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AN OBSERVATIONAL STUDY OF SYNCHRONOUS/METACHRONOUS TUMOURS IN MICROSATELLITE UNSTABLE/MISMATCH REPAIR DEFICIENT ENDOMETRIAL CARCINOMAS (2009-2015)

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Student number:1445177

A research report in 'submissible format' submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine (MMed) in the specialty of Anatomical Pathology.



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Johannesburg, 2021

DECLARATION

Declaration: Student's contribution to article and agreement of co-author

I, Suzanne Kotze, student number 1445177, declare that this research report is my own work and that I contributed adequately towards research findings in the article stated below which are included in my research report. It is being submitted in fulfillment of the requirement for the degree of Master of Medicine in the branch of Anatomical Pathology at the University of the Witwatersrand, Johannesburg. Assistance that I have received has been declared in the acknowledgements section of the article. This work has not been submitted previously for any degree or examination at this or any other university.

I certify that the protocol has been approved in accordance with the Human Research Ethics Committee (Medical) at the University of the Witwatersrand, Johannesburg (Appendix 3.4: Ethics Clearance Certificate M190939)

Signature of Student **Date** 2021/07/19

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Date 2021/07/19

Agreement by co-author: By signing this declaration, the co-author listed below agree to the use of the article by the student as part of her research report.

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1st author	Suzanne Kotze		2021/07/19
2nd author	Reubina Wadee		2021/07/19

In loving memory of my dad, who gave me wings.

Japie Kachelhoffer

(1943 – 2015)

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

The findings of this project have been presented as a poster presentation, poster number: PRC 21-318, at NHLS PathReD Congress, 19 – 21 August 2021.

PUBLICATIONS ARISING FROM THIS RESEARCH PROJECT

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ABSTRACT

Objective

Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma (EEC). Microsatellite unstable endometrial carcinomas may be associated with multiple primary malignancies. We aimed to document the incidence and type of associated tumours in MSI/mismatch repair deficient (MMR-d) endometrial carcinomas at a single institution in South Africa.

Methods

The study assessed EECs for the period 2009-2015 at a Johannesburg hospital and followed on previous research undertaken in our department in which 66/145 MSI/MMR-d EECs were identified using immunohistochemistry and polymerase chain reaction (PCR). After ethical clearance was granted, using the microsatellite unstable/MMR-d endometrial carcinoma case-specific laboratory reference numbers, a retrospective search and cross-reference for associated histologically proven tumours, was performed.

Results

Three patients (4.5%) out of 66 confirmed MSI/MMR-d endometrial carcinoma cases had associated tumours, including an invasive squamous cell carcinoma of the cervix and mucinous breast carcinoma. One patient had two tumours, namely uterine leiomyosarcoma and rectal adenocarcinoma. The incidence of EEC patients with an associated tumour is significantly lower ($p=0.0045$) than that documented in the United States of America (USA).

Conclusion

Our study's incidence of associated tumours in MSI/MMR-d endometrial carcinoma patients was significantly lower than studies from the USA. The associated tumours in our study included traditional Lynch syndrome tumours such as colonic adenocarcinoma and demonstrated less commonly associated tumours, namely cervical carcinoma and uterine leiomyosarcoma. These findings highlight the need for further research in our population to assess risk factors and the true incidence of Lynch syndrome-associated tumours.

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NOMENCLATURE

AIDS	Acquired Immunodeficiency Syndrome
DNA	Deoxyribonucleic Acid
EEC	Endometrioid Endometrial Carcinoma
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
IACR	International Association of Cancer Registries
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MMR-d	Mismatch Repair Deficient
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability-High
MSI-L	Microsatellite Instability-Low
MSS	Microsatellite Stable
NHLS	National Health Laboratory Services
PCR	Polymerase Chain Reaction
PMS2	Post-meiotic Segregation Increased 2
POLE	DNA Polymerase Epsilon
TCGA	The Cancer Genome Atlas Research Network
USA	United States of America

