False Positive + False Negative)}}$

$$= \frac{(29+4)}{(29+4+0+1)}$$

$$= \frac{29}{34}$$

$$= 85.29\%$$

The average between the specificity and sensitivity or balanced accuracy is:

$$\frac{(100+96.67)}{2}$$

$$= 98.34\%.$$

Thus, a total of 29 (85.29%) cases demonstrated methylation by the OneStep qMethyl™ kit out of 34 and 5 (14.71%) cases showed no methylation. These results demonstrate a concordance of 85.29% between EpiTYPER methylation and methylation using the OneStep qMethyl™ kit.

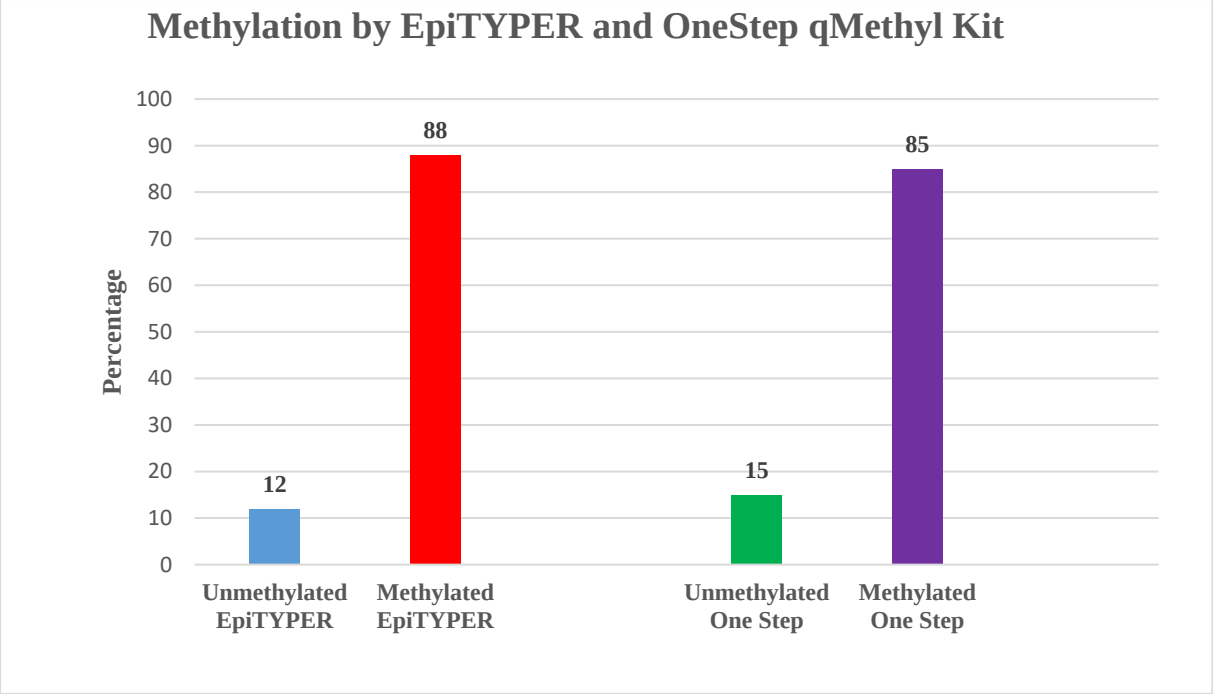

Figure 47. Comparison of methylation by EpiTYPER and the OneStep qMethyl™ Kit.

In addition to the 37 MLH1 deficient cases undergoing methylation analysis, the 25 cases that had discordant IHC and PCR results were subjected to methylation analysis. When the number of cases that did not have sufficient DNA for analysis were excluded, there was a total of 52 cases that could be evaluated by the OneStep qMethyl™ kit (table 62).

Table 62. Frequency of methylated cases which had discordant IHC MMR and MSI PCR results.

Degree of methylation	Frequency	Percentage
<10%	16	30.77
≥10%	36	69.23
Total	52	100

Table 62 illustrates that more than two-thirds of cases that had discordant IHC and PCR and had MLH1 deficiency by IHC results were methylated by both EpiTYPER and the OneStep qMethyl™ kit.

Table 63. Discordant IHC and PCR cases using EpiTYPER and the OneStep qMethyl™ kit.

Specimen Type	No. of cases	Poor tissue preservation	Methylated	Unmethylated	Meth Variable IHC	Unmeth Variable IHC	Variable IHC	Strong IHC staining	Insufficient tissue
Excision	13	9	7	6	7	3	10	3	0

Curetting	12	0	10	2	5	1	6	6	1
Total	25	9	17	8	12	4	16	9	1

The 25 cases that had discrepant results between IHC and PCR were also subjected to methylation analysis by EpiTYPER and the OneStep qMethyl^{TM} kit. Thirteen of these 25 cases were excisions, of which 9 showed poor preservation and 7 were hypermethylated. Seven of the 7 methylated cases showed variable IHC staining, but in total, 10 cases (hyper and unmethylated) showed variable IHC staining (table 63).

The remaining 12 cases were endometrial curettings, 1 of which showed very little residual tissue. There were 10 cases that were hypermethylated. Of the 10 methylated cases, 5 showed variable IHC staining. There was 1 unmethylated case that showed variable staining.

Therefore, of the 25 discordant cases, 17 were hypermethylated (68%). Of the 17 methylated cases, 12 (71%) showed variable IHC staining. Of the remaining 8 cases, 7 were unmethylated, of which 4 (57%) cases showed variable staining. One case had insufficient amounts of tissue from the paraffin embedded tissue block and could not be assessed by for methylation. Thus these 4 cases may have had missense or truncation mutations resulting in varied IHC staining but had PCR detected mutations. One unmethylated case was poorly preserved, but still showed some nuclear staining. It is plausible that the mutations in these cases were detected by PCR due to the ability of the mononucleotide markers to identify small changes in base pairs.

Table 64. Possible cases of Lynch syndrome.

Possible LS Case Number	**IHC Mutation**	**MSI Status**	**Methylation status**
1	MHS2/MSH6	MSI-High	Methylation status not assessed
2.	MHS2/MSH6	MSI-Low	Methylation status not assessed
3.	MSH6	MSS	Not enough DNA
4.	MSH6	MSS	Hypermethylated
5.	MLH1	MSI-Low	Unmethylated
6.	MLH1	MSI-Low	Unmethylated
7.	MLH1	No reaction	Unmethylated

8.	MLH1	MSS	Unmethylated
9.	MLH1	MSS	Not enough DNA
10.	MLH1	MSS	Unmethylated
11.	No mutation	MSI-Low	Unmethylated
12.	No mutation	MSI-Low	Unmethylated
13.	No mutation	MSI-Low	Unmethylated
14.	No mutation	MSI-Low	Unmethylated
15.	No mutation	MSI-Low	No result
16.	No mutation	MSI-Low	No result
17.	No mutation	MSI-Low	No result
18.	No mutation	MSI-High	No result

Table 64 shows 18 cases out of 145 (12.41%) that had deficiency/mutation by IHC or PCR and suggests that further investigation is warranted. Of these, 4 have MSH2/MSH6 or isolated MSH6 loss. One MLH1 mutated tumour did not have enough DNA for further methylation assessment and 9 cases were unmethylated, of which 5 showed MLH1 deficiency by IHC whilst the remaining 4 cases were MLH1 proficient. A further 4 cases yielded no result by methylation assessment which is likely due to insufficient DNA volumes. Furthermore, the true methylation status of these 4 cases cannot be ascertained. As such, a total of 5 out of 18 cases (27.78%) do not have a true methylation assessment. Therefore, the remaining 13 (8.97%) of 145 cases are potential germline carriers based on available results in this study and are suspected of having Lynch syndrome.

Table 65. Methylation status using the OneStep qMethyl^{TM} kit stratified by age.

Factor	Not Methylated (<10%)			Methylated (≥10%)			**Total**		**P-value**
	N= 16	**30.77%**	**95% CI: 58.10767 68.01733**	**N=36**	**69.23 %**	**95% CI: 65.23467 70.98756**	**N=52**	**100%**	
Age (years) (Mean)	63.0625			68.11111					0.0302*
<60	6	37.50%		9	25.00%		15	28.85%	
≥60	10	62.50%		27	75.00%		37	71.15%	

*T-Test

The table above shows that over 60% of unmethylated cases were derived from patients over the age of 60 years; whilst three-quarters of patients with methylated tumours were over 60 years of age, with a statistically significant p-value of 0.0302.

Table 66. Methylation status using the OneStep qMethyl™ kit according to tumour FIGO grade.

FIGO grade	Not methylated <10%	Methylated ≥10%	Total
1	4 (25.00%)	7 (19.44%)	11 (21.15%)
2	9 (56.25%)	22 (61.11%)	31 (59.62)
3	3 (18.75%)	7 (19.44)	10 (19.23%)
Total	16 (100%)	36 (100%)	52 (100%)

Pearson chi2(2) = 0.2083 Pr = 0.901

The majority of unmethylated tumours showed FIGO grade 2 histology whilst FIGO grade 3 features were seen in less than one-fifth of unmethylated tumours. In the methylated group of tumours, most cases were histologically FIGO grade 2 whereas one-fifth of tumours showed FIGO grade 1 or 3 features (table 66).

Table 67. Agreement between the OneStep qMethyl™ kit and IHC deficiency.

ONESTEP qMETHYL™ ZYMO KIT METHYLATION				
IHC		**No Methylation (<10%)**	**Methylation (≥10%)**	**Total**
	Retained	11 (68.75%)	7 (19.44%)	**18 (34.62%)**
	Deficient	5 (31.25%)	29 (80.56%)	**34 (65.38%)**
	Total	**16 (100%)**	**36 (100%)**	**52 (100%)**

Pearson chi2(1) = 11.8978 Pr = 0.001

Table 67 shows that approximately two-thirds of unmethylated cases had retained IHC staining and 5 unmethylated cases had IHC deficiency; whilst over 80% of methylated cases demonstrated IHC deficiency, with statistical significance (p-value = 0.0001).

Table 68. Agreement between the OneStep qMethyl™ kit and IHC deficiency according to age.

Agreement	Age	Kappa	Std. Error	p-Value
80.00%	<60	0.5714	0.2556	0.0127
75.68%	>60	0.4365	0.1615	0.0034

There is a weak degree of agreement between OneStep qMethyl™ kit detected methylated cases and IHC deficiency, with statistical significance in patients under the age of 60 years (kappa=0.5714, p-value = 0.0127) and similarly in patients over the age of 60 years (kappa=0.4365, p-value=0.0034) as shown in table 68; whilst there was moderate agreement

between OneStep qMethyl^{TM} detected methylated cases and IHC mutations in FIGO grade 2 tumour (kappa=0.5953; p-value= 0.0004 (table 69).

Table 69. Agreement between the OneStep qMethyl^{TM} kit and IHC by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	72.73%	0.3774	0.2949	0.1004
2	83.87%	0.5953	0.1790	0.0004
3	60.00%	0.3103	0.2290	0.0877

Tables 70, 71 and 72 show that there is no degree of agreement between OneStep qMethyl^{TM} detected methylated cases and PCR mutations (kappa= -0.1839, p-value = 0.9055); (table 70) between OneStep qMethyl^{TM} kit detected methylated cases and PCR mutations in cases from all patients under and over 60 years of age (kappa=-0.2162, p-value=0.8010; and -0.2143, p-value=0.9013 respectively) as shown in table 71, and between OneStep qMethyl^{TM} kit and PCR irrespective of tumour FIGO grade, with no statistical significance (table 72).

Table 70. Agreement between the OneStep qMethyl^{TM} kit and PCR mutations.

Agreement	Kappa	Std. Error	p-Value
49.02%	-0.1839	0.1400	0.9055

Table 71. Agreement between the OneStep qMethyl^{TM} kit and PCR mutations according to age.

Agreement	Age	Kappa	Std. Error	p-Value
40.00%	<60	-0.2162	0.2558	0.8010
52.78%	>60	-0.2143	0.1662	0.9013

Table 72. Agreement between the OneStep qMethyl^{TM} kit and PCR by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	45.45%	-0.1786	0.3015	0.7232
2	46.67%	-0.1940	0.1805	0.8588
3	60.00%	-0.1765	0.2557	0.7549

Table 73. A confusion matrix between the OneStep qMethyl™ kit methylation and PCR mutations.

ONESTEP qMETHYL™ KIT METHYLATION				
		No Methylation (<10%)	**Methylation (≥10%)**	**Total**
PCR	**No Mutation**	3 (18.75%)	13 (37.14%)	**16 (31.37%)**
	Mutation	13 (81.25%)	22 (62.86%)	**35 (68.63%)**
	Total	**16 (100%)**	**35 (100%)**	**51 (100%)**

Pearson chi2(1) = 1.7253 Pr = 0.189

This table (table 73) shows that over 80% of the unmethylated cases had PCR mutations and nearly two-thirds of methylated cases were mutated by PCR. One PCR reaction yielded no result on an endometrial carcinoma and was thus excluded for comparison.

Table 74. A confusion matrix between the OneStep qMethyl™ kit methylation and PCR mutational status.

ONESTEP qMETHYL™ KIT METHYLATION				
		No Methylation	**Methylation**	**Total**
PCR	**Microsatellite stable**	3 (18.75%)	13 (37.14%)	**16 (31.37%)**
	MSI-Low	10 (**62.50%**)	17 (**48.57%**)	**27 (52.94%)**
	MSI-High	3 **(18.75%)**	5 **(14.29%)**	**8 (15.69%)**
	Total	**16 (100%)**	**35 (100%)**	**51 (100%)**

Pearson chi2(2) = 1.7259 Pr = 0.422

Of the unmethylated cases, over 60% demonstrated MSI-Low by PCR whilst under one-fifth of cases were MSI-High or were microsatellite stable; whereas nearly half of the methylated cases were MSI-Low and over a third were microsatellite stable (table 74).

Table 75. A confusion matrix between the OneStep qMethyl™ kit methylation and PCR microsatellite low and high.

ONESTEP qMETHYL™ METHYLATION				
		No Methylation	**Methylation**	**Total**
	MSI-Low	10 (**37.04%**)	17 (**62.96%**)	**27 (100%)**

PCR	**MSI-High**	3 **(37.50%)**	5 **(62.50%)**	**8 (100%)**
	Total	**13 (37.14%)**	**22 (62.86%)**	**35 (100%)**

Pearson chi2 (1) = 0.0006 Pr=0.981

Fisher's exact = 1.000

1-sided Fisher's exact = 0.645

Table 75 shows that most of the methylated cases (62.96%) demonstrated MSI-Low by PCR and similar results were obtained between methylated cases that showed MSI-High by PCR.

Table 76. Agreement between the OneStep qMethyl^{TM} kit and MMR/MSI PCR overall mutational status.

ONESTEP qMETHYL^{TM} KIT METHYLATION				
		No Methylation (<10%)	**Methylation (≥10%)**	**Total**
Overall MMR deficiency/ PCR mutation	**Mutation/ Abnormality**	16 (**100%**)	35 (**100%**)	**51 (100%)**
	Total	**16 (100%)**	**35 (100%)**	**51 (100%)**

Of the 51 cases assessed (table 76), 16 were not methylated but were deficient/mutated by IHC or PCR respectively and 35 of the methylated cases were deficient/mutated by either IHC or PCR respectively.

Table 77. Agreement between the OneStep qMethyl^{TM} kit and overall abnormality/mutational status.

Agreement	**Kappa**	**Std. Error**	**p-Value**
69.23%	0.0000	0.0000	-

There is no agreement between OneStep qMethyl^{TM} kit and overall abnormality/mutational status (table 77); the OneStep qMethyl^{TM} kit and overall abnormality/mutational status by patient age (table 78); and abnormality/mutational status according to tumour FIGO grade (table 79).

Table 78. Agreement between OneStep qMethyl^{TM} kit and overall abnormality/mutational status according to age.

Agreement	**Age**	**Kappa**	**Std. Error**	**p-Value**
60.00%	<60	0.0000	0.0000	0.5000
72.97%	>60	0.0000	0.0000	0.5000

Table 79. Agreement between the OneStep qMethyl™ kit and overall abnormality/mutational status by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	63.64%	0.0000	-	-
2	70.97%	0.0000	-	-
3	70.00%	0.0000	-	-

Table 80. Agreement between the OneStep qMethyl™ kit and BRAF IHC.

Agreement	Kappa	Std. Error	p-Value
17.65%	0.0104	0.0247	0.3367

Table 80 shows that there is no agreement or statistically significant relationship between methylation using the OneStep qMethyl™ kit and BRAF IHC. This agreement is based on the exclusion of 3 cases which had no methylation using the OneStep qMethyl™ kit but could be assessed using the BRAF IHC stain (table 80). Furthermore, that there is no agreement or statistically significant relationship between methylation using the OneStep qMethyl™ kit and BRAF IHC and patient age (table 81).

Table 81. Agreement between the OneStep qMethyl™ kit and BRAF IHC stratified by age.

Agreement	Age	Kappa	Std. Error	p-Value
30.00%	<60	0.0541	0.1026	0.2991
12.50%	≥60	0.0000	0.000	-

The calculations (for both tables 81 and 82) were based on test tissue that did not include the 3 cases as noted in table 80.

Table 82. Agreement between the OneStep qMethyl™ kit and BRAF IHC stratified by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	25.00%	0.0000	0.0000	-
2	17.39%	0.0135	0.0342	0.3461
3	0.00%	0.0000	0.0000	-

Table 82 demonstrates that there is no statistically significant relationship between the methylation using the OneStep qMethyl™ kit and BRAF IHC with respect to tumour FIGO grade. Similarly, there is no agreement or statistically significant relationship between methylation using the OneStep qMethyl™ kit and BRAF PCR (table 83). There were 4 BRAF PCR mutations identified. Of these, 3 showed positive methylation using the OneStep

qMethyl™ kit. This number was used in the calculations for the next two tables (tables 84 and 85).

Table 83. Agreement between OneStep qMethyl™ kit and BRAF PCR.

Agreement	Kappa	Std. Error	p-Value
20.59%	-0.0315	0.0508	0.7320

Table 84 Agreement between the OneStep qMethyl kit and BRAF PCR stratified by age.

Agreement	Age	Kappa	Std. Error	p-Value
30.00%	<60	0.0541	0.1026	0.2991
16.67%	≥60	-0.0667	0.0572	0.8783

Tables 84, 85 and 86 show that there is no association between methylation using the OneStep qMethyl™ kit and BRAF PCR stratified by age; no agreement or statistically significant relationship between methylation using the OneStep qMethyl™ kit and BRAF PCR according to tumour FIGO grade and no agreement between methylation using the OneStep qMethyl™ kit and BRAF sequencing. These calculations were performed on 34 cases only as there were 3 cases in which no result was obtained using the OneStep qMethyl test but had undergone BRAF sequencing. Of these 3 cases, 2 showed no mutation by BRAF sequencing whilst 1 demonstrated a BRAF mutation. As such, the tables 86, 87 and 88 reflect calculations for 34 cases.

Table 85. Agreement between the OneStep qMethyl™ kit and BRAF PCR stratified by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	12.50%	-0.2727	0.1473	0.9680
2	26.09%	0.0440	0.0612	0.2360
3	0.00%	0.0000	0.0000	-

Table 86. Agreement between the OneStep qMethyl kit and BRAF Sequencing.

Agreement	Kappa	Std. Error	p-Value
14.71%	0.0000	-	-

Table 87. Agreement between the OneStep qMethyl™ kit and BRAF Sequencing stratified by age.

Agreement	Age	Kappa	Std. Error	p-Value
20.00%	<60	0.0000	0.0000	-
12.50%	≥60	0.0000	0.0000	-

Table 87 to 91 demonstrate that there is no association between methylation using the OneStep qMethyl™ kit and BRAF sequencing according to patient age (table 87), tumour FIGO grade (table 88), overall mutational status (table 89), overall mutational status by age (table 90) and overall mutational status stratified by tumour FIGO grade (table 91).

Table 88. Agreement between the OneStep qMethyl™ kit and BRAF Sequencing stratified by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	25.00%	0.0000	0.0000	-
2	13.04%	0.0000	0.0000	0.5000
3	0.00%	0.0000	0.0000	-

Table 89. Agreement between the OneStep qMethyl™ kit and BRAF overall mutational status.

Agreement	Kappa	Std. Error	p-Value
23.53%	-0.0208	0.0574	0.6413

Table 90. Agreement between the OneStep qMethyl™ kit and BRAF overall mutational status by age.

Agreement	Age	Kappa	Std. Error	p-Value
40.00%	<60	0.1176	0.1488	0.2146
16.67%	≥60	-0.0667	0.0572	0.8783

Table 91. Agreement between the OneStep qMethyl™ kit and BRAF overall mutational status by tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	12.50%	-0.2727	0.1473	0.9680
2	30.43%	0.0612	0.0718	0.1970
3	0.00%	0.0000	0.0000	-

Table 92. A confusion matrix between the OneStep qMethyl™ kit and EpiTYPER.

ONESTEP qMETHYL™ KIT METHYLATION				
		No Methylation (<10%)	**Methylation (≥10%)**	**Total**

EpiTYPER Methylation	**No Methylation**	9 (**56.25%**)	3 (**8.33%**)	**12 (23.08%)**
	Methylation	7 (43.75%)	33 (91.67%)	**40 (76.92%)**
	Total	**16 (100%)**	**36 (100%)**	**52 (100%)**

Pearson chi2(1) = 14.3271 Pr = 0.001

Table 92 illustrates that over half the number of unmethylated cases assessed by EpiTYPER were also not methylated by the OneStep qMethyl^{TM} kit. Under half the number of methylated EpiTYPER cases were not methylated by the OneStep qMethyl^{TM} kit. Less than 10% of EpiTYPER unmethylated cases were methylated by the OneStep qMethyl^{TM} kit whilst the vast majority of cases (91.67%) were methylated by both test methodologies, which is statistically significant (p-value = 0.001). Using the confusion matrix (table 92) and in light of EpiTYPER being a validated test (against which other methods are tested due to its high precision)[114], it is possible to assess sensitivity, specificity, positive and negative predictive values for the OneStep qMethyl^{TM} kit for all 52 cases that underwent methylation assessment.

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{(True Positive+False Negative)}} = \frac{33}{(33+3)} = 91.67\%$$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; p=percentage, n=sample size

$$= 91.67 \pm 1.96\sqrt{\frac{91.67(100-91.67)}{52}}$$

$$= 91.67 \pm 1.96\sqrt{\frac{14.68482885}{52}}$$

$$= 91.67 \pm 7.510874683$$

$$= 84.16 \quad 99.18$$

$$\text{Specificity} = \frac{\text{True Negative}}{\text{(True Negative+False Positive)}} = \frac{9}{(9+7)} = 56.25\%$$

95% Confidence Interval = $p \pm 1.96\sqrt{\frac{p(100-p)}{n}}$; where p=percentage, n=sample size

$$=56.25\pm1.96\frac{56.25(100-56.25)}{52}$$

$$=56.25\pm1.96\frac{2460.9375}{52}$$

$$=56.25 \pm 13.48346098$$

$$=42.77 \qquad 69.73$$

Positive Predictive Value = $\frac{\text{True Positive}}{\text{(True Positive+False Positive)}} = \frac{33}{(33+7)} = 82.5\%$

95% Confidence Interval = $p\pm1.96\frac{p(100-p)}{n}$; where p=percentage, n=sample size

$$=82.5\pm1.96\frac{100(100-82.5)}{52}$$

$$=82.5\pm1.96\frac{1750}{52}$$

$$=82.5 \pm 11.37033928$$

$$71.12 \qquad 93.87$$

Negative Predictive Value = $\frac{\text{True Negative}}{\text{(True Negative+False Negative)}} = \frac{9}{(9+3)} = 75\%$

95% Confidence Interval = $p\pm1.96\frac{p(100-p)}{n}$; p=percentage, n=sample size

$$=75\pm1.96\frac{75(100-75)}{52}$$

$$=75\pm1.96\frac{1875}{52}$$

$$=75 \pm 6.004805768$$

$$=69.00 \qquad 81.00$$

Thus, the sensitivity of the OneStep qMethyl^{TM} kit is 91.67%; CI (84.16-99.18), the specificity is 56.25%, 95% CI (42.77-69.73). The positive predictive value (PPV) is 82.5%, 95% CI (71.12-93.87) and the negative predictive value (NPV) is 75%, 95% CI (69.00-81.00).

The overall accuracy = (True Positive + True Negative)

(True Positive + True Negative+ False Positive + False Negative)

= (33+9)

(33+9+7+3)

= 42

52

= 80.77%

The average between the specificity and sensitivity or balanced accuracy is:

(56.25+91.67)

2

= 74%.

The concordance level of the OneStep qMethyl™ kit relative to EpiTYPER methylation assessment is 80.77%.

Table 93. Agreement between EpiTYPER and the OneStep qMethyl™ kit.

Agreement	Kappa	Std. Error	p-Value
80.77%	0.5149	0.1360	0.0001

Table 93 demonstrates a statistically significant level of agreement between the 2 methylation analysis methods, with a weak kappa score (kappa =0.5149, p-value = 0.0001); whereas there is no agreement in patients under the age of 60 years (table 94 below). This is not statistically significant (kappa=0.1935; p-value = 0.1024). In patients over the age of 60 years however, there is moderate agreement and statistical significance between the OneStep qMethyl™ kit and EpiTYPER methylation (kappa= 0,6679; p-value=0.0001).

Table 94. Agreement between methylation using the OneStep qMethyl™ kit and EpiTYPER analysis stratified by age.

Agreement	Age	Kappa	Std. Error	p-Value
66.67%	<60	0.1935	0.1527	0.1024
86.49%	>60	0.6679	0.1640	0.0001

Table 95. Concordance between EpiTYPER and the OneStep qMethyl^{TM} kit according to tumour FIGO grade.

FIGO grade	Agreement	Kappa	Std. Error	p-Value
1	90.91%	0.8136	0.2962	0.0030
2	80.65%	0.4593	0.1675	0.0031
3	70.00%	0.2105	0.3051	0.2451

Table 95 shows that the greatest degree of agreement between the 2 methylation methodologies is in FIGO grade 1 tumours (kappa=0.8136, p-value = 0.0030) and FIGO grade 2 tumours (kappa 0.4593 and p-value= 0.0031) whilst there is no statistical significance between the two tests and FIGO grade 3 carcinomas (kappa=0.3051, p-value =0.2451).

4.14. SUMMARY OF RESULTS

- Almost two-thirds of patients with EEC were over 60 years of age.
- Most tumours (43.45%) demonstrated FIGO grade 2 histological features.
- Out of 145 EEC cases, 41 showed MMR deficiency by IHC, of which MLH1 losses were the most common.
- MSI by PCR was detected in 46 cases, of which, most were microsatellite low with a minority being MSI-high.
- Using both IHC MMR and PCR MSI, a total of 45.51% of 145 cases showed deficiency/mutations.
- Six BRAF mutations were identified out of 37 MLH1 deficient cases; of which 1 each was detected by BRAF V600E IHC and BRAF V600E Sanger sequencing whilst 4 BRAF mutations were detected by PCR.
- Of the 4 BRAF PCR identified mutations, 3 were BRAF V600E mutations and 1 was a V600D mutation.
- The 37 MLH1 deficient cases also underwent methylation assessment, of which more than 80% showed hypermethylation.

- A further 25 cases that demonstrated discordant results between IHC MMR and MSI PCR were also subjected to methylation assessment, of which 68% were hypermethylated.
- Out of 52 comparable cases, EpiTYPER MassARRAY showed methylation in 76.92% of cases; whilst the OneStep qMethyl^{TM} kit showed methylation in 69.23% of cases.
- Most MMR deficient cases could therefore be ascribed to hypermethylation.
- Thirteen cases out of 145 (8.97%) however, are suspected of having possible germline mutations.

CHAPTER 5

5.0. DISCUSSION

This study was conducted with the aim of identifying cases of microsatellite instability in endometrial carcinomas with the view to introducing screening modalities at our institution for the identification of patients with possible Lynch syndrome.

5.1. STUDY LIMITATIONS

The exact race/ethnicity of each of the patients is not known. This may be explained by the fact that the clinical request form on which the patient's details are recorded by the treating physician does not always have the patient's race group recorded. As South Africa is a culturally diverse nation, it is not possible to assume a patient's race based purely on their name.

Many patients in the public health sector of South Africa are unfortunately lost to follow up for various reasons that range from financial to cultural beliefs on Western medicine. Thus, not all patients who had undergone an endometrial curettage, had presented for hysterectomy. It is therefore not possible to correlate each patient's hysterectomy specimen result with the results from an endometrial curettage specimen. In addition, it is not possible to stage each of the tumours in this study as many patients did not undergo formal hysterectomies. Furthermore, as many specimens were endometrial curettings and not excision specimens, it was not possible to assess cases for histological features that have been said to be suggestive of Lynch syndrome in endometrial carcinomas; such as the occurrence of tumour within the lower uterine segment, peri-tumoural lymphocytes of more than 42 per 10 high powered field, undifferentiated tumour or tumour heterogeneity as documented by Garg *et al*[94]

Confirmatory germline mutational assessment is currently not available at the Department of Human Genetics, Braamfontein, NHLS. Thus, the cases in this study that have been

identified as having mismatch repair deficiencies by immunohistochemistry, in addition to microsatellite instability by PCR cannot undergo confirmatory, definitive testing for Lynch syndrome. This has not been budgeted for by me, as such testing is not within the scope of the current study. Furthermore, such testing would require consent from the patients concerned; and in light of some patients having been lost to follow up, which is not an uncommon occurrence in our context, this would not be a feasible task. Following on discussions with consultants at the Department of Human Genetics, it is hoped that this study may provide sufficient evidence for the need to include such testing in the state sector so that patients serviced by the public health system may be afforded additional management.

5.2. DISCUSSION OF RESULTS

Endometrial carcinomas are frequently occurring tumours that are currently on the increase in western societies[1–4,7–9] Endometrioid endometrial carcinoma, the most common histological variant, is the prototypic Type I tumour and is strongly associated with high estrogen levels.[11] One of the most frequently identified molecular abnormalities in this histological subtype is microsatellite instability.[17,18,30,35] Most tumours associated with Lynch syndrome are microsatellite unstable, as are up to 30% of sporadically occurring tumours.[17,38]

From Table 6, the mean age of the 145 patients with endometrial carcinoma in this study was 65.24 (±9.2) years. This is slightly older than the mean age of patients in a study undertaken at The University of Texas MD Anderson Cancer Center in which a mean of 61.3 (±10.6) years was noted. Approximately 66.9% of patients in the current study were menopausal and were 60 years or older. Furthermore, 30.34% were FIGO grade 1 tumours, 43.45% were FIGO grade 2 tumours and 26.21% were FIGO grade 3 tumours. Studies undertaken by Haruma *et al*[115] grouped FIGO grade 1 and 2 tumours together which accounted for 90.79% of their cases; whilst less than 10% of endometrial carcinomas in their study showed FIGO

grade 3 histological features. Whilst the majority of cases in the present study comprised FIGO grades 1 and 2 tumours, more than a quarter of the cases demonstrated 3 histological features.

The remainder of data from table 6 table 5 are elaborated on in detail in later sub-sections.

5.2.1. IMMUNOHISTOCHEMICALLY DETECTED MISMATCH REPAIR DEFICIENCIES

Immunohistochemical staining for the four mismatch repair antibodies, namely MLH1, MSH2, MSH6 and PMS2 is often the first step in screening for patients with suspected Lynch syndrome; thus, accurate evaluation and analysis of these markers is required.[116] A binary pattern of staining is used in interpretation whereby a positive nuclear signal in tumour nuclei is widely accepted as retained mismatch repair; whilst an absence of nuclear staining of tumour cells is considered deficient (figures 15-26).[26,31,59,117] Immunohistochemical staining of nuclear or cytoplasmic membranes or nucleoli are anomalous and should be not be interpreted as retained mismatch repair.[116] The present study illustrated deficient staining in 28.28% (41 of 145) cases (table 6) and were thus regarded as demonstrating mismatch repair deficiency. Table 7A (table 10) shows that MLH1 was the most frequently identified deficient stain (25.52%) in this study; whilst MSH2 was the most uncommonly identified deficient stain (1.38%). Per patient sample (table 7B), MLH1 and a combination of MLH1 and PMS2 were most often deficient (11.03% and 13.79% respectively). There were no cases of isolated loss of PMS2 (table 7B). These results mirror those found in other studies. [65,94] The synchronous loss of both MLH1 and PMS2 may be explained by MLH1 being the obligatory partner of the heterodimer which is needed for expression. A mutation in MLH1 culminates in breakdown of the dimer and loss of staining of both components.[56] Similarly, this explains the dual loss of MSH6 and MSH2. Only 1 patient sample in this study (0.69%)

showed 3 IHC losses (MLH1, PMS2, MSH6). These stained tissue sections demonstrated complete loss of staining in tumour nuclei with their respective antibody markers, whilst adequate internal controls such as endothelial cells, lymphocytes and stromal cells demonstrated retained nuclear positivity. This provided evidence that the loss of staining of tumour nuclei was indeed a valid result. Any degree of positive staining in tumour nuclei was considered retained/mismatch repair proficient. There were 22 cases out of 145 in the present study that demonstrated a weak or focal nuclear signal and were therefore considered mismatch repair intact. Four of these cases demonstrated weak or focal staining in all four IHCs. The distribution of variable staining patterns across all four stains is illustrated in figure 27. Figure 28 demonstrates the relationship of these variable IHC staining patterns to their MSI PCR status and their methylation status. Pai *et al*[116] state that weak intensity staining may be challenging but that evaluation of the internal control is imperative so as to exclude technical matters resulting in weak intensity staining.[116] The technical problems that may be responsible include a delay in placement of the tissue specimen in formalin, the type of formalin used for fixation, prolonged periods of formalin fixation which affect antigenicity of tissue specimens or decalcification treatment.[118] Watson *et al*[117]consider the binary pattern of staining as indicative of a "flawed definition" and state that without information on a patient's MSI status that an erroneous diagnosis may be reached in some patients at risk for LS.[28] There are authors such as Pai and colleagues[116] and Stelloo *et al,* [20] who acknowledge that there may actually be three patterns of staining namely: complete negativity, diffuse positivity as well as heterogenous staining whereby scattered neoplastic cells display loss of staining.[20,116] Joost *et al,*[119] in their studies on colorectal carcinomas have recognised three heterogeneous patterns of staining which include clonal, intraglandular and compartmental; all of which were noted within the same neoplasm with the extent of tumour involvement having varied.[119] A number of factors are believed to be involved in this heterogeneity such

as unpredictable expression of epitopes on tumour cells, methylation of specific tumour clones, varied tumour differentiation with variable epitope expression and second-hit mutations. In addition, there may be localised tissue hypoxia accounting for heterogeneous IHC staining.[119] Stelloo and colleagues[20] found that heterogeneous loss of MSH6 together with complete loss of MLH1/PMS2 could be ascribed to MSH6 undergoing additional secondary mutations.[20] These authors also identified MLH1 methylation and MSI in subclones which suggests a sporadic occurrence of intra-tumoural heterogeneity. This may explain the varied IHC staining patterns noted in cases with MSI mutations and methylation noted in the current study (figure 28). Stelloo and colleagues also suggest that tumours with >10% heterogeneous sub clonal IHC staining be considered MMR deficient despite the tumours demonstrating microsatellite stability in regions where IHC staining is retained.[20] Watson *et al*[117] discuss the heterogeneous patterns of staining and in their correspondence to the Editor-in-Chief,[120] further elaborate that the heterogeneous IHC staining noted in their initial article[117] suggests that individuals should undergo additional MSI testing.[117] In the study by Watson *et al*,[117] approximately 10% of the patients in their study exhibited heterogeneous patterns of staining and were thus initially interpreted as showing normal mismatch repair activity. These cases were subsequently microsatellite unstable by PCR and demonstrated germline mutations. Thus, the initial IHC interpretation of intact MMR machinery was incorrect and had this been the only screening test employed in their study, patients with LS would have been missed.

Of the IHC diagnosed MMR deficient tumours in the present study, the mean age at diagnosis was 65.3 (± 9.7) years (Table 8A) which is higher than that noted in studies by Buchanan *et al*[65] and Kato *et al*,[121] but is fairly similar (67.1 years) to that seen by McConechy *et al*.[31] Of the IHC mutated cases, FIGO grade 2 histological features were seen in 66.9% of samples in the present study; whilst the minority of tumours (12.2%) showed FIGO grade 3 features

(Table 8A) FIGO grade 2 histological features were also identified as being the most common subtype in a study by Bruegel *et al*[122] and McConechy *et al*.[31] This may be due to microsatellite unstable endometrial carcinomas being coupled to higher FIGO grade and stage.[41] In contrast, a study undertaken by Buchanan *et al*[65] demonstrated that FIGO grade 1 histological features were the most commonly occurring subtype (46.6%).[65]

Table 8B and Table 8C will be discussed in their respective subsections.