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In the developed world, endometrial carcinoma is the most common malignancy of the female genital tract.[1] According to the GLOBOCAN 2020 database, uterine carcinoma was the 16th most common cancer diagnosed worldwide.[2] In the United States of America (USA), endometrial carcinoma is the sixth leading cause of death among females patients with cancer.[3] New cases of endometrial carcinoma in the USA have been slowly increasing, and this is partially ascribed to the increase in population age and obesity, both of which are risk factors for certain subtypes of endometrial carcinoma.[3] The American Cancer Society estimates a 7% incidence of uterine carcinoma in 2021.[4] In 2018 the incidence of uterine cancer was 3.2% in sub-Saharan Africa.[5] According to the latest published National Cancer Registry statistics in South Africa, cancer of the uterine corpus had a similar incidence of 3.77%.[2,6]

Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma (EEC) and is associated with both inherited and sporadic endometrial carcinomas.[1,7] Microsatellite unstable endometrial carcinomas may be associated with multiple primary malignancies, especially in the setting of underlying Lynch syndrome.[1,8,9]

The incidence of Lynch syndrome in Africa is not well reported.[10] A 2019 population-based study from Zimbabwe assessed hereditary colorectal carcinomas and found a 3.3% prevalence of Lynch syndrome.[11] A survey in Libya assessing the incidence of synchronous and metachronous tumours reported an incidence of 1.14%.[12] In South Africa, Wadee and Grayson's study showed that 6.9% of patients with EEC could have underlying Lynch syndrome.[1]

1.2 Overview of Microsatellites and Microsatellite instability:

Microsatellites or short tandem repeats (STR) are defined as small DNA sections, approximately 10 to 60 base pairs in length, containing repeat sequences of 1-5 base pairs or more.[1,13,14] The

repeat of the base pair cytosine and adenine (CA), is most frequently seen.[1] Microsatellites are present throughout coding and non-coding areas within the human genome, including telomeres and centromeres.[1,14,15]

Microsatellites are prone to DNA replication errors during the cell cycle.[16] The errors occur secondary to DNA slippage during replication.[17] Errors in replication are repaired by one of the body's DNA repair mechanisms, known as the mismatch repair (MMR) system.[14] which corrects replication errors such as "base-base mismatches" and "insertion-deletion" loops in the microsatellite areas.[1] Several genes encode the group of proteins that form part of the MMR system, including “mutL homologue 1 (MLH1), mutL homologue 3 (MLH3), mutS homologue 2 (MSH2), mutS homologue 3 (MSH3), mutS homologue 6 (MSH6), postmeiotic segregation increased 1 (PMS1) and post-meiotic segregation increased 2 (PMS2)”.[1,13] The MMR system has both major and minor genes with different functions.[1] Major genes include *MSH2* and *MLH*, and these genes are responsible for protein expression.[1] The minor genes include *MSH3*, *MSH6* and *PMS2*, which support and stabilize the major genes in their function.[1] For example, *MSH6* and *MSH2* form a complex called "MutSα"which functions to identify base-pair mismatches and can repair insertion-deletion and single base-pair mutations.[1] These genes have a "proofreading" function with their corresponding proteins, ensuring DNA integrity and accurate replication before cell division.[18]

Loss of function of one or more MMR system members causes accumulation of mutations within the microsatellites.[13] Loss of MMR gene function may be secondary to a genetic mutation, which may be inherited, may occur sporadically, or may be due to epigenetic hypermethylation of the *MLH1* gene.[15,16] The most commonly affected gene is *MLH1*.[13] Loss of mismatch repair gene function on its own does not directly cause malignant transformation of cells. However, their loss does lead to the accumulation of mutations or errors in other genes, including microsatellites, during the normal cell cycle, resulting in a strong mutator phenotype.[18,19] The range of mutations that follows after the MMR system's loss of function is broad, causing missense mutations, silent mutations or deletions/insertions that may result in frameshift mutations.[1,18] These mutations may then accidentally inactivate tumour suppressor genes or activate proto-oncogenes.[18] When affected cells gain these driver mutations (oncogenes or inactivated tumour suppressor genes),

this may result in cancer development.[18] This genetic instability and accumulation of mutations throughout the genome in microsatellites contributes to cancer development and is termed microsatellite instability.[17–19]

MLH1 promotor hypermethylation is an epigenetic process that leads to gene silencing by adding a methyl group to cytosine which then blocks transcription factors attaching to DNA and thus prevents DNA transcription.[1]