5.2.3. MICROSATELLITE INSTABILITY DECTECTED BY PENTAPLEX PCR

Table 6 shows that approximately one third of endometrial carcinomas in the present study were microsatellite unstable (figures 29-33). A study by Bruegl *et al*[122] highlighted MSI in roughly 28% of their patients, whilst a fifth of patients in a study by Black *et al*[28] were microsatellite unstable. A possible explanation for the lower percentage of MSI cases noted by Black *et al*[28]could be due to tumours in their cohort including non-endometrioid subtypes, such as serous carcinomas and clear cell carcinomas, in which microsatellite instability does not feature as a main pathogenetic pathway in contrast to endometrioid endometrial carcinomas.[28,30] The current study confirmed a higher percentage of MSI-low (24.1%) cases compared to MSI-high (7.6%) cases. Bruegl *et al*[122] and Wang *et al* [59] however found the converse; with a quarter of their patients being MSI-High and just 6% being MSI-low.[59,122]

Table 8B exhibits that approximately two thirds of patients with microsatellite unstable (mutated) tumours were over the age of 60 years. Similar results (66%) were obtained for patients with microsatellite stable tumours over the age of 60 years. The mean age of patients with MSI tumours in a study by Black *et al*[28] was 62 years whilst the mean age of patients with microsatellite stable tumours was 63 years.[28] The mean age of patients with microsatellite unstable tumours in a study by Kanopiene *et al*[123] was 65.7 years whilst the mean age in patients with microsatellite stable tumours was 63.5years.[123] These two studies,

as observed in the current one, do not show statistical significance in age between microsatellite stable and unstable tumours (p-value = 0.95). These findings are corroborated by Karamurzin *et al*[41] during their analysis of multiple studies.[41]

Table 8B shows that half of the mutated or microsatellite unstable tumours in the present study displayed FIGO grade 2 histological features, with 28% showing FIGO grade 3 features. Tumours with FIGO grade 1 histology accounted for the least amount of cases. These results show that there is no statistically significant association between PCR diagnosed mutations and tumour FIGO grade (p-value =0.30) which is in contrast to findings of overall mutational status in relation to tumour FIGO grade (p-value = 0.030) (Table 8C). A number of studies, as stated by Karamurzin *et al*[41] have examined the FIGO grade of tumours that have MSI, with some studies documenting a higher FIGO grade whilst others have not shown the same. Karamurzin and colleagues[41] state that none of the studies have recorded an association between MSI and a lower histological tumour FIGO grade.[41] Shia *et al*[124] however, noted that tumour FIGO grade did not have a significant association with PCR MSI-high status in their study.[124] Thus, it may be seen that various studies have demonstrated contrasting findings with some illustrating an association between PCR MSI tumours with high tumour FIGO grade and others showing the opposite. The present study shows that most tumours displaying mutation irrespective of the method of detection (that is to say, overall mutation), were diagnosed as FIGO grade 2 tumours. There is a strong correlation between overall mutational status and tumour FIGO grade identified in this study (p-value=0.030). However, to the best of the investigator's knowledge, studies have not examined the overall mutational status with respect to age or tumour FIGO grade.

5.2.4. MSI HIGH TUMOURS

From the MSI-high group in the current study, 75% of cases had 2 mutations, whereas 3 (25%) cases had 3 mutations. (Figure 34). The current study demonstrated that BAT25 was the most commonly mutated marker, followed by NR27, NR21, BAT26 and NR24 (table 9 and figure 35). A study by Wong *et al*[125]also showed BAT25 to be the most commonly mutated allele, followed by NR27, NR24, BAT26 and NR21.[125] The differences in frequencies of the various alleles may be ascribed to the different populations between the two studies as the incidence of genetic variation of polymorphism for each of the markers varies between populations.[126] Buhard *et al*[126] have identified that African populations demonstrate the greatest rate of pleomorphism for each marker excluding NR24. In their study, the frequency of variant alleles for each marker in individuals from Africa in decreasing order was NR27, NR21, BAT25, BAT26. [126] In light of the South African population being so culturally diverse, with patients from countries throughout the African continent being assessed at our local clinics and hospitals with their respective specimens being diagnosed at Charlotte Maxeke Johannesburg Academic Hospital, it is not unexpected that a varied frequency of mutated alleles be seen, as reflected by the results of the present study.

5.2.5. A COMPARISON OF RESULTS OBTAINED BY IHC AND PCR

There were 41 MMR deficient stains identified by IHC whilst 46 MSI cases were detected by PCR. Five cases yielded no result by PCR and were thus excluded or comparative purposes (table 8C). Of the abnormal cases, detected by either IHC or PCR, approximately two-thirds of cases were from patients over the age of 60 years (Table 8C). The mean age at which endometrial carcinomas were diagnosed in patients with abnormalities and those without abnormalities is not statistically significant (p-value = 0.6005). Similar results have been documented in other studies using either IHC or PCR.[28,41,123] However, significant differences in age have been documented in cases of sporadically occurring endometrial tumours versus

those in patients with Lynch syndrome.[41] Whilst most endometrial carcinomas in LS patients tend to occur over the age of 50 years, up to a quarter of such cases may arise in patients under 50 year of age. As such, some authors advocate the need for Lynch syndrome screening tests in endometrial carcinomas developing in younger females. [41,127] This would undoubtedly be beneficial in identifying patients with suspected LS to offer appropriate genetic counselling, confirmatory testing and to ensure that the patient and her relatives are afforded continuous surveillance for the development of possible neoplasias. To the best of the investigator's knowledge, studies (such as work by McConechy *et al*[31]) have examined either IHC MMR or PCR MSI in relation to age and tumour FIGO grade, but have not looked at overall abnormality/mutational status with respect to these biological parameters. As such it is not possible to compare the findings of the present study to those in the literature.

5.2.6. AGREEMENT OF ABNORMAL/MUTATIONAL DIAGNOSIS BETWEEN IHC AND PCR

The present study revealed an IHC sensitivity of 45.65%, a specificity of 80.85%, a positive predictive value (PPV) of 53.85% and a negative predictive value (NPV) of 75.25% relative to PCR (table 10). These results are in contrast to studies undertaken by others such as McConechy *et al*[31] and Takehara *et al.*[128] In the study by McConechy and colleagues,[31] a sensitivity of 88.5%, specificity of 95.2%, PPV of 88.5% and NPV 95.2% was achieved for MMR IHC's relative to MSI by PCR.[31] In addition, Takehara *et al*,[128] using a tetraplex PCR system obtained a sensitivity of 92.7% relative to MMR deficient IHCs; and a specificity of 97.9% for MMR proficient IHCs relative to PCR.[128] Furthermore, Modica *et al* [129] showed a sensitivity level of 91% and a specificity level of 83% using MMR IHC to predict MSI-H phenotype. [129]The overall accuracy in the present study is 69.29% and the balanced accuracy is 69.25%. From table 11, the concordance or level of agreement was 69.29%. There were 25 cases that showed MSI by PCR but were MMR proficient, which are regarded as false

negatives when PCR is regarded as the gold standard.[110] These 25 cases subsequently underwent hypermethylation analysis which showed that 17 (68%) cases were methylated, thus accounting for some of the discrepant IHC/PCR results (figure 36). In 4 of the unmethylated cases, variable IHC staining was seen and these cases were therefore regarded as mismatch proficient whilst PCR identified mutations. PCR MSI may recognise mutations that are not identifiable by the current IHC antibodies used and would thus not be detected immunohistochemically.[56] Furthermore, PCR may be able to detect small changes in base pairs which may not be detected on a protein or immunohistochemical level. Of the 25 discordant cases, 9 cases (36%) showed strong staining for all 4 IHC markers, 5 (20%) demonstrated variable staining with MLH1/PMS2, 4 cases (16%) had variable staining of all 4 IHC antibodies and the tissue showed poor preservation. There were 5 cases (20%) that showed variable and weak PMS2 staining, 1 case (4%) showed variable MSH6/PMS2 staining and 1 case (4%) showed variable/focal staining of MLH1/MSH6 and PMS2. Hypermethylation may account for the discrepant MMR/MSI results. Another possible explanation for discordance includes heterogeneity of tumour within the tissue sections thereby accounting for focal/weak staining in tumour nuclei. Furthermore, tumour heterogeneity may result in microsatellite instability identified in a subclone of tumour which may have been negative immunohistochemically in an area of tumour whilst the remaining tissue section showed nuclear positivity and was thus interpreted as MMR proficient.[31] A recent study by Sari *et al*[130] has suggested that these cases should be reported as mismatch repair deficient as these neoplasms may be MSI-high tumours which may be associated with germline mutations.[130] Other possibilities for discordant results between IHC and PCR include used of archived material with non-standard fixation methods, degradation of antigens in tissue sections as well as the possibility of existent, but non-functional MMR genes.[31,131] Furthermore, it is documented that patients who are carriers of MSH6 mutations

have lower occurrences of MSI with PCR showing either MSS or MSI-low.[31] Joost *et al* [119] state that there may be mixed expression of epitopes within a tumour which may result in clonal heterogeneity or intraglandular heterogeneity.[119] Furthermore, areas of variable tumour differentiation may display varied expression of an immunohistochemical stain. Furthermore, there may be additional methylation or second hit mutations in certain clones of tumour cells[119] These 25 discordant cases are also discussed in the methylation subsection.

From a technical aspect, there are a number of possible explanations for a false positive immunohistochemical stain such as the interval from biopsy or excision to placement in formalin, the type of formalin used for tissue fixation, the pH of the formalin and the duration of tissue fixation.[118,131–133] The interval from surgical biopsy to formalin fixation is a variable that cannot be accounted for by laboratory personnel and is a confounding factor. Furthermore, prolonged fixation in formalin of specimens derived from peripheral hospitals is another variable that cannot be justified as this is beyond our control. In the early 2000's our laboratory produced 10% neutral buffered formalin in-house whereas the laboratory currently purchases formalin from a commercial supplier. This may account for the discrepant cases noted on archived material from 2009. Our laboratory can provide the formalin used currently to theatres and wards at the Charlotte Maxeke Johannesburg Academic Hospital, but it is uncertain what type of formalin is used by peripheral clinics and hospitals which submit biopsies and excision specimens to our department as they do not obtain formalin directly from our laboratory. Additional technical factors that may result in a false positive result include non-specific background staining, the antigen retrieval method used, the concentration of antibody used, the period of incubation time of the primary antibody and the detection kit used.[132,134] One of the most common technical causes of false positive staining is a very high concentration of antibody.[132] In the present study, a high concentration of antibody can be excluded as a cause for false positive staining as the concentration of

antibody used was the same for each of the individual markers. In addition, control tissue sections were run with test tissue sections and were examined for adequacy of staining. The remaining factors described by Nuovo[132] can be disregarded as a cause of a false positive staining as only nuclear staining of cells was interpreted as positive and any background staining if identified, was disregarded. Furthermore, the antigen retrieval method, the period of incubation time of the primary antibody and the detection kit used were the same for all cases evaluated and were thus standardised in this regard. Lastly, the antibody clones used have been considered as possible factors contributing to discrepant results. The MLH1 (clone ES05) antibody was assessed to perform the best amongst a number of clones in an evaluation of MLH1 by NordIQC.[135] Whilst the clones for the remaining antibodies PMS2 (clone MOR4G), MSH2 (clone 25D12) and MSH6 (clone PU29) have not been assessed to be the best clones for their respective antibodies in assessments by NordIQC,[136–138] these antibodies have been optimised for diagnostic use in our laboratory and have demonstrated adequate staining of internal controls such as endothelial cells, stromal cells and lymphocytes in control and test tissue samples. As such, the antibody clones have been excluded as possible causes for discrepant results.

It must be borne in mind that IHC is not able to differentiate between wild-type occurring polypeptides and missense mutations that give rise to abnormal proteins. Approximately 33% of *MLH1* mutations are missense mutations which produce abnormal proteins that are ultimately non-functional, but still retain their antigenicity and are thus able to produce a false-positive signal immunohistochemically.[56,129,139] In addition, in cases where *MLH1* truncating mutations are identified or in-frame deletions are present, the resultant protein may still react with the MLH1 antibody and therefore yield a false positive IHC signal.[56,139] Research undertaken by de Leeuw *et al*[140]demonstrated that only in some of their cases which had germline *MLH1* mutations was MLH1 IHC completely lost.[140,141] With regard to the

latter, Zighelboim *et al*[142] have identified a deletion mutation in exon 14-15 of *MLH1* which had caused an epitope-stable carboxyl terminal of *MLH1* to be deficient in the region of the carboxyl terminal domain which is required for adequate interplay between *MLH1* and *PMS2* with *PMS2* stabilization[117]. This resulted in failure of the *MLH1-PMS2* heterodimer with resultant deficient PMS2 staining whilst MLH1 protein was still expressed as the epitope was stable and therefore immunoreactive. [142] This study identifies a shortcoming of IHC in the initial screening of suspected Lynch syndrome in patients who have PMS2 deficient tumours but no identifiable *PMS2* mutation.[142]

In the current study, had PCR not been undertaken on all 145 cases, cases of MSI diagnosed by PCR would not have been identified as these cases demonstrated intact staining, regardless of the quantity of nuclear positivity, by IHC. In contrast however, Kato *et al*[47] state that heterogeneous staining patterns are difficult to interpret and that this has resulted in abandonment of staining interpretation other than to note whether or not there is nuclear positivity.[47] It has been stated that such variation in staining patterns may be documented in MLH1 and that germline mutations in *MLH1* may be responsible for such staining.[20,47,143] However, in laboratories where germline mutational analysis is not available, it is not possible to determine if a germline mutation is the cause of variable staining. As such, any degree of nuclear staining would be interpreted as demonstrating retained mismatch repair. Nevertheless, it does raise concern for the possibility of missing an opportunity at identifying an individual who may be at risk for LS. The study by Watson *et al*[117] quotes a value of approximately 10% of patients in whom a false positive result was noted by IHC, which is similar to that noted in the present study where 18 false positive (12.41%) IHC results were identified when compared to MSI PCR results. As raised by Watson *et al*[117] in resource constrained settings additional MSI testing by PCR may not be warranted; yet, this could

imply missing diagnoses of patients and possibly their family members, who may develop a hereditary tumour syndrome. The question therefore is if we are willing to pay such a price?

Despite BAT26 being the best marker to identify MSI-high tumours, Cicek *et al*[144] have noted that there may be somatic deletions of the BAT26 MSI marker in scenarios whereby there have been germline mutations in the MSH2 gene. This may then result in a tumour being incorrectly assigned microsatellite stable if only this marker is selected. Research undertaken by Pastrello *et al*[145] showed that despite intragenic MSH2 deletions being detected in 10 families with Lynch syndrome who developed unstable tumours, approximately two thirds of these cases were stable for BAT26. It is believed that due to there being no BAT26 target sequences in the tumours, there was only amplification of normal DNA in which wild type BAT26 sequences were present. As such, it is suggested that this marker should not be used in isolation but rather as part of a panel in the work-up of MSI.[145]

5.2.7. ADVANTAGES AND DISADVANTAGES OF IMMUNOHISTOCHEMISTRY AND PCR

5.2.7.1. ACCESSIBILITY

Immunohistochemistry is readily available in many pathology departments and allows for rapid assessment of these markers by general pathologists.[139] Clinical features of Bethesda or Amsterdam criteria are often not noted along with details by the submitting clinician. Pathologists may identify morphological features such as location of the tumour and lymphocytic infiltration that may suggest features of possible Lynch syndrome and as such, this allows for the pathologist to evaluate IHC staining as a screening tool for LS at the time of the initial biopsy.[124,139] In contrast, PCR MSI is limited to centres where a molecular unit performing such testing is available. In the public sector setting, there are limited numbers of

Anatomical Pathology departments that provide such a service. This, however, is easily circumvented by the existence of a referral system where one laboratory, such as ours; may serve the needs of all other state pathology departments requiring such testing. In addition, this would serve to increase our experience in the diagnosis of such cases and may instil a greater sense of confidence in clinicians knowing that a molecular diagnosis on their patient has been rendered by a centre in which the pathologists are proficient in assessing results of different molecular assays. Referral for molecular testing will, however, affect the overall turn-around time for a specimen result.

5.2.7.2. INTERPRETATION OF RESULTS

Pathologists are required to be cognisant of staining variability which may result in ambiguity in interpretation of the tumour. Furthermore, it is suggested that the immunohistochemical stains be interpreted by a pathologist who is experienced with such stains and their possible staining variations. [139,146] Such staining variations may be interpreted differently amongst those with less experience. The sensitivity and specificity of the antibody panel used (in terms of 4 antibodies versus just 2 antibodies) varies and this too, may affect overall assessment by the reporting pathologist.[146] Zhang,[146] however argues that MSI testing by PCR has an advantage over IHC as PCR does not require an experienced pathologist to interpret IHC stains and that the manager of a molecular laboratory may be taught to read and interpret PCR results which are highly reproducible.[146]

5.2.7.3. COST

The cost of immunohistochemical staining varies across countries and laboratories. The cost of IHC also depends on whether 2 or 4 antibodies are used. Shia[56] and others have noted that using just MSH6 and PMS2 allows for the prediction of all 4 antibodies.[56] These authors state that should a mutation in either MSH6 or PMS2 be identified, then an additional antibody

(namely MSH2 in the occurrence of MSH6 mutation or MLH1 in the event of a PMS2 mutation) may be performed to identify which is mutated so as to ultimately guide gene mutation analysis.[56] However, a recent study by Pearlman *et al*[147] has highlighted the fact that staining of just 2 antibodies does not identify all patients with LS as the markedly reduced intensity in staining of MSH6 may be interpreted as intact staining and they had also found that patchy or weak MSH6 staining may be present even though MSH2 is absent. They thus advocate use of all 4 antibodies as a screening measure for Lynch syndrome.[147]

The cost of MSI in centres where this is available may be reduced by use of pentaplex or multiplex PCR which allows for a decrease in cost of consumables and labour costs whilst increasing throughput of the assay.[148] The cost of a pentaplex MSI PCR reaction in our department is currently approximately 30% the cost of 4 IHC stains.

5.2.7.4. IDENTIFICATION OF THE MUTATED GENE

A deficient IHC result on 1 of the 4 stains suggests that its corresponding gene is mutated.[56] This may then direct gene mutational analysis. MSI PCR as a screening tool is however, not able to direct gene mutational testing.[56]

Whilst MSI is identified in LS, the occurrence of MSI is not diagnostic of LS as microsatellite instability may be identified in up to 30% of sporadically occurring endometrial carcinomas. [23] This is often due to methylation of the *MLH1* promoter region which results in gene silencing without changes at the DNA level.[146] The occurrence of *MLH1* methylation does not however, exclude the possibility of an *MLH1* germline mutation. Methylation of *MLH1* may actually be the second hit of a wild-type allele.[146]

Studies by Bartley *et al*[149] suggest that when MMR deficiencies are identified immunohistochemically, there is a high possibility of a germline mutation being detected. In

contrast however, in cases where tumours are MMR intact but show MSI-high by PCR, it is far less likely that a germline mutation will be detected.[149]

5.2.7.5. IDENTIFICATION OF MMR DEFICIENT CASES POTENTIALLY MISSED BY MSI PCR

Hampel and colleagues[58,150] have shown that up to 50% of endometrial carcinomas with mutated MSH6 do not exhibit MSI-H by PCR.[58,150] IHC is able to detect mutations in MSH6 which may be microsatellite stable (and thus not detected by PCR) or may be MSI-Low.[56] Such a phenomenon may be explained by the redundant nature of MSH6 and MSH3 proteins; such that when *MSH6* is mutated, *MSH2/MSH3* is still functional. The *MSH2/MSH3* (*MutSβ)* complex tends to identify insertion-deletion mismatches that are larger than one nucleotide. Therefore, MSI may be limited to a single nucleotide when *MSH6* is mutated but the *MutSβ* complex is functioning. Therefore, use of additional mononucleotide markers may allow reclassification of MSS tumours to MSI-Low or MSI-Low tumours to MSI-High.[146]

5.2.7.6. TUMOUR QUANTITY

In circumstances where there is a limit to the amount of tumour tissue present, PCR requires only a single representative tumour section with reasonable DNA quantity, instead of 4 sections which are needed for MMR IHC testing.[146] As IHC staining may be focal in tumours, loss of staining of IHC's on small biopsies should not be erroneously interpreted as reflective of tumour biology of the entire tumour and such findings should be treated with caution.[56]

Thus, it may be seen that there are advantages and disadvantages to both test methods and whilst there may be cost implications, complimentary testing would be most advantageous from a patient benefit point of view.

5.2.8. LOGISTIC REGRESSION COMPARING ABNORMALITIES/MUTATIONS IDENTIFIED BY IHC AND PCR

Table 12 shows that the likelihood of a mutation being identified by PCR is 3.5 times that in patients who have IHC MMR in contrast to those who do not have a mutation. In light of studies by McConechy *et al*[31] and others demonstrating levels of concordance over 90%, the likelihood of mutations being identified by both methods are high.[31,151]

5.2.8.1. IHC VERSUS PCR STRATIFIED BY AGE

Table 13A shows a concordance level of 71.74% and a p-value of 0.0009; between IHC and PCR in patients over the age of 60 years. This suggests that IHC may be a good screening tool to use in patients over the age of 60 years, but not in patients under the age of 60 years. Watson *et al* [117]respond to a letter to the Editor[120] in which Watson and colleagues stated that some younger patients may harbour mutant genes that may not be detected if IHC is used as the only screening tool.[117,120] In the present study, a similar conclusion may be drawn for patients under the age of 60 years.

5.2.8.2. IHC VERSUS PCR BY TUMOUR FIGO GRADE

From 13B, evaluation of the association between IHC and PCR with regards to FIGO grade 1 tumours shows a high degree of agreement (76.19%) which is the highest amongst the three tumour FIGO grades. The kappa score (0.2953) shows minimal agreement[108] whilst the p-value is statistically significant (p=0.0266). In contrast, there is a concordance level of 61.67% between IHC and PCR in FIGO grade 2 carcinomas, with minimal agreement (kappa = 0.2087) and a p=value tending towards significance (p=0.0520). Examination of IHC and PCR in relation to FIGO grade 3 tumours shows high concordance (73.68%). The kappa score is indicative of only minimal agreement, with a statistically significant p-value (p=0.0103). This suggests that in FIGO grade 1 and 3 tumours, there is a high degree of

correlation between IHC and PCR. McConechy *et al*[31] identified most IHC and MSI cases in tumours with FIGO grades 2 and 3 histological features.[31]

5.2.9. LOGISTIC REGRESSION COMPARING MUTATIONS IDENTIFIED BY IHC AND PCR STRATIFIED BY AGE

Table 14 demonstrates that with an increase in age, there is a 0.6% increased likelihood of having a PCR mutation. Studies by Garg *et al*[94]have shown that losses of MLH1/PMS2 occurred more frequently in older patients (mean age of 58 years) ; whereas losses of MSH2/MSH6 were noted in slightly younger patients (mean age of 54 years). [94] Tumours may be regarded as an age-related phenomenon in which promoter hypermethylation is identified together with diffuse hypomethylation.[60] Methylation causes loss of MMR gene expression thereby resulting in destabilisation of microsatellites in genomic material.[152] Thus, an increase in age would suggest an increase in the likelihood of promoter-hypermethylation which may account for the increase in MLH1/PMS2 loss in older patients noted in the study by Garg *et al*.[94] Harroldsdottir has stated that hypermethylation of MLH1 has been demonstrated in 4 out of 5 cases that show MLH1/PMS2 loss.[138]

Table 15A suggests that the likelihood of a PCR detected mutation decreased from approximately 2 times to 1.7 times in moving from FIGO grade 2 to FIGO grade 3 whilst table 15B (table21) shows that there is 40% decreased odds of having a PCR mutation with FIGO grade 1 endometrial carcinomas when compared to FIGO grade 3 tumours despite there being no statistically significant relationship. With respect to FIGO grade 2 tumours, there is an approximately 20% likelihood of a PCR mutation occurring in tumours with FIGO grade 2 histological features in comparison to FIGO grade 3 carcinomas. This is indicated by studies undertaken by Black *et al*[28] who identified that most of their MSI mutated tumours

demonstrated FIGO grade 2 histological features. Furthermore, Pendergast *et al*[22] have noted that MSI-High tumours are associated with higher FIGO grade endometrial carcinomas.[22]

5.2.9.1. IHC VERSUS PCR IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Assessment of concordance between IHC and PCR with respect to tumour FIGO grade, stratified by age shows a 75% level of agreement for FIGO grade 3 tumours in patients under 60 year of age (table 16). The kappa score shows a weak[108] level of association whilst the p-value is statistically significant. This suggests that for patients under the age of 60 years with high FIGO grade tumours, either IHC or PCR may be used as a screening tool. In contrast, in patients over the age of 60 years, FIGO grade 2 tumours showed an agreement level of 69.23% between IHC and PCR. A minimal degree of association is noted with a kappa score of 0.3500, p-value =0.0144. Nagle *et al*[153] identified a higher tumour FIGO grade and stage in patients who had germline mutations in contrast to tumours demonstrating retention of MMR staining. However, in somatically mutated MMR tumours, tumour FIGO grade was only slightly higher than in MMR proficient tumours.[153]

Table 17 shows that there is no statistically significant relationship between age and tumour FIGO grade and the odds of a tumour harbouring a PCR mutation amongst those without MMR IHC mutations; whereas there is a strong, statistically significant relationship between the possibility of a tumour being PCR MSI in patients who have IHC deficiency (p-value =0.002). This is substantiated by McConechy *et al*[31] who found a high concordance between tumours that were MSI by PCR and MMR deficient.[31]

5.2.10. IHC VERSUS OVERALL SCREENING ABNORMALITY/MUTATIONAL STATUS

Table 18 shows a moderate degree of concordance between IHC and overall abnormality/mutational status with statistical significance (kappa score =0.6288, p-value=0.0001). This result suggests that IHC is a good screening test overall. Whilst the current literature provides information on the agreement of IHC in comparison to PCR, to the best of the author's knowledge there is no information on either IHC or PCR in comparison to overall screening abnormality/mutational status.

Hechtman *et al*[154] have noted that IHC and PCR have equivalent sensitivities and specificities and may be complementary.[154] It is important to remember that IHC MMR testing is a screening modality. However, mutations in any of the 4 MMR stains point toward the possibility of an underlying germline mutation.[121] Mills and Longacre[155] have stated that that MMR IHC stains have an approximately 90% sensitivity for the detection of germline mutations whilst Kheirelsaid *et al*[156] noted that the IHC stains have sensitivities ranging between 27%-100% and specificity levels from 43-100% for the determination of germline mutation.[155,156] In contrast, reviews of other studies showed a sensitivity of 74% (95% CI: 54-87) and specificity of 77% (95% CI: 61-88).[156] Garg and Soslow[26] have noted that the positive predictive value for the detection of suspected germline mutation with absent staining of IHC, is high.[26] In contradistinction, Dierssen[141] identified just 3 out of 11 (27.27%) cases in which an MLH1 IHC was able to correctly detect a germline mutation; whilst MSH6 IHC was able to identify germline mutations in 9 out of 12 (75%) tumours and MSH2 IHC was able to detect possible mutations in all 8 of 8 (100%) endometrial carcinomas. Despite these findings, Dierssen[140,141] suggests that MMR IHC should not be used in isolation to guide germline assessments as it is felt that the staining patterns used to predict an MLH1germline mutation may depend on the expertise of the investigator and that

the identification of mutations in MSH2 and MSH6 may not be overtly conspicuous.[140,141] Whilst it is not possible to assess the sensitivity and specificity of results of IHC staining in the present study to germline mutation, as the latter has not been performed as it is beyond the scope of the current study, this lends itself to a future study.

5.9.10.1. IHC VERSUS OVERALL ABNORMALITY/MUTATIONAL STATUS STRATIFIED BY AGE

From Table 19A, the comparison of IHC and overall abnormality/mutation in patients under 60 years of age demonstrates concordance of 81.25%, a moderate kappa score[108] (0.6250) and statistical significance (p-value =0.0001), which indicates that IHC is a good screening test to use in patients under 60 years of age. Resnick *et al*[157] have predicted that up to 12% of patients with endometrial carcinoma are under the age of 50 years and that approximately 9% of such patients have Lynch syndrome. Their studies have illustrated that immunohistochemistry with subsequent directed genetic testing is a valuable and worthwhile exercise in attempt to identify LS afflicted individuals. [157] The current study shows an agreement of 82.61% between IHC and overall abnormality/mutational status in patients over 60 years of age. There is a moderate kappa score of 0.6290[108] and p-value = 0.0001, which is suggestive of IHC being a good test to evaluate abnormality/mutational status in patients over the age of 60 years. Results of this study illustrate that irrespective of the age of the patient with endometrial carcinoma, IHC is a good screening utensil.

5.9.10.2. IHC VERSUS OVERALL SCREENING ABNORMALITY/MUTATIONAL STATUS STRATIFIED BY TUMOUR FIGO GRADE

From 19B, it may be seen that tumours with FIGO grade 1 histology showed a high degree of non-random statistically significant concordance (kappa= 0.6400; p-value = 0.0001) between IHC and overall abnormality/mutation. Similarly, endometrial carcinomas with FIGO grade 2

morphological features demonstrated a non-random statistically significant relationship between IHC and overall abnormality/mutation. (kappa= 0.6753; p-value =0.0001). This suggests that in low FIGO grade endometrial carcinomas, IHC is a good screening modality for the assessment of possible mutation.

5.9.10.3. IHC VERSUS OVERALL ABNORMALITY/MUTATIONAL STATUS STRATIFIED BY AGE IN RELATION TO EACH TUMOUR FIGO GRADE

Table19 C shows a statistically significant concordance level of 86.67% between IHC and overall abnormality/mutational status for FIGO grade 1 tumours in cases from patients under the age of 60 (kappa score = 0.5946, p-value=0.0059). There is a statistically significant 80.95% agreement level between IHC and overall abnormality/mutational status in FIGO grade 2 carcinomas (kappa =0.6111; p-value = 0.0012). Similarly, a statistically significant concordance level of 75.00% between IHC and overall abnormality/mutational status for FIGO grade 3 tumours in cases from patients younger than 60 years was seen. (p-value=0.0334). These results all suggest that in FIGO grades 1 and 2 carcinomas, IHC is a good screening method for patients under the age of 60 years.

In patients over the age of 60 years, a statistically significant non-random association was noted between IHC and overall abnormality/mutational status in all 3-tumour FIGO grade. These results are indicative of IHC being a good screening modality for abnormality/ mutational status in all histological FIGO grades in patients over 60 years of age.

Table 19C thus suggests that regardless of the patient age and histological tumour FIGO grade that IHC should be considered a screening tool to detect abnormality/mutations in endometrial carcinomas.

5.2.10. VALIDITY OF PCR MUTATIONS IN ENDOMETRIAL CARCINOMAS

5.2.10.1. PCR VERSUS OVERALL MUTATIONAL STATUS

Table 20 shows that there is a concordance level of 87.14% between PCR and overall abnormality/mutational status (kappa =0.7351; p-value = 0.0001). Similar to IHC as noted above, this result suggests that PCR is a good screening utensil for the identification for microsatellite unstable tumours. However, as stated previously, there is no current literature comparing IHC or PCR to overall abnormality/mutational status.

5.2.10.2. PCR AND OVERALL ABNORMALITY/MUTATIONAL STATUS ACCORDING TO AGE

From Table 21A, it may be seen that there is a statistically significant concordance level of 89.13% between PCR and overall abnormality/mutational status in patients over the age of 60 years (p-value of 0.0001). In patients under the age of 60 years, there is a non-random statistically significant level of agreement of (83.3%; p-value =0.0001). Both these sets of results suggest that irrespective of the age of the patient, PCR is a valuable screening test for abnormalities/mutations. Zighelboim *et al*[44]showed that patients with microsatellite unstable tumours were only marginally older than those with MSS tumours, but the mean age for patients with MSI and MSS tumours was over 60 years.[44]The results of the present study mirror results obtained using immunohistochemistry as a screening tool. However, as previously noted, IHC and PCR have not to the best of the author's knowledge been compared to overall abnormality/mutational status with respect to age or tumour FIGO grade.

5.2.10.3. PCR AND OVERALL ABNORMALITY/MUTATIONAL STATUS IN RELATION TO TUMOUR FIGO GRADE

Table 21B shows that with FIGO grade 1 endometrial carcinomas, there is non-random, statistically significant level of concordance of 90.48% between PCR and overall abnormality/mutational status (kappa=0.7692; p-value = 0.0001). There is concordance of 78.3% for PCR and overall abnormality/mutational status for FIGO grade 2 tumours (kappa score = 0.5860; p-value = 0.0001). For FIGO grade 3 tumours however, there is a 97.37%

level of concordance between mutations detected by PCR versus overall abnormality/mutational status, with “almost perfect”[108] score of 0.9426; and p-value = 0.0001.These results indicate that irrespective of the tumour FIGO grade, that PCR is a good screening modality, which is consistent with the ideology by some of PCR being the gold standard of mutational detection.[110,131,158] These results however, show a greater degree of association with FIGO grade 1 and FIGO grade 3 tumours whereas Wang *et al*[59] have noted a correlation between MSI-High endometrial carcinomas having FIGO grade 2 features. Zighelboim and colleagues[44] have demonstrated a strong association between microsatellite instability and high tumour grade. The results of the current study have compared all microsatellite low and high tumours against tumour FIGO grades in contrast to the study by Wang *et al* [59] where only microsatellite high tumours have been evaluated against tumour FIGO grades. Studies by Catasus *et al*[16] showed a correlation between MSI and FIGO grade 3 endometrioid endometrial carcinomas, similar to findings of the current study. However, their research indicates a greater correlation with FIGO grade 2 tumours as opposed to FIGO grade 1 tumours.[16] This is in contradistinction to the present study in with there was a greater association with FIGO grade 1 carcinomas rather that FIGO grade 2 tumours.

5.2.10.4. PCR AND OVERALL ABNORMALITY/MUTATIONAL STATUS STRATIFIED BY AGE FOR EACH TUMOUR FIGO GRADE

Table 21C shows the highest degree of association between MSI and overall abnormality/mutational status in patients under the age of 60 years who had FIGO grade 3 tumours (kappa 1.000, p= 0.0003). Whilst Zighelboim and co-workers[44] identified a strong association between MSI and tumour FIGO grade, their mean patient age was 65.2 years whereas the present study demonstrated high concordance in FIGO grade 3 carcinomas from patients under the age of 60 years. For patients over the age of 60 years, there was strong concordance (kappa = 0.9128; p-value = 0.0001) between MSI and overall

abnormality/mutational status for FIGO grade 3 tumours and age over 60 years. Studies undertaken by Zighelboim *et al* [44] show high correlation between MSI and tumour FIGO grade.[44] However, these authors only evaluated MSI PCR but did not contrast such findings with IHC and overall abnormality/mutational status. In the present study, the lowest degree of association between MSI and overall abnormality/mutational status was noted in FIGO grade 2 carcinomas in patients under the age of 60 years (kappa = 0.3951; p-value = 0.0115). The results of the present study therefore suggest that irrespective of tumour FIGO grade or age of patients with endometrial carcinomas, that MSI by PCR is a good predictor of mutational status.

5.2.11. BRAF ASSESSMENT OF ENDOMETRIAL CARCINOMAS

BRAF mutations offer a route by which activation of the MAP-kinase pathway may occur.[159] In colonic carcinomas, the occurrence of MMR together with identification of a *BRAF* mutation or hypermethylation points toward a sporadically occurring tumour.[63] *BRAF* mutations have been associated with an aggressive clinical course with unfavourable outcomes.[160] There are currently over 70 known *BRAF* mutations; of which *V600E* is the most common.[160] At nucleotide 1799, adenine replaces thymine which, at position 600 of the amino acid sequence, results in the replacement of glutamate (E) for valine (V). [160]The presence of a *BRAF V600E* mutation in a tumour offers the possibility of targeted therapy being a treatment option for patients with such a mutation.[161] Whilst there have been varied reports of the incidence of *BRAF V600E* mutations in endometrial carcinoma in the literature, to the best of the investigator's knowledge, this has not been documented in the South African population. [61,62,161,162] Furthermore, the present study evaluated 3 test methodologies namely immunohistochemistry, PCR and Sanger sequencing for the detection of BRAF mutations in endometrial carcinomas in patients in the greater Johannesburg area.

5.2.11.1. BRAF V600E IHC DIAGNOSIS IN ENDOMETRIAL CARCINOMA

In the present study, only a single case (2.7%) out of 37 MLH1 negative cases, demonstrated weak cytoplasmic staining of tumour cells (figure 37). Studies in which BRAF analysis was performed on endometrial carcinomas have used molecular tests such as PCR or sequencing.[62,163] It is likely that due to the low numbers of positive cases identified in endometrial carcinomas in some studies that BRAF V600E IHC has not been validated for use in endometrial carcinomas. In addition, it has been documented that the IHC antibody may not perform well on different tumour types.[164] Five (13.5%) of the 37 cases in the present study showed nuclear staining which is considered negative for a BRAF mutation (figure 38). Thus, 36 out of 37 cases were negative by BRAF IHC (figure 39). The aberrant nuclear staining pattern has been identified by authors who have ascribed its presence to cross-reactivity between a nuclear protein and the 11-amino acid peptide that is used in the production of the VE1 antibody.[165] It is believed that this non-specific binding may be due to raised antibody concentration which allows for increased amounts of antibody to bind to the nuclear protein.[165] In addition, retrieval solutions which have a more basic pH level may have increased nuclear staining as the basic pH prevents the peptide from folding and thus ensures greater antigenicity.[165] Kuan *et* al[165] noted variability of staining upon using new reagents or a new antibody lot.[165] Such factors may contribute to some authors stating that the VE1 antibody is difficult to optimise.[164,165] Dvorak *et al*[160] conducted one of the largest studies to validate use of the BRAF V600E IHC stain in colorectal carcinomas and papillary thyroid carcinomas.[160] In this study, the BRAF V600E IHC was tested against Sanger sequencing which is regarded as the gold standard. These authors found a high concordance between IHC staining and Sanger sequencing for colorectal carcinomas and papillary thyroid carcinomas. In contrast, Estrella *et al*[166] found that the sensitivity and specificity for the VE1 stain in colorectal carcinoma was not ideal.[166] Such findings were mirrored by studies of Løes *et*

al.[167] It has been stated that various validation tests are necessary for different tumour types for example, identification of BRAF V600E staining in a pituitary adenoma does not in fact imply a BRAF V600E mutation.[160] Furthermore, Dvorak and co-workers have indicated that optimal tissue fixation is necessary for reliable results. As such, it is recommended that tumour tissue be placed in 10% neutral buffered formalin within 2 hours of removal and that the tissue be fixed in formalin for a period of 12 to 24 hours.[160]

A major benefit that has been documented for the VE1 stain is its low-limit of detection which allows for mutations to be identified at the level of a single cell.[168] In contrast, a major disadvantage of the IHC stain is the possibility of false negatives in cases where there is low tumour volume or large amounts of non-tumoural cells within the tissue.[169] Tissue ischaemia is another possible reason for a false negative as hypoxia may affect protein synthesis.[170] Furthermore, the IHC stain is not able to detect mutant variants of V600 such as V600D. In addition, the stain detects proteins and as such, may result in discordant results when compared to DNA-based technologies.[169]

5.2.11.2. BRAF PCR

BRAF mutations were identified by PCR in 4 cases of endometrial carcinoma (figure 40). Three (8.11%) out of these 4 cases had V600E mutations by PCR (figure 41). These 3 mutations were identified at exon 15, codon 600 of the *BRAF* gene. PCR analysis performed on this region by Kawaguchi and colleagues[63] in their studies on endometrial carcinomas, yielded no mutations.[63] Standard PCR was performed in a case report by Moschetta *et* al[161]on a patient who had metastatic endometrial carcinoma that was shown to be MSI-high and BRAF V600E mutated. The patient was subsequently treated with target therapy and had therapeutic response. Whilst many authors such as Chen *et al*[171] and Kawaguchi and colleagues[63] note that there is a low incidence, if any, of *BRAF* V600E mutations in their

patient populations, and that the cost of BRAF testing may not be warranted, the possibility of a patient harbouring such a mutation exists, as indicated by the case report by Moschetta *et al* [161] In cost-restrained hospitals the cost-to benefit ratio is always a concern, sometimes overshadowing the human life affected by such decisions.

A single case (2.7%) out of 37 endometrial carcinomas in the present study had a BRAF V600D mutation (figure 41). This test was repeated so as to ensure a reproducible result. V600D are uncommon mutations and have only been detected in up to 1.3% of tumours including melanomas and colonic carcinomas.[172] In this mutation, adenine-thymine is substituted for thymine-guanine (TG) at position 1799-1800 resulting in valine being changed for aspartic acid. To the best of the author's knowledge, such a mutation has to date, not been identified in endometrial tumours.

5.2.11.3. SANGER SEQUENCING OF *BRAF*

Sanger sequencing has for a long duration been considered the gold standard against which other methodologies are tested.[160,169] Sanger sequencing offers a number of advantages such as availability, reliable results and reagents that are readily available; whilst its major disadvantage is low sensitivity.[169] Only one (2.7%) out of 37 cases of endometrial carcinoma in the current study showed a mutation by Sanger sequencing (figures 42 and 43). The majority of studies examining *BRAF* mutations in endometrial carcinomas and in other neoplasms, have used sequencing.[160] With the passage of time however, next generation sequencing is being used more frequently.[168] He *et al*[173] identified *BRAF V600E* mutations in 3 cases of endometrial carcinomas in their series.[173] Mills *et al*[66] began examining their cases of endometrial carcinoma for *BRAF* mutations but abandoned this leg of their study due to a complete lack of identifiable *BRAF* mutations in the cases evaluated.[66] Feng and colleagues[62] have however, identified *BRAF* mutations in a staggering 21% of endometrial carcinomas. In

their study, a number of newly documented missense mutations were identified, such as V600G. These authors suggest a number of reasons for the differences in their results in comparison to other studies, which include a build-up of *BRAF* mutations amongst the ethnic group investigated.[62] Metcalf and colleagues[61] however, negate such a theory as studies on Japanese patients by other researchers had been conducted which did not yield results similar to that of Feng and colleagues.[61,63] Furthermore, Metcalf and Spurdle[61] state that there are a number of techniques used in which the tests have high sensitivities and specificities and as such these tests should identify mutations irrespective of the population being evaluated.[61] However, a South African study on colorectal carcinomas by Cronjé *et al*[174]demonstrated a *BRAF* mutation in a single patient, whilst in the same cohort of patients up to 32% had KRAS mutations. In contrast, over 60% of colorectal carcinomas that are deficient for MLH1 on immunohistochemistry and negative for *MLH1* mutation have BRAF mutations identified.[61] Therefore, as there has been little investigative work-up on the South African population for endometrial carcinomas, it is possible that our culturally mixed population may harbour mutations not identified or at frequencies not documented previously. Feng *et al*[62] also suggest the discrepant results obtained between their studies and the work of others, may be attributed to dilution of tumour DNA concentration at the time of macrodissection. This, however, seems unlikely, as stated by Metcalf and co-workers as other mutations were identified when macrodissection was used by other investigators, but *BRAF* V600G mutations were not documented.[61] Thus, the exact reasoning behind the high percentage of BRAF mutations documented by Feng *et al*[62]is not entirely certain.

5.2.12. AGREEMENT OF BRAF IHC AND SANGER SEQUENCING

From Table 22, it may be seen that BRAF IHC has a sensitivity of 0%, a specificity of 97.22%, a positive predictive value of 0% and a negative predictive value of 97.22%. In stark contrast, Kuan *et al*[165] and Capper *et al*[175]in their studies, using colorectal carcinomas to test

IHC against sequencing, obtained sensitivities and specificities of 100%, 94% and 100%, 98.8% respectively. It is possible that the IHC whilst adequately optimised for use on colorectal carcinoma and melanoma in our laboratory, is not deemed suitable for use on endometrial carcinomas. This would corroborate Bellizzi's belief that not all tumour types stain adequately with the BRAF V600E IHC stain.[164] In addition, in light of the results obtained by PCR and Sanger sequencing, it may be seen that there are only a few cases in our patient cohort in whom BRAF mutations were identified and as such, there are very few cases to compare the IHC staining results to.

5.2.13. AGREEMENT OF BRAF PCR AND SANGER SEQUENCING

Table 23 shows that the sensitivity, specificity, positive predictive value and negative predictive value for BRAF PCR when compared to sequencing was 0%, 88.89%, 0% and 96.97% respectively. In comparison, however, work undertaken by Benlloch *et al*[176] revealed both a sensitivity and specificity of 100% when comparing real-time BRAF PCR to sequencing in colorectal tumours.[176] Despite the very poor sensitivity and positive predictive value, use of PCR enabled identification of BRAF mutations in 4 cases that would not have been identified by Sanger sequencing alone.

5.2.14. INCIDENCE OF BRAF MUTATIONS USING IHC, PCR AND SEQUENCING

Table 24 demonstrates that when all three test methodologies were used, a total of 6 (16.21%) BRAF mutations were identified out of 37 cases. Of these 6 mutations, 50% were identified in patients under the age of 60 years and 50% were over the age of 60 years. Of the 31 patients who did not have BRAF mutations, less than one-third of patients were under 60 years of age whilst the remaining patients were above 60 years of age. Two of the 3 patients in the study by He *et al*[173] were over the age of 60 years.

From the current study, in the group of patients with BRAF mutations, only 1 patient had a FIGO grade 1 tumour whilst the remainder of patients had tumours demonstrating FIGO grade 2 histological features. In contrast, in the study by Feng *et al* [62] 22% of patients with BRAF mutations had FIGO grade 1 histological features, 26% demonstrated FIGO grade 2 features and 22% showed FIGO grade 3 features; whilst in the study by He and colleagues,[173] 66.6% of their patients were diagnosed as having FIGO grade 1 tumours and one third of their cases were histologically FIGO grade 2 tumours[62,173] Thus, the findings of the present study are similar to that by He *et al*[173] in which the majority of patients with BRAF mutations showed FIGO grade 2 histological features. This may suggest that tumours with BRAF mutations are associated with an intermediate tumour FIGO grade.

5.2.14.1. AGREEMENT OF BRAF ASSESSMENT USING IHC, PCR AND SEQUENCING

Although there was a high degree of agreement (86.49%) between both IHC and PCR as well as between PCR and sequencing, the results were not statistically significant and the kappa score demonstrated no agreement (kappa= -0.0452, p=0.6379) (table 25). In a study by Szymonek *et al*,[170] assessing IHC, PCR and Sanger sequencing on papillary thyroid carcinomas, there was a concordance level of 94.2% between IHC and PCR, whilst a concordance of 76.2% was detected between IHC and sequencing. Any discordant case in their study subsequently underwent next generation sequencing, and based on these results, these authors deemed PCR as their gold standard for the identification of *BRAF* mutations. These researchers noted that IHC testing was superior to sequencing for identification of mutations. Capper *et al*[175] however, demonstrated a 100% concordance between IHC and sequencing in their study examining *BRAF* mutations in colorectal carcinomas. In the current study, it is plausible that sequencing and immunohistochemistry may not have detected the mutations that were identified by PCR as 5 of the 6 mutated cases were derived from

endometrial curettings in which there was limited amounts of tissue and hence reduced amounts of DNA. Szymonek *et al*[170] noted that PCR was more resistant to the quality of DNA. Thus, this may offer a possible explanation for an increased number of mutations detected by PCR than IHC and PCR. Furthermore, the remaining case was derived from an excision specimen in which there was poor preservation, and as such, tissue hypoxia may have affected the DNA which could have contributed to mutations not being detected by sequencing. Estrella *et al* [166] mention that there may be specific factors in colonic mucosa that may result in a particular modification of BRAF proteins[166]. Similarly, the same may apply to endometrial tumours. Moreover, it is very likely that most endometrial carcinomas do not have BRAF mutations as documented by other researchers.[61,63,66] As the present study consisted of multiple steps of investigation, tissue sections were simultaneously cut and prepared for hypermethylation studies and as such, there was not sufficient tissue to consider testing by next generation sequencing. Furthermore, financial provisions for an additional test method had not been considered when the budget was drawn up.

5.2.15. BRAF IHC MUTATIONS IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 26 shows that only a single patient under the age of 60 years was identified as harbouring a BRAF mutation. The tumour morphologically exhibited FIGO grade 2 histological features. To the best of the investigator's knowledge there are no studies demonstrating BRAF IHC usage on endometrial carcinomas.

5.2.15.1. BRAF IHC VERSUS OVERALL BRAF MUTATIONAL STATUS STRATIFIED BY AGE AND TUMOUR FIGO GRADE

From table 27 it may be seen that agreement between BRAF IHC and overall BRAF mutational status was statistically significantly but showed low concordance (p=0.0175,

kappa = 0.2857) for patients under the age of 60 years who had FIGO grade 2 tumours. It may be argued that the one positive BRAF IHC was a chance occurrence as this result was not confirmed on Sanger sequencing and was also not detected by PCR. Whilst there was a statistically significant association between BRAF IHC and overall BRAF mutational status in FIGO grade 1 tumours in patients over the age of 60 years, (p=0.0001) the kappa score is 0.0000. This suggests that this was a random occurrence. Due to the small number of cases that underwent BRAF examination, further statistical analyses could not be performed.

5.2.16. BRAF PCR MUTATIONS IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 28 shows that 25% of patients who had a BRAF mutation detected by PCR were under 60 years of age; whilst the remaining 75% were older than 60 years of age. Similarly, one patient was assessed as having a FIGO grade 1 tumour whereas 3 of the 4 patients had histologically diagnosed FIGO grade 2 tumours. Despite these results not being statistically significant, it suggests that there may be an association between BRAF mutated endometrial tumours occurring in older females. This is corroborated by studies in which BRAF mutations were associated with an older age in patients with colonic carcinomas.[177] Furthermore, it is suggested that BRAF mutations confer a worse prognosis for patients who have the mutation and extrapolating from this, it is surmised that the poorer prognosis may be ascribed to a higher tumour FIGO grade; as was noted in 3 of the 4 patients in the current study who had a BRAF mutation by PCR.[178]

5.2.16.1. BRAF PCR VERSUS OVERALL MUTATIONAL STATUS STRATIFIED BY AGE AND TUMOUR FIGO GRADE

There is a statistically significant relationship with poor kappa score (kappa = 0.2857; p-value = 0.0175) between BRAF PCR and overall BRAF mutational status in patients under

the age of 60 years who had FIGO grade 2 endometrial carcinomas (table 29). These findings suggest that PCR is a good test to identify BRAF mutations, as corroborated by Szymonek *et al*[170] who used PCR as their gold standard for BRAF analyses.[170] However, in light of the poor kappa score and small sample size, it is possible that this may have been a random occurrence.

There is statistically significant agreement between BRAF PCR and overall BRAF mutational status in patients over the age of 60 years who had FIGO grade 1 or FIGO grade 2 tumours (0.4467; p-value=0.0023 and kappa=0.4615; p-value=0.0001 respectively). These results suggest that BRAF mutations are associated with older age. Additionally, BRAF mutations have a slightly greater association with FIGO grade 2 tumours in contrast to FIGO grade 1 tumours. Colorectal carcinomas with BRAF mutations are often poorly differentiated and as such, there is an association between BRAF mutations and higher tumour FIGO grade.[179] This may be a possible explanation for the association noted in the current study despite the low numbers of BRAF mutations identified.

5.2.17. BRAF SEQUENCING MUTATIONS IN RELATION TO TUMOUR FIGO GRADE STRATIFIED BY AGE

Table 30 reveals that the only patient who had a BRAF mutation detected by sequencing was under the age of 60 years and that this patient had a FIGO grade 2 tumour. This result is not statistically significant (p-value = 1.00). In the study by Feng *et al,*[62] 26% of patients diagnosed with endometrioid endometrial carcinomas who had BRAF mutations, were found to have FIGO grade 2 tumours. In contrast, there were BRAF mutations in 22% of patients who had both FIGO grades 1 and 3 endometrioid endometrial carcinomas.[62] Feng and co-worker's study did not stipulate the age of patients who had BRAF mutations. The results of Feng's[62] study are in contrast to those described by He *et al*[173] where 66.6% of their patients

with BRAF mutations had FIGO grade 1 tumours and were over 60 years of age; whereas only one-third of their patients had a FIGO grade 2 tumour and were under the age of 60 years.[173] In a study by Salvesen's group, the only patient with a BRAF mutation had a FIGO grade 2 tumour.[159] The age of the patient is however, not stated. McConechy *et al*[162] identified a higher percentage of cases with BRAF mutations in FIGO grade 3 endometrioid endometrial carcinomas. Whilst there is not much information on endometrial carcinomas and BRAF mutations due to the rarity of such mutations in these tumours, the collective data suggests that sequenced BRAF mutations are associated with intermediate FIGO grade tumours.

5.2.18. *BRAF* SEQUENCING VERSUS OVERALL MUTATIONAL STATUS STRATIFIED BY AGE AND TUMOUR FIGO GRADE

Table 31 highlights agreement between results obtained by *BRAF* sequencing versus overall mutational status for FIGO grade 2 tumours in patients below the age of 60 which is tending to statistical significance (p=0.0537)., but shows a weak kappa score There is good agreement between *BRAF* sequencing and overall BRAF mutational status in patients with FIGO grade 1 and 2 tumours who were over 60 years of age but there is no kappa score and no p-value. As such, these results suggest that that these are random occurrences.

5.2.19. CONCORDANCE OF BRAF IHC, PCR AND SEQUENCING VERSUS OVERALL BRAF MUTATIONAL STATUS

Table 32 shows that agreement between results obtained by IHC versus overall BRAF mutational status as well as Sequencing and overall BRAF mutational status, was 86.49%, but the kappa score shows no level of agreement, kappa =0.15, p-value<0.005. Furthermore, there was concordance of 94.59% between PCR and overall BRAF mutational status. There was statistically significant, moderate, non-random concordance[108] (kappa=0.77, p=0.0001).

These results demonstrate that PCR is a good test methodology for identifying BRAF mutations. Whilst the agreement levels between both IHC and sequencing in relation to overall mutational analysis was the same on endometrial carcinomas in this study, there have been conflicting reports on different tissue types; for example Zagzag *et al*[180] noted that sequencing is more sensitive than IHC in the detection of BRAF mutations in papillary thyroid carcinomas whilst Szymonek *et al*[170] document lower sensitivity levels for sequencing in comparison to IHC on papillary thyroid carcinomas. There are obvious differences between the current study and those undertaken by Zagzag *et al*[180] and Szymonek *et al*[170] such as the present study being undertaken on endometrial carcinomas; and importantly; these tumours in western studies have shown low frequencies of BRAF mutations. Shia *et al*[181] have stated that immunohistochemical problems such as decreased intensity staining of tumour cells especially with an MLH1 stain, patchy and decreased intensity staining of internal control cells seem to occur with increased frequency and pose more of a concern than in colorectal carcinomas. It is postulated that these problems arise due to a lower rate of cellular proliferation in endometrium in contrast to the colon.[181] It is possible that such factors are not restricted to the MMR IHCs but may also affect staining with BRAF IHC.

5.2.20. HYPERMETHYLATION ANALYSIS

Human tumours undergo changes such as gains in methylation at promoter regions or they may undergo hypomethylation.[182] Methylation takes place on cytosine bases at CpG islands which are areas that are rich in CpG dinucleotides such that these regions have over 50% GC and are found in the 5'region of a gene (such as *MLH1*) including the promoter, exon 1 and untranslated region.[32,182,183] It is currently believed that more than half of human protein-coding regions ae associated with CpG regions. In normal cells, such regions are not methylated. In neoplasia, however, the occurrence of methylation, either partial or complete,

of the promoter region results in silencing of the associated gene.[182] It is this process that is key to development of carcinogenesis. Histone deacetylase and DNA methyltransferase (DNMT) regulate DNA methylation.[183] During methylation methyl groups adhere to cytosine bases and result in configurational changes by projecting into the major groove of the DNA helix thereby preventing transcriptional factors from binding and therefore inhibit transcription from occurring.[32,182] Methyl groups attract methyl-adhering proteins which result in compaction of chromatin.[32] Gene expression or silencing is intimately associated with chromatin changes and methylation.[184]

Whilst the exact activator of methylation is not entirely known, it is currently believed to arise because of aging and low levels of spontaneous methylation of CpG islands have been demonstrated in normal tissue with an increase occurrence with an increase in age.[185] It has been suggested by Klutstein *et al*[185] that in neoplastic tissue there are two changes that occur in contrast to normal cells; these comprise methylation of CpG islands which simultaneously occurs with demethylation of multiple sites within the genome. Whilst initial work on methylation proposed that hypermethylation concentrated on the promoter regions of tumour suppressor genes; the current view is that this is a diffuse phenomenon that may be facilitated by polycomb complex targeting, in which active chromatin is converted into inactive heterochromatin. It is currently believed that this polycomb complex initiates methylases that culminates in methylation. It is hypothesized that the process of methylation is due to separation of the methylase complex from the replication components to preferentially identify CpG islands. As a consequence of CpG islands being targeted by the polycomb complex, the methylation pattern is fairly uniform in all tissue subtypes, but the intensity varies between different tumour types.[185] It is postulated that abnormal methylation in tumours may already have been present in normal cells before the progression of oncogenesis. Inflammation and oxidative stress have been linked to carcinogenesis and it has

been shown that both these environmental factors attract DNA methyltransferases to polycomb target areas which then culminates in methylation similar to that seen in tumours. Studies have shown that a multitude of genes that are mutated in tumours are in fact implicated in chromatin structural control or are associated with metabolism of DNA methylation. Thus, a multitude of mutations exist in the process of carcinogenesis. Whilst these abnormalities may affect DNA methylation, it is important to remember that DNA methylation is a process that occurs with an increase in age and may result in tumour development, and is independent of gene mutations.[185]

Studies have demonstrated that *MLH1* promoter methylation has been identified in approximately 70-90% of sporadically occurring endometrial carcinomas.[186] Studies by Esteller and fellow researchers[187] have demonstrated that *MLH1* promoter hypermethylation occurs early and is identified in cases of atypical hyperplasia. These authors have noted that *MLH1* promoter hypermethylation may be detected prior to identification of microsatellite instability.[187] It should be borne in mind that methylation is a reversable phenomenon and as such, this provides an attractive possible target for treatment.[188] Furthermore, epigenetic changes may serve as biomarkers which may aid in the identification of tumours.[183]

Studies by Deng and co-workers[189] using colorectal cell lines, identified a region situated -248 to -178 base pairs upstream from the transcription site of the *MLH1* promoter region which has been linked to the absence of MLH1.[189] The promoter region is divided into 4 regions, namely A (-711 to +15) which has 23 CpG sites, B (-552 to -266) which has 19 CpG sites, C (-248 to -178) that has 8 CpG sites and D (-109 to +15) which has 7CpG sites. Their research demonstrated that methylation of the C region was directly associated with *MLH1* silencing. In addition, they found that methylation regions C and D had greater significance that regions A and B. Thus, they concluded that methylation of sites just proximal to the starter region, especially region C, is crucial to the expression of *MLH1*.[189] Furthermore,

Metcalf and Spurdle have noted that region C has a specificity for loss of *MLH1*.[61] It is for this reason that the present study focused examination of the *MLH1* promoter to just the C and D regions.

5.2.21. EpiTYPER MassARRAY HYPERMETHYLATION ANALYSIS

EpiTYPER is a bisulphite-conversion that uses mass-spectrometry to allow for quantitative DNA methylation of a specified region. This technology has facilitated evaluation of differences in DNA methylation to a low percentage and has been used for assessment of neoplasms.[190]Figure 44 illustrates that 31 out of 36 (86%) of cases that were negative for MLH1/PMS2 IHC stains were methylated. These results therefore account for the negative staining identified with MLH1/PMS2 immunohistochemical stains, as documented by Haraldsdottir.[72] Furthermore, the presence of *MLH1* promoter methylation suggests the sporadic nature of endometrial carcinogenesis as opposed to germline mutation in which there is a negative result for *MLH1* methylation studies. Figure 45 illustrates that the average percentage methylation for all the positions examined in region C was 40% whilst the average percentage of positions examined in region D was 37%. These results were run with negative controls and a level of methylation above 10% was interpreted as positive. Different methods of methylation analysis are available including qualitative and quantitative studies and the method employed varies amongst different studies. Pérez-Carbonell *et al*[100] for example, evaluated regions C and D of the *MLH1* promoter region and regarded a value of >15% as methylated.[100] Leenen *et al*[163] however, used methylation specific multiplex ligand-dependent probe amplification (MS-MLPA) to assess methylation status of their cohort of endometrial carcinomas. As stated by Metcalf and Spurdle,[61] there are various methods of methylation analysis used in endometrial carcinomas, with different thresholds used to assess positive versus negative methylation status; in addition to whether a test is quantitative or qualitative.[61]

Table 33 demonstrates that a total of 62 cases underwent EpiTYPER methylation analysis. These 62 cases were composed of the 37 MLH1 IHC deficient cases and 25 cases that showed discordant IHC MMR and MSI PCR results. One case did not have enough tissue in the paraffine embedded tissue block and therefore not enough DNA for methylation analysis. This case was therefore excluded from further analysis for comparative analysis. Approximately 72% of these 62 cases showed methylation levels of more than 10% and were hence considered hypermethylated.[190] As stated previously, Salvesen *et al*[186] have noted that between 70-90% of sporadically occurring endometrial carcinomas show *MLH1* methylation. The results of the present study are thus in accordance with such findings.

5.2.21.1. EPITYPER METHYLATION STRATIFIED BY AGE AND TUMOUR FIGO GRADE

Table 34 highlights that of the 72% of methylated endometrial carcinomas, the mean age of patients was 65 years, of which nearly 40% were below the age of 60 years whilst the remainder of patients in this category were over the age of 60. This result is consistent with the findings of Jiang *et al*[183] who stated that there are a number of genes which demonstrate an increase in methylation with an increase in age.[183] Similarly, most of the patients with unmethylated tumours were over the age of 60 years. In the present study, as was noted by Salvesen *et al*[186], MLH1 methylation did not statistically correlate with patient age.[186]

A statistically significant association is noted between EpiTYPER methylation status and tumour FIGO grade (p=0.036). Most of the methylated cases demonstrated FIGO grade 2 histological features. The next most frequent histological FIGO grade was 3 and lastly, FIGO grade 1. This contrasts with the unmethylated cases in which FIGO grade 1 was the most common FIGO grade identified, followed by FIGO grades 2 and 3 respectively. Studies by Salvesen *et al*[186] have identified that the majority of MLH1 hypermethylated endometrial

carcinomas demonstrate FIGO grade 2 histological features, similar to that noted in the present study.[186] Their study showed that FIGO grade 1 and 3 features were identified with equal distribution in hypermethylated cases.[186] They had concluded that *MLH1* methylation was not significantly correlated with tumour FIGO grade.[186] Similar to the current study, Peterson *et al*[2] exhibited the greatest frequency of methylation in FIGO grade 2 tumours, followed by FIGO grade 3 and then FIGO grade 1.[2] Bruegl *et al*[191] grouped FIGO grades 1 and 2 together; which demonstrated a total of 74% of cases that were hypermethylated.

5.2.21.2. METHYLATION BY EPITYPER AGAINST IHC MMR STATUS

Table 35 shows statistically significant results in that approximately 70% of cases that demonstrated hypermethylation by EpiTYPER were MMR deficient and thus demonstrated mutations (p-value = 0.003). Similarly, over 70% of cases that were MMR intact by IHC were not methylated. These findings are similar to those by Bruegl *et al*[191] who showed that, 54 patients in their cohort had MLH1 loss on IHC and of these 54 patients, 40 (74%) demonstrated MLH1 hypermethylation.[191] Metcalf and Spurdle[61] demonstrated that 82% of cases that were methylated showed MLH1 loss by IHC and were MSI-high on PCR.[61] These results are indicative of a strong association between loss of staining by IHC and methylation of the *MLH1* promoter region which is supported by the epigenetic phenomenon in which the *MLH1* gene is silenced by methylation of the promoter gene with loss of function of MLH1.[72,189] With respect to approximately 30% of cases in the current study that were methylated but showed no deficiency by IHC, that is they demonstrated retention of nuclear staining, it is possible that such cases had missense or truncated mutations which resulted in antigenically identifiable epitopes by IHC but did in fact harbour *MLH1* methylation as suggested by Shia.[181] The cases which showed MMR deficiencies but no methylation may be ascribed to possible mutations occurring in *MSH2/MSH6* as well as *MLH1* mutations. These

findings are suggestive of possible germline mutations as documented by Wang and co-workers.[59]

5.2.21.3. AGREEMENT BETWEEN EPITYPER METHYLATION AND PCR MUTATIONS

Table 36 and table 37 show the association between *MLH1* methylation and MSI status. More than two-thirds of cases showed concordance for methylation and MSI; whilst a third of cases were methylated but showed no MSI by PCR. These results support previous reports of a strong correlation between *MLH1* methylation and MSI.[182,186] However, the association between *MLH1* methylation and MSI status is not statistically significant in the present study (p=0.123) In contrast, a strong statistical association was obtained in the comparison between methylation status and IHC MMR status (table 35). Salvesen showed a strong correlation between MSI-High and methylation status.[186] From table 37, the presence of only 12.5% MSI negative cases being methylated in the current study is consistent with the findings of other researchers such as Salvesen *et al.*[186] In contrast, only 20.93% of cases in the present study were MSI-high and hypermethylated, whilst almost half the number of cases showed MSI-Low and methylation and were tending toward statistical significance (p=0.057); whereas in the study by Salvesen and colleagues[186] over two-thirds were MSI-High and methylated. These authors have acknowledged that hypermethylation does play an important role in tumours showing lower levels of microsatellite instability.[186] In table 38 hypermethylation status was assessed in relation to PCR unstable cases which shows that more than two-thirds of hypermethylated cases were MSI-Low whilst a third were hypermethylated and MSI-High. It is interesting to note that Levine *et al*[192] have established that MSI-High tumours, most of which were due to *MLH1* hypermethylation have been linked to an improved prognosis in contrast to microsatellite stable tumours.[192] It is thus prudent and beneficial to the patients whom we serve, to fully assess the molecular make-up of their tumours.

The results of the present study showed that a third of hypermethylated cases were microsatellite stable, which is less than the 44% seen in the study by Peterson *et al.*[2] Possible causes for the MSS/hypermethylation discordant results included a single allele producing adequate quantities of *MLH1* or that possibly only a small percentage of cells had abnormal MLH1 protein expression due to hypermethylation.[2]

Table 39 illustrates that there is significantly low, non-random concordance (kappa=0.3586, p-value = 0.0017) between IHC deficiency and hypermethylation, which may be explained by the fact that a large number of MMR deficient cases were due to MLH1 loss; and of the MMR deficient cases, 70% demonstrated hypermethylation (table 35) These results are corroborated by Bruegl *et al.*[191]

Table 40 shows statistically significant, non-random agreement between IHC MMR and methylation (kappa = 0.4757; p-value= 0.0011) in patients over the age of 60 years. These results are similar to those noted by Cosgrove *et al*[193] who stated that methylated associated MMR defects are associated with an increase in age. It is currently hypothesised that the actual process of aging instigates abnormal methylation.[185] The process of methylation that occurs with carcinogenesis is associated with aging, and occurs irrespective of the presence of gene mutations.[185]

Table 41 shows that there is a weak and moderate degree of association which is statistically significant, between EpiTYPER methylation status and IHC with respect to FIGO grades 1 and 2 tumours respectively. These results are similar to those found by Bruegl *et al.*[191]In contrast, Broaddus *et al*[29] have shown that hypermethylation is almost exclusively associated with higher FIGO grade endometrial carcinomas; which is similar to work by Kim and colleagues who demonstrated that hypermethylation was strongly associated with FIGO grade 3 tumours and a worse outcome for patients.[29,194]

Table 42 shows that there is no statistically significant relationship between EpiTYPER methylation and MSI by PCR. Peterson *et al*[2] showed that 83% of MSI-H cases were methylated. Such findings were corroborated by studies undertaken by Trimarchi *et al*[195] and Hirasawa *et al*[196] who demonstrated a strong correlation between MSI and *MLH1* methylation which suggests that MLH1 methylation contributes to MSI-High status of tumours. The occurrence of tumours being MSI-High but unmethylated may be ascribed to factors such as somatic mutations occurring in *MLH1*, a possible germline mutation of *MLH1* or that mismatch repair genes other than *MLH1* being deficient.[2] These 3 factors may all be considered as possibilities for there not being a statistically significant relationship between methylation and PCR MSI in the present study.

Table 43 demonstrates that the relationship between MSI and methylation in patients over the age of 60 years is tending toward significance (p-value=0.5262). Zighelboim *et al*[44] showed that patients with MSI tumours and hypermethylation were approximately 10 years older than patients who had MSI tumours that were not methylated. These findings, similar to those of the present study may be explained by the fact that methylation is an age-related phenomenon as discussed by Klutstein *et al.*[185]

Table 44 shows that there is no statistically significant relationship between methylation status, MSI and all three tumour FIGO grades. Cosgrove *et al*[193] however established that tumours demonstrating epigenetic MSI or MMR by IHC are associated with a higher tumour FIGO grade and worse clinical outcome.[193] It has been suggested that hypermethylation of the promoter region results in instability of the genome which may result in a clinically aggressive tumour.[194]

Table 45, 46 and 47 show that there is no statistically significant relationship between EpiTYPER methylation status and overall MSI/MMR status irrespective of age or tumour

FIGO grade. Cosgrove *et al*[193] however demonstrated a strong correlation between methylation, older age and higher tumour FIGO grade in MMR deficient carcinomas.[193] It is possible that the present study did not show statistical significance in contrast to the study by Cosgrove and co-workers[193] as the present study examined a smaller cohort of patient samples and as such, assessed a smaller number of MSI-MMR deficient cases.

5.2.21.4. EPITYPER METHYLATION STATUS IN RELATION TO BRAF MUTATIONS

Tables 48, 49 and 50 show that there is no association between methylation using EpiTYPER and BRAF IHC; stratified by age or tumour FIGO grade. Similar results were obtained for assessment between methylation using EpiTYPER and BRAF PCR and sequencing; in addition to such assessment with respect to age or tumour FIGO grade (tables 51-56) respectively. Furthermore, these results were mirrored by the assessment of methylation using EpiTYPER in relation to BRAF overall mutational status; stratified by age or tumour FIGO grade (tables 57-59).

5.2.21.5. ONESTEP qMETHYL^{TM} KIT METHYLATION KIT ANALYSIS

Of these 37 cases, only 34 had enough DNA for methylation analysis to be performed. Figure 46 shows that 85% of the 37 MLH1 immunohistochemically deficient cases demonstrated hypermethylation. The results of the two methylation analyses are shown in figure 47 which shows that 88% of cases were methylated using EpiTYPER and 85% of cases were methylated using the OneStep qMethyl^{TM} kit. These results recapitulate those obtained by Haraldsdottir and fellow researchers who noted that over 80% of MLH1/PMS2 immunohistochemically deficient cases were hypermethylated.[72] A comparison of results obtained by EpiTYPER versus the OneStep qMethyl^{TM} kit shows only 1 discrepant result (table 60). This isolated case had a low positive result by EpiTYPER but was negative using

the OneStep qMethyl™ kit. It is possible that the DNA yield for the OneStep qMethyl™ kit was low as the DNA for this test was extracted after EpiTYPER testing had been performed. Data could not be obtained by the OneStep qMethyl™ kit for 2 cases (cases 8 and 17 in table 60) which were not methylated using EpiTYPER analysis. It is again possible that there were inadequate volumes of tumour DNA to assess for methylation. One case had insufficient tissue for methylation analysis. Of 34 cases, 24 (70.59%) demonstrated methylation of ≥33% relative to normal control samples used, which according to personal communication with the Zymo Research Group, and using the instruction manual, is considered strongly methylated.[103,197] This frequency of methylation (70.59%) is consistent with the findings of Bruegl *et al*[122]who documented that 65-97% of endometrial carcinomas that show MLH1 IHC deficiency, are in fact methylated.[122] Figure 47 compares results obtained using EpiTYPER and the OneStep qMethyl™ kit on the 34 cases which shows similar methylation results.

EpiTYPER is validated for use of methylation analysis as discussed by Olkov-Mitsel and Bapat[101] and as such, EpiTYPER was used to validated results obtained on the 25 discrepant MMR IHC and MSI PCR results by the OneStep qMethyl™ kit. Using the confusion matrix in table 61 the sensitivity of the OneStep qMethyl™ kit was 96.67%; CI (90.64-102.70), the specificity is 100%, 95% CI (100). The positive predictive value (PPV) is 100%, 95% CI (100) and the negative predictive value (NPV) is 80%, 95% CI (66.55-93.45). Thus, the OneStep qMethyl™ kit has a concordance of 85.29% when compared to methylation by EpiTYPER. Therefore, the OneStep qMethyl™ may be considered a good test for the assessment of methylation status in endometrial carcinomas and it is possible that with time, that this test may be implemented for use in the molecular unit of our laboratory.

Table 62 shows that more than two-thirds of the 52 cases tested by the OneStep qMethyl™ kit were hypermethylated. These results corroborate findings by Goodfellow *et al*[198] who noted that a high percentage of endometrial carcinomas show MLH1 hypermethylation.[198]

5.2.21.6. EPITYPER METHYLATION STUDIES ON DISCREPTANT IHC MMR AND PCR MSI CASES

Table 63 shows that the 25 cases that displayed discordant IHC and MSI PCR results underwent methylation analysis. Seventeen (68%) of these cases demonstrated hypermethylation, which clarifies the loss of staining for MLH1/PMS2 with microsatellite stability on PCR. Further analysis of the tissue preservation, type of tissue (namely excision specimen or curettage) and evaluation of IHC staining intensity/variability of these 25 cases revealed variable staining in 10 (59%) of methylated cases, whilst 4 of the 8 remaining cases demonstrated variable IHC staining. Thus these 4 cases may have had missense or truncation mutations resulting in varied IHC staining whilst PCR detected the mutations in these cases. One of these 8 cases had insufficient DNA for methylation assessment. One unmethylated case was poorly preserved, but still showed some nuclear staining. It is plausible that the mutations identified in the 8 remaining (7 unmethylated and 1insufficient DNA) cases were detected by PCR due to the ability of the mononucleotide markers to identify small changes in base pairs. These cases would have been missed had IHC testing been the only test method applied. It has recently been noted by Cho and co-workers that up to half the number of patients with MMR- deficiencies do not in fact harbour germline mutations and it is postulated that several factors may be responsible for this such as false positive MMR IHC stains, somatic inactivation of both MMR genes or the presence of unidentified mutations within mismatch repair genes. Next generation sequencing has identified that more than two-thirds of tumours have acquired biallelic somatic mutations with or without MMR genes showing loss of heterozygosity.[25]

Table 64 demonstrates 13 (8.97%) cases out of the total cohort of 145 that are suspected of harbouring possible germline mutations. Of the 18 cases, 5 (27.78%) did not have a true methylation assessment. Therefore, 13 of these (8.97%) are suspected of having Lynch syndrome. These results are almost double to those obtained by Cosgrove *et al* who identified 5% of their cohort as being probable germline mutations.[193] Furthermore, Wang *et al*[59] have indicated that similar to colorectal carcinomas, endometrial carcinomas also have MMR germline mutations in up to 5% of patients.[59] Vergouwe *et al*[199] have estimated that 10.5% of colorectal carcinomas in a South African population in the Northern Cape have germline mutations in MMR genes. In addition, Blokhuis *et al*[200] have identified that 14% of female patients with an *hMLH1* c.C1528T mutation in a South African study had extracolonic carcinomas, of which breast and endometrial carcinomas were the most frequently occurring tumours.[200] These studies suggest that there is a greater incidence of germline mutation associated neoplasia in South African patients. Two of the cases from the present study showed MSH2/MSH6 deficiencies and 2 cases showed isolated MSH6 loss, which according to work undertaken by Wang *et al*,[201] suggests that these 4 patients be further investigated for germline mutations as isolated losses of immunohistochemical expression for MSH2, MSH6 and PMS2 suggest germline mutations in the respective genes. Furthermore, these authors state that if loss of staining of both MSH2/MSH6 is noted, then MSH2 is considered the primary defect and therefore further testing for *MSH2* should be performed.[201] In addition, 5 cases of MLH1 IHC loss of staining were noted, which did not show hypermethylation by EpiTYPER or the OneStep qMethyl™ kit and three cases showed low levels of methylation such that they were considered unmethylated. Wang and co-workers[59] have recommended that germline mutational analysis for *MLH1* be performed in cases where methylation does not account for deficient MLH1 IHC staining.[201] These 13 patients should be identified and

their results be relayed to the relevant gynaecologist so that these patients may be offered genetic testing with a view to possible germline testing.

Table 65 shows that two-thirds of cases that underwent methylation assessment were hypermethylated and that 75% of these patients were over the age of 60 years, which is statistically significant (p=0.0302). This is consistent with the methylation findings using EpiTYPER (table 34) and supports studies conducted by Jiang *et al*[183] who documented the association between methylation and an increase in age.[183] In addition, table 66 shows that most methylated carcinomas demonstrated FIGO grade 2 histological features. Unlike findings from table 34 and studies by Salvesen *et al*[186], methylation by the OneStep qMethyl™ Zymo kit did not show a statistically significant association with tumour FIGO grade.

Table 67 shows statistical significance (p=0.001) with over 80% of methylated cases demonstrating IHC MMR mutations. This relationship mirrors the relationship between EpiTYPER methylation analysis and MMR IHC (table 35) and is similar to the findings of Metcalf and Spurdle.[61] This association may be ascribed to silencing of the *MLH1* promoter region by methylation. The 69% of cases that were not methylated or deficient on IHC showed MSI by PCR; whilst 31% of cases which had deficient staining by IHC but were not methylated may be attributed to possible mutations in *MSH2/MSH6* as well as *MLH1*; all of which may imply possible germline mutation as suggested by Wang *et al*.[59]

Table 68 shows that there is a weak, non-random, statistically significant relationship between methylated endometrial carcinomas using the OneStep qMethyl™ Zymo kit and IHC MMR deficiency in patients under and over the age of 60 years (kappa=0.5714, p-value = 0.0127 and kappa=0.4365, p-value=0.0034 respectively). These findings are supported by

researchers Zighelboim *et al*[44] and Cosgrove *et al*[193] who documented methylation in older patients.[44,193]

Table 69 demonstrates a weak, non-random but statistically significant association between methylation using the OneStep qMethyl™ kit and IHC MMR deficiency and FIGO grade 2 tumours (kappa=0.5953, p-value = 0.0004). There was no statistically significant relationship noted for FIGO grade 1 and 3 tumours. Whilst Bruegl *et al*[191] have stated that there is no statistically significant relationship between methylation, IHC loss and tumour FIGO grade; Kim *et al*[194] have documented that epigenetic abnormalities are associated with higher tumour FIGO grades and Cosgrove *et al*[193] have demonstrated that epigenetically associated MMR deficient tumours are associated with a higher tumour FIGO grade.[191,193,194] In the present study, the greatest association is noted with regard to FIGO grade 2. It is possible that this is due to the majority of cases in the current cohort being of intermediate FIGO grade. However, despite FIGO grade 1 tumours accounting for the next greatest frequency of tumour grades, FIGO grade 3 tumours may be seen to have a greater statistical significance than that for FIGO grade 1 tumours. It is thus possible that this suggests a greater association between methylation and higher-grade tumours.

Table 70 shows that there is no association between methylation using the OneStep qMethyl™ kit and PCR MSI (kappa= -0.1839, p-value = 0.9055). These results are similar to the degree of association between PCR MSI and methylation using EpiTYPER, (table 42). Contrasting results were noted by Peterson *et al* [2] who noted that 83% of their patients had MSI-High tumours. Factors stated previously such as possible MLH1 somatic mutations, mismatch repair genes besides MLH1 being affected or germline mutations of MLH1 may be considered as reasons for there not being a statistically significant relationship between methylation and PCR MSI in the present study.[2]

Table 71 shows that there is no statistically significant relationship between the OneStep qMethyl^{TM} kit and PCR MSI with respect to patient age. These findings are in contrast to methylation using EpiTYPER in which the p-value was tending toward statistical significance.

Table 72 shows that there is no statistically significant relationship between the OneStep qMethyl^{TM} kit and PCR MSI with respect to tumour FIGO grade. These results are similar to those noted in Table 43 which compared EpiTYPER to MSI PCR and tumour FIGO grades. These results contrast with those of Peterson *et al*[2] who documented that over 80% of MSI cases are methylated.[2]

Table 73 shows that over 60% of methylated cases were mutated by PCR; whilst more than 80% of unmethylated cases were mutated on PCR, with no statistical significance (p-value = 0.189). Table 74 shows that there is no statistically significant relationship between PCR MSI and methylation status using the OneStep qMethyl^{TM} kit (p-value =0.422). Almost half the number of methylated cases (48.57%) were MSI-Low on PCR; whilst over 60% of unmethylated case demonstrated MSI-Low phenotype. Under one-fifth (14%) of methylated cases were MSI-High and similarly, less than one-fifth of unmethylated cases were MSI-High by PCR. In contrast, the association between EpiTYPER methylation and PCR MSI status was tending toward significance (table 36). Whilst there is no statistically significant relationship between EpiTYPER methylation and MSI-Low and MSI-High, most of the methylated cases (62.96%) demonstrated MSI-Low by PCR and similar results were obtained between methylated cases that showed MSI-High by PCR (table 75). Kim *et al*[194] have however documented a strong association between methylation status and higher tumour grade. Using both EpiTYPER and the OneStep qMethyl^{TM} Zymo kit, most of the cases in the present study demonstrated MSI-Low on PCR; whilst Salvesen *et al*[186] have shown a strong correlation between methylation status and MSI-High features. However, as stated

previously, it has been noted that methylation is involved in MSI-Low tumours.[186] It is possible that the relationship between the OneStep qMethyl™ Zymo kit and MSI status is less strong than EpiTYPER and MSI status as in the OneStep qMethyl™ kit there were a few cases in which no result was obtained as there was less DNA available in some endometrial carcinomas as the samples had undergone IHC, PCR, BRAF analysis and EpiTYPER testing prior to the OneStep qMethyl™ kit.

Table 76 demonstrates that all cases that underwent methylation assessment were abnormal or mutated by IHC or PCR. This is explained by the fact that cases underwent methylation analysis if there was loss of staining with an MLH1 stain or if there were discordant results between IHC and PCR MSI results. It is thus evident that all the cases on which methylation was performed were abnormal/mutated by either IHC or PCR.

Tables 77-79 show that there is no statistically significant relationship between the OneStep qMethyl™ kit methylation status and overall MSI/MMR abnormality/mutational status irrespective of age or tumour FIGO grade. These findings are similar to those noted in the comparison between EpiTYPER methylation status and overall MSI/MMR abnormality/mutational status irrespective of age or tumour FIGO grade. Cosgrove and co-workers[193] showed a strong correlation between methylation, older age and higher tumour FIGO grade.[193] In the present study it is possible that no statistically significant relationship was noted in contrast to the study by Cosgrove and co-workers[193] as the current study evaluated a smaller cohort of patient samples and consequently assessed a smaller number of MSI-MMR deficient cases.

Tables 80-82 show that there is no statistically significant relationship between methylation using the OneStep qMethyl™ kit and BRAF IHC according to age or tumour FIGO grade. Similarly, tables 83-85 show no statistically significant association between methylation

using the OneStep qMethyl™ kit and BRAF PCR according to patient age or tumour FIGO grade. Furthermore, tables 86-88 show no statistically significant relationship between methylation using the OneStep qMethyl™ kit and BRAF sequencing. In addition, similar results were obtained when comparing methylation using the OneStep qMethyl™ kit and overall BRAF status according to age and tumour FIGO grade (tables 89-91). Studies have assessed both *BRAF* and *MLH1* methylation status in colorectal carcinomas and Parsons *et al*[202] have reported that BRAF status is a better, more sensitive indicator of a tumour's MMR gene mutation status than methylation.[202] As *BRAF* mutations have been thought to occur too uncommonly in endometrial carcinomas, *BRAF* mutational analysis has not been considered as part of the work-up and triaging of endometrial carcinoma mutational status.[65] Such sentiment has been echoed by Metcalf and Spurdle as well as Bruegl and co-workers.[61,191] Whilst Feng *et al*[62] reported that one-fifth of their patients with endometrial carcinomas had *BRAF* mutations, they did not assess the *MLH1* methylation status of these tumours.[62] Thus, the present study illustrates that there is no statistically significant relationship between BRAF mutations, irrespective of the test methodology and *MLH1* methylation status of endometrial carcinomas; and despite a slight increase in the number of BRAF mutations identified in contrast to Western studies, it is evident that BRAF mutations in the South African population assessed are not useful in identifying which patients may be suspected of having a germline mutation.

Table 92 shows a confusion matrix assessing the relationship between EpiTYPER and the OneStep qMethyl™ kit. The sensitivity of the OneStep qMethyl™ kit for the 52 comparable cases is 91.67%; CI (84.16-99.18), the specificity is 56.25%, 95% CI (42.77-69.73), the positive predictive value (PPV) is 82.5%, 95% CI (71.12-93.87) and the negative predictive value (NPV) is 75%, 95% CI (69.00-81.00). Table 93 confirms the mathematical equation calculating the concordance level and demonstrates statistical significance (p=0.0001). Thus,

the OneStep qMethyl^{TM} kit may be considered a good test for the assessment of methylation status in endometrial carcinomas in cases of discrepant IHC and PCR MSI results as well as in instances of MLH1 IHC loss. This may be a useful, rapid, bisulphite-free test that may be performed in-house on cases of endometrial carcinomas diagnosed in our department.

Table 94 demonstrates a moderate degree of non-random, statistically significant concordance (kappa = 0.6679; p=0.0001) between methylation using the OneStep qMethyl^{TM} kit and EpiTYPER in patients over the age of 60 years. This illustrates a strong relationship between methylation status and an increase in age which supports results obtained by Cosgrove *et al*[193]and Klutstein *et al*.[185] Again, the association between methylation and older age may be attributed to epigenetic phenomena which have a propensity to occur in older patients.[183,185] For patients under the age of 60 years there is no agreement between the two methylation tests (kappa = 0.1935, p-value = 0.1024).

Table 95 shows a statistically significant relationship between the OneStep qMethyl^{TM} kit and EpiTYPER methylation assessment with respect to tumour FIGO grades 1 and 2 (p-value = 0.003 and p=0.0031 respectively). It is interesting to note that whilst Bruegl *et al*[191]have shown that there is no relationship between hypermethylation and tumour FIGO grade, other authors such as Cosgrove *et al*[193] have identified a strong association between epigenetic or methylated tumours and a higher tumour FIGO grade which corroborates results from the current study.[191,193] In the present study, the weakest relationship between methylation status and FIGO grade 3 tumours was observed. A possible explanation for this may be that most of the tumours in the present cohort demonstrated FIGO grade 2 or intermediate FIGO grade histological features whilst FIGO grade 3 tumours comprised the minority of cases.

CHAPTER 6

CONCLUSION

Endometrial carcinomas are common malignancies of the female genital tract that are on the rise in developing and developed nations.

It has been demonstrated by Hampel and co-workers[150] that sensitivity of family history in identifying patients with possible Lynch syndrome is an ineffective screening tool. In the context of the South African population, it is possible that language differences in our multicultural society may be a limiting factor in obtaining a thorough familial carcinoma history and as such, this is likely not to be a useful screening method for LS. It has been illustrated by work of Ryan *et al*[203] that over half the number of endometrial carcinomas from carriers of germline mutations did not have morphological features that have been previously noted as being suggestive of hereditary mutations such as occurrence of tumours within the lower uterine segment, tumour heterogeneity, peri-tumoural lymphocytes and tumour infiltrating lymphocytes.[94,203] Shia also states that histopathology does not provide promise of being an effective screening tool.[181] The histological features in the current cohort of patient samples could not be assessed as not all patients underwent total abdominal hysterectomy, and as such it is not possible to comment on the findings of the local population. Yet, anatomical pathologists occupy unique positions to identify possible germline mutations in endometrial carcinomas. This may be accomplished by adopting an endometrial carcinoma screening program.

The results of the present study demonstrate that there were 25 cases of discordant results between IHC MMR and MSI PCR on endometrial carcinomas seen in our department which is distinctly different to studies undertaken by McConechy *et al.*[31] Factors implicated in these discrepant results included hypermethylation of the *MLH1* promoter region, possible

germline mutations of the MMR genes, somatic mutations in *MLH1*, use of archived material which had been fixed with non-standard fixation methods and the possible occurrence of subclones of tumours which may exhibit immunohistochemical negativity in contrast to the surrounding tumour. Furthermore, small changes in base pairs may have been identified by PCR.[2,31,131] Whilst Resnick *et al*[157] have established that use of IHC as a screening modality followed by directed genetic testing is a cost-effective process by which to identify patients with Lynch syndrome. The results of the present study demonstrate that 25 (17.24%) out of 145 cases would not have had mutations identified if PCR MSI was not performed. The majority (68%) of these 25 cases were hypermethylated. For the remaining 8 (5.52%) cases, 1 case did not have sufficient DNA for methylation assessment, whilst 7 were unmethylated. Of these 7 cases, 4 cases may have shown false positive/retained staining immunohistochemically as these showed variable staining patterns. Thus, these 7 cases may have had small changes in base pairs which were only detected by PCR (table 64). Had these cases not been subjected to PCR MSI they would not have been detected immunohistochemically and would not have been identified as having a possible germline mutation. Thus, PCR had identified 5.52% of cases out of a total of 145 that would have been missed on IHC testing alone. Whilst this may not seem like a high percentage, the morbidity and mortality implications for the affected individuals and their families are significant.

Garg and Soslow[26] have suggested that as there is a possibility of immunohistochemistry not detecting mutations in the 4 marker panel, it is proposed that both IHC and PCR be used to screen patients with endometrial carcinoma for Lynch syndrome.[26]

Six BRAF mutations were identified in the current cohort of patients using 3 tests. Whilst BRAF sequencing is the current gold standard against which other methods are tested, sequencing only detected a single BRAF mutation. Similarly, BRAF IHC also only detected a single mutation. BRAF PCR identified 4 mutations, of which 3 were V600E mutations and a

single BRAF V600D mutation was found; which to the best of the investigator's knowledge has not been previously identified in endometrial carcinomas. Using all 3 methods increased the total number of BRAF mutations detected in our patient population which shows a slight increase over the number of *BRAF* mutations detected in western societies, but less than that found in an Eastern study. However, results of the present study indicate that similar to that of studies in the west, *BRAF* mutations are not commonly encountered in endometrial carcinomas in the South African population assessed and BRAF assessment is thus not useful in triaging patients with suspected Lynch syndrome.

Methylation analysis by both EpiTYPER and using the OneStep qMethyl™ kit illustrated that most (68%) of the discordant MMR IHC and MSI PCR results could be attributed to *MHL1* promoter hypermethylation which is similar to reports noted in the west.[2,61] The presence of hypermethylation in these patients points toward a likely sporadic occurrence of carcinogenesis. These patients would then not undergo further molecular testing.

Possible cases of Lynch syndrome in this study include 4 cases which had MSH2/MSH6 or isolated MSH6 loss of staining. One MLH1 deficient tumour did not have enough DNA for further methylation assessment. Nine cases were unmethylated, of which 5 showed MLH1 loss by IHC whilst the remaining 4 cases were MLH1 proficient. A further 4 cases yielded no result by methylation assessment which is likely due to insufficient DNA volumes. Furthermore, the true methylation status of these 4 cases cannot be ascertained. A total of 5 out of 18 cases (27.78%) are considered to not have a true methylation assessment. Thus, the remaining 13 (8.97%) of 145 cases are potential germline carriers based on available results. These patients should be identified, and their treating gynaecologists contacted with the view to genetic counselling and possible germline assessment. This is higher than the frequency (approximately 4%) of suspected Lynch syndrome noted in the west.[198] However, an unpublished study on colorectal carcinomas with suspected Lynch syndrome conducted in

our department suggests that over 30% of cases point toward a germline mutation. In addition, a South African study on colonic tumours calculated an expected frequency of Lynch syndrome in 10.5% of all colorectal carcinomas.[199]This therefore suggests that there may be a higher incidence of Lynch syndrome in the South African population than is documented in the west.

The present study illustrated that the OneStep qMethyl™ kit fared well in comparison to results obtained by the EpiTYPER Massarray system, The OneStep qMethyl™ kit is an attractive option that circumvents the need for a bisulphite conversion. In light of the good concordance rates found between these two tests, this kit would be a suitable method to incorporate into the molecular unit of our department with a view to assessing methylation status of endometrial carcinomas.

By providing a histopathological and molecular diagnosis of endometrial carcinoma which suggests possible germline mutation, we may empower our patients by including them in decisions for their holistic management which encompasses genetic counselling and possible germline assessment; in addition to surgery and/or chemoradiation. Identification of mismatch proficient versus deficient tumours may assist in treatment options as tumour cells of mismatch repair proficient tumours are sensitive to methylating agents whilst mismatch repair deficient tumours are not.[204] Furthermore, with the advent of targeted therapy the presence of mismatch repair mutations offers the opportunity for use of an antibody directed against programmed cell death (PD-1) which has shown promising results[10,205] Recently, the Food and Drug Administration (FDA) have approved use of the anti PD-1 drug Pembrolizumab, for either metastatic or unresectable malignancies that are mismatch repair deficient or microsatellite-high for patients whose neoplasms have progressed despite previous therapy and in whom there are no alternate treatment options.[206] This is the first

instance in which anti-tumour treatment has been approved based solely on biomarker results instead of the primary site of tumour origin.[206]

IHC is a screening modality and not a confirmatory test. Thus, it should not be construed as being of questionable ethics to perform such tests on patient specimens.[181] Rather it should be considered how unethical it would be to not identify patients who may have germline mutations as without screening modalities there would be no indication of the need for further testing and surveillance of future tumour development.

6.1. KEY POINTS OF THE STUDY

1. Twenty-five cases in the present study showed discordant MMR IHC and MSI PCR results, possible reasons for these include:
    a. Hypermethylation of the *MLH1* promoter region.
    b. Possible germline MMR gene mutations.
    c. Somatic *MLH1* mutations.
    d. Use of archived material which had been fixed by non-standard fixation methods.
    e. Possible subclones of tumour which may show loss of IHC staining relative to the rest of the tumour.
    f. Small changes in base pairs only detectable by PCR.
2. Most (68%) of the 25 discordant cases showed hypermethylation.
3. Seven of the remaining 8 cases were not methylated and 1 case did not have enough DNA for methylation assessment.

4. These 8 (5.52%) cases had mutations detected by PCR with retained MMR IHC staining; of which 7 showed no methylation; therefore, raising the possibility of a germline mutation.

5. BRAF mutations were detected in 6 out of 37 cases using 3 tests; namely IHC, PCR and Sanger sequencing.

6. Despite Sanger sequencing being the current gold standard against which other tests are assessed, 4 mutations were identified using PCR. Of these 4 mutations, 3 showed BRAF V600E mutations whilst 1 case showed a BRAF V600D mutation which has not been previously detected in endometrial carcinomas.

7. *BRAF* mutations in endometrial carcinomas of South African females are uncommon and do not assist in the triaging of patients with suspected LS.

8. Methylation assessment showed that 68% of cases showing discrepant MMR IHC and MSI PCR results were due to hypermethylation, thus pointing to a sporadic tumour occurrence.

9. A possible 13 out of 145 patients with EEC have possible germline mutations, suggesting that there may be a higher incidence of LS in South African patients.

10. Thus, most MMR deficient or MSI EECs in the present study were sporadic in nature as demonstrated by methylation assessment. In contrast, the 13 cases suspected of having possible germline mutations should be further investigated. As such, both MMR IHC and MSI PCR should be used as screening tests together with methylation assessment of EEC in the workup of EECs. However, BRAF testing is not indicated.

6.2. RECOMMENDATIONS

Reflex testing of all endometrial carcinomas occurs in some but not all centres worldwide. The present study suggests a need to screen possible Lynch syndrome patients in our population. Whilst Cho *et al*[25] currently advocate use of PMS2 and MSH6 immunohistochemical stains, Dierssen[141] as well as Garg and Soslow[26] recommend use of immunohistochemistry and PCR testing as screening modalities. [25,26,141] In light of the cases of discordant IHC MMR/PCR MSI results and the fact that 5% of patients would not have been identified had they not undergone PCR MSI analysis, it is recommended that endometrial carcinomas undergo both screening tests at our institution. However, in an attempt to curtail costs, it is suggested that screening be performed in patients under the age of 70 years and that biopsies from such patients undergo IHC testing for MSH6 and PMS2, as suggested by Cho *et al*[25] together with PCR MSI testing. The rationale for this is as follows:

1. Most patients in the present study were over the age of 60 years and therefore if screening is only for patients under the age of 60 years, many patients with possible Lynch syndrome may not be detected.
2. Some MSI PCR detected mutations were not identified by IHC in the present study and as such, this suggests that there may be mutations or small base pair defects that are not detected by IHC but may be picked up by PCR.
3. MSH6 IHC should be performed as it is known that a mutation in this gene is often not detected by MSI PCR.
4. Should deficiencies in MSH6 and/or PMS2 IHC stains be observed, then these may be followed by testing of MSH2 and/or MLH1 with reflex testing for promoter methylation when indicated.

The cases demonstrating *MLH1* promoter methylation suggest a sporadic event and no further molecular assessment is required. The cases which are suspected of harbouring a germline mutation, however, may then be offered genetic counselling with a view to mutational assessment. It is envisaged that this study will heighten awareness of the possible occurrence of Lynch syndrome in endometrial carcinomas from patients at our institution; and elsewhere throughout the country. It may also allow for the submission of samples from patients in whom Lynch syndrome is suspected, for IHC and PCR testing, with or without methylation analysis, to our department for testing so as to have one centralised unit performing such tests. Obtaining samples from other parts of the country will help establish the incidence of possible Lynch syndrome in South Africa as a whole. Furthermore, it is anticipated that future germline mutational assessment in a state hospital may be established so that patients with these suspected germline mutations may be afforded therapy targeted to their specific tumours.

CHAPTER 7.0.

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APPENDIX 1. PUBLISHED ARTICLE

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Review Article

A potpourri of pathogenetic pathways in endometrial carcinoma with a focus on Lynch Syndrome

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ARTICLE INFO

Keywords:
Endometrioid endometrial carcinoma
Lynch syndrome
Microsatellite instability

ABSTRACT

Endometrial carcinoma is the most frequently occurring female genital tract malignancy in developed nations, with a rising annual incidence. Endometrioid endometrial carcinoma (EEC), the most common histological variant, differs in morphologic and molecular characteristics from serous carcinomas but morphological distinction of high-grade EECs from serous carcinomas may prove difficult. Thus, molecular categorization of tumors may allow for better tumor classification with greater insight into the underlying biology of endometrial carcinomas with new therapeutic options.

Microsatellite instability (MSI) is a commonly occurring molecular aberration in EECs and has been identified in most Lynch Syndrome (LS) associated tumors. This tumor syndrome predisposes afflicted individuals to a myriad of tumors including endometrial carcinoma. Herein, the molecular signature of endometrial tumors as well as LS, and its clinical manifestations are reviewed. Understanding of the pathogenetic pathways allows for greater comprehension of occurrences at a molecular level which are then appreciated at a cellular and tissue level, by the histopathologist.

The molecular classification of endometrial tumors allows for further targeted therapeutic options for affected patients. Screening tests for patients with suspected LS enables surveillance of other tumors in the affected patient and her family with the potential to decrease morbidity and mortality. It is envisioned that this overview will allow for enhanced comprehension of genetic pathways by practicing pathologists, oncologists, gynecologists and other members of the multidisciplinary team, all of whom are involved in the management of the patient with an endometrial malignancy.

1. Background

Endometrial carcinoma is the most frequently occurring female genital tract malignancy in developed nations; and in western societies, is one of the most common malignancies in females [1-4]. Endometrial carcinoma is anticipated to affect approximately 7% of females in the United States of America alone, in 2018. [1-4] Worldwide, endometrial carcinoma accounts for roughly, 4% of all tumors [4]. This article will delve into the molecular aberrations of endometrial tumors and will highlight the recent advances in genetic profiling of these neoplasms. Furthermore, this article discusses microsatellite instability, which has been identified in approximately 90% of endometrial carcinomas associated with LS [5] and provides the practising histopathologist with an in-depth understanding of the underlying fundamental mechanisms that culminate in DNA mismatch repair, which have also been documented in up to 30% of sporadic endometrial carcinomas [2]. This will facilitate a more thorough comprehension of the basis of the hereditary tumor predisposition syndrome, known as Lynch Syndrome (LS). It must be borne in mind that it is a mutation of MLH1, MSH2, MSH6 or PMS2 genes that defines LS and not the absence of staining of one the immunohistochemical stains. Lynch Syndrome and its clinical correlates are reviewed in detail.

There is currently a rising incidence of endometrial carcinoma in the western world which has been ascribed to an increased usage of hormonal therapy such as Tamoxifen for breast carcinoma, the associated occurrence of obesity, as well as a prolonged life span [6-10]. Tamoxifen has been shown to possess anti-estrogenic effects in breast parenchyma but in endometrial tissue, Tamoxifen provides an estrogen rich environment [9]. This may then set the scene for endometrial hyperplasia and possible neoplastic transformation. Another source of

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Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Reubina Wadee, student number [9900264F], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article stated below which are included in my Thesis.

Signature of Student ..... **Date** 23 April 2019.

Name of Primary Supervisor Professor Wayne Grayson

Signature of Primary Supervisor ..... **Date** 23 APRIL 2019

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of her Thesis. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 1: Title: .....A potpourri of pathogenetic pathways in endometrial carcinoma with a focus on Lynch Syndrome.....

Journal name, year, volume and page numbers: Annals of Diagnostic Pathology, 39 (2019) 92-104.....

Authors	Name	Signature	Date
1st author	Reubina Wadee		
2nd author	Wayne Grayson		
3rd author			
4th author			
5th author			
6th author			

Comments by primary supervisor:

I AGREE TO THE SUBMISSION OF THIS WORK.

1

APPENDIX 2. ETHICAL CLEARANCE CERTIFICATE

R14/49 Dr Reubina Wadee

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M151051

NAME: (Principal Investigator)	Dr Reubina Wadee
DEPARTMENT:	Division of Anatomical Pathology Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Services
PROJECT TITLE:	Endometrial Carcinomas: Microsatellite Instability and Suspected Lynch Syndrome in the Greater Johannesburg Area (2009-2015)
DATE CONSIDERED:	Adhoc
DECISION: CONDITIONS:	Approved unconditionally
SUPERVISOR:	Prof Wayne Grayson

APPROVED BY: Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 25/11/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

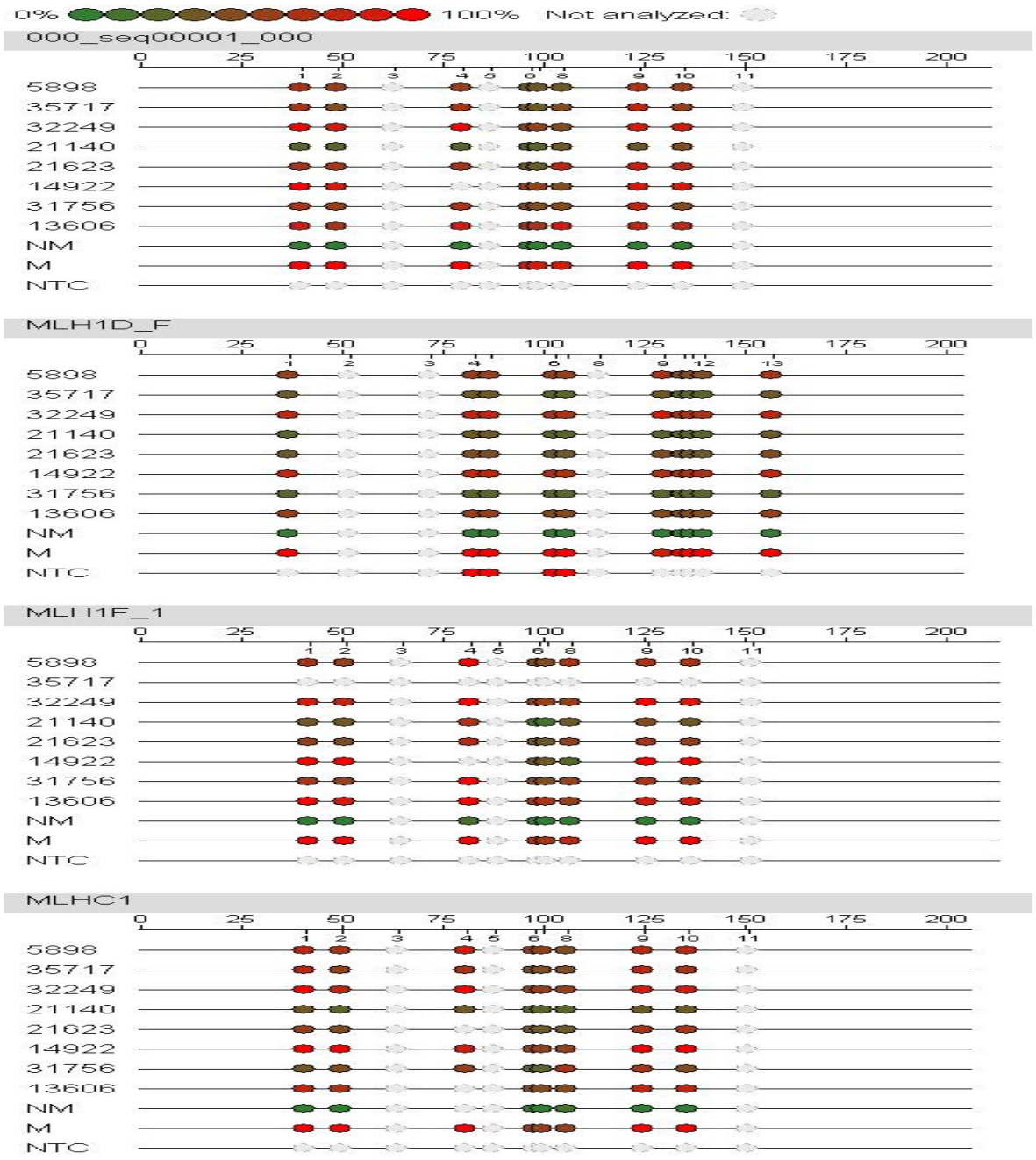
DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/2nd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

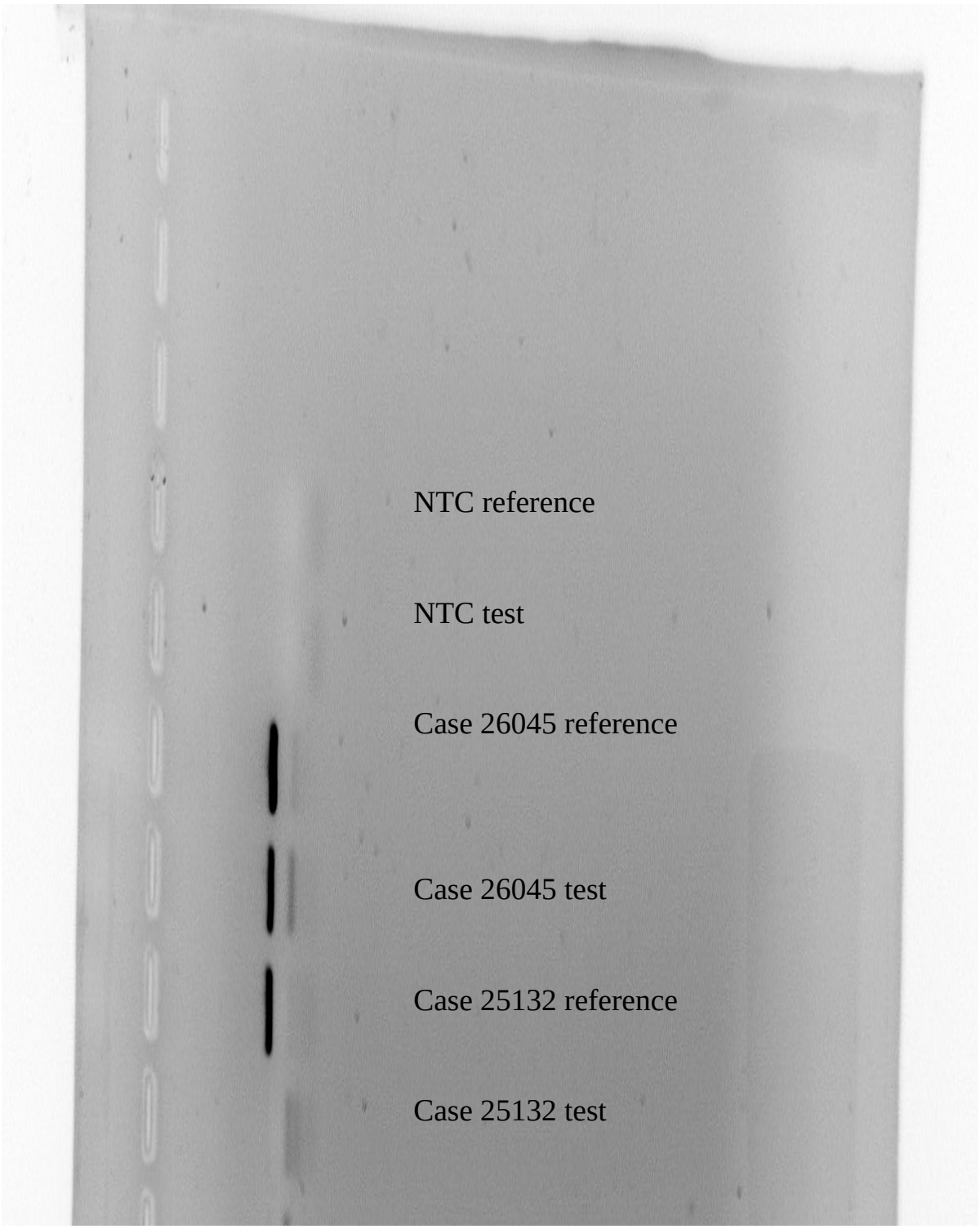
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 3. EPIGRAM



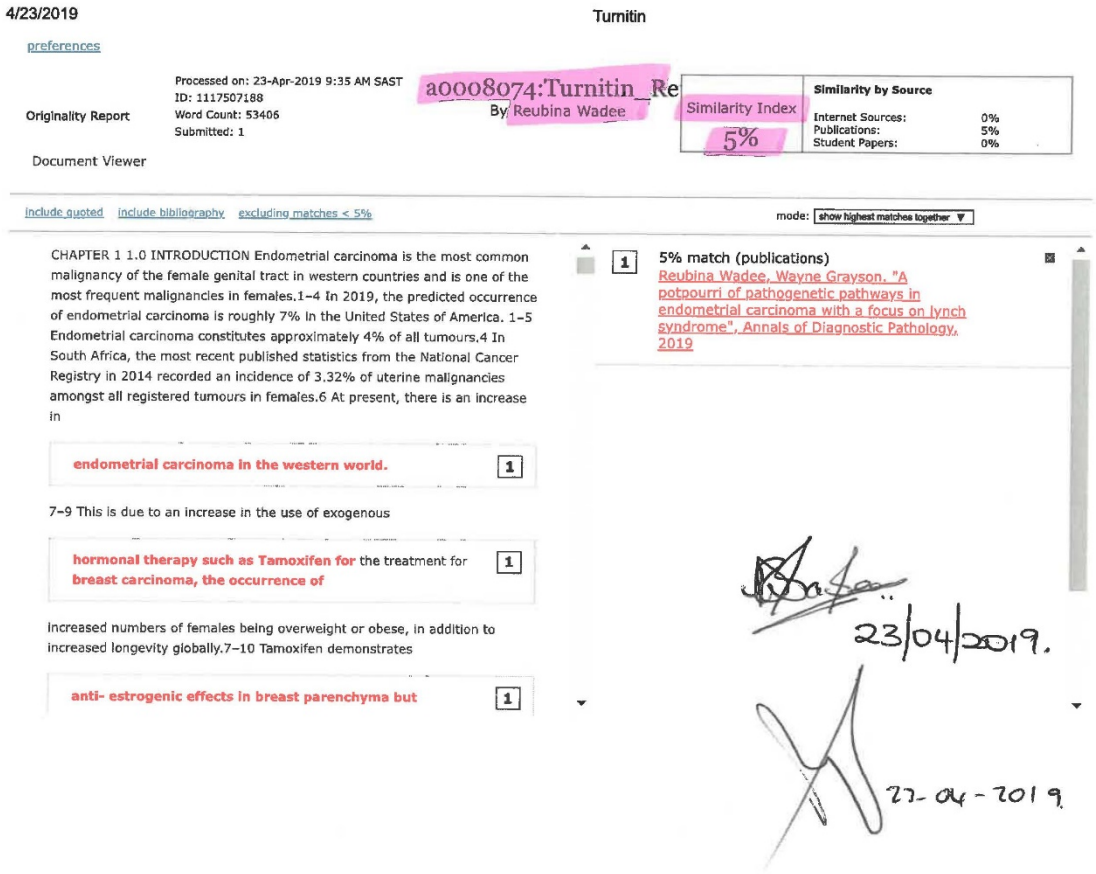
This epigram demonstrates varying degrees of methylation for a few representative samples from the present study, where 0% methylation is represented by green circles and 100% is represented by red circles with varying shades in-between red and green for varying percentages of methylation.

APPENDIX 4. PCR GEL.



This PCR gel, using the OneStep qMethyl™ PCR kit, shows a methylated case (26045) and an unmethylated case (25132) with reference tests and no template control (NTC).

APPENDIX 5. Turnitin Report.



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CHAPTER 1 1.0 INTRODUCTION Endometrial carcinoma is the most common malignancy of the female genital tract in western countries and is one of the most frequent malignancies in females.1–4 In 2019, the predicted occurrence of endometrial carcinoma is roughly 7% in the United States of America. 1–5 Endometrial carcinoma constitutes approximately 4% of all tumours.4 In South Africa, the most recent published statistics from the National Cancer Registry in 2014 recorded an incidence of 3.32% of uterine malignancies amongst all registered tumours in females.6 At present, there is an increase in

endometrial carcinoma in the western world. 1

7–9 This is due to an increase in the use of exogenous

hormonal therapy such as Tamoxifen for the treatment for breast carcinoma, the occurrence of 1

increased numbers of females being overweight or obese, in addition to increased longevity globally.7–10 Tamoxifen demonstrates

anti- estrogenic effects in breast parenchyma but 1

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APPENDIX 6. PLAGIARISM FORM

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FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I REUBINA WADEE (Student number: 9900264F) am a student registered for the degree of DOCTOR OF PHILOSOPHY in the academic year 2019.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
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