Microsatellite instability and mismatch repair deficiency can be detected predominantly by using polymerase chain reaction (PCR) analysis and/or immunohistochemistry (IHC).[16] A case is identified as microsatellite unstable by PCR if one or more markers show a difference in the size of the alleles.[20] Several markers are used to detect microsatellite instability and different centres choose to use different panels; however, a commonly used mononucleotide panel includes BAT-25, NR-21, NR-24, BAT-26 and NR-27.[13] A case is classified as MSI-H (microsatellite instability-high) if two or more markers show dissimilarity in allele size, MSI-L (microsatellite instability-low) if one marker shows difference in allele size, and microsatellite stable (MSS) if none of the markers show a difference in allele size.[1] A case is identified as mismatch repair deficient if one or more of the MMR antibodies (PMS2, MLH1, MSH6 and MSH2) show loss of nuclear staining.[20] A study by McConechy *et al* showed a high level of concordance (93.3%) between MMR protein immunohistochemistry and MSI PCR analysis, and as such, IHC may be used instead of PCR.[21]

1.3 Classification of Endometrial Carcinoma:

Endometrial carcinoma can be classified according to pathogenesis, histological appearance or molecular subtype.[1,3] There is, however, significant overlap between the groups, and all three classifications should be used to help predict risk and prognosis more accurately.[1,3] The current recommendation is to use molecular classification in combination with histology for risk stratification and is the best prediction of prognosis.[7]

1.3.1 Pathogenetic classification

Bokhman divided endometrial carcinomas into the traditional oestrogen dependent (Type I) endometrial carcinomas and oestrogen independent (Type II) pathway.[1,22] Sources of oestrogen can be either be exogenous or endogenous. Tamoxifen, used as a treatment for breast carcinoma, and hormonal therapy without opposing progesterone, are both sources of exogenous oestrogen.[1] Tamoxifen is used as a treatment for breast carcinoma as it has an anti-oestrogenic effect on the breast.[1] However, in contrast, tamoxifen has a pro-oestrogenic impact on the endometrium, causing abnormal endometrial proliferation.[1] Oestrogen therapy in the absence of progesterone, also causes abnormal endometrial proliferation.[1] Both give rise to endometrial hyperplasia and thus possible evolution to cancer.[1] Endogenous oestrogen sources include adipose tissue in obese females and ovarian pathology, such as certain ovarian neoplasms and polycystic ovarian syndrome.[1]

Type I tumours (oestrogen dependent) develop in relatively younger peri- or premenopausal women.[1,3,15,17] These tumours may be preceded by endometrial hyperplasia as a precursor lesion.[17] Type II tumours (non-oestrogen dependent) are also called non-endometrioid endometrial carcinoma (NEEC) and occur in older, non-obese, post-menopausal women.[1,3,15,17] The background non-neoplastic endometrium of Type II tumours tends to be atrophic.[17] As these tumours are not oestrogen-dependent, the tumour is thought to arise from intraepithelial carcinoma.[17] Type II tumours generally show a more aggressive clinical behaviour than Type I tumours.[7,15,17]

1.3.2 Histological classification

The World Health Organization (WHO) Blue book of Female Genital Tumours currently classifies endometrial carcinomas according to the light microscopic features, dividing them into endometrioid, serous, clear cell, undifferentiated/dedifferentiated, mixed and other.[3,7] A small subset of endometrial carcinomas shows mesenchymal differentiation, and these are termed carcinosarcomas.[3] Amongst newly diagnosed cases of endometrial cancer, the endometrioid subtype is the most common, accounting for approximately 85% of tumours.[3]

Endometrioid carcinoma derives its name from the fact that the neoplastic glands closely resemble normal proliferative endometrium.[3] Endometrioid carcinoma can show different histological patterns depending on the type of cellular differentiation present.[3] Typically, this subtype is characterized by neoplastic gland formation with a complex architecture showing branching and papillary structures.[3] A smooth luminal border is maintained.[3] The neoplastic cells lining the glands show pseudostratified, elongated to round nuclei with a variable degree of atypia.[3,7] Other histological patterns associated with endometrioid carcinoma, include squamous, mucinous, and secretory patterns.[3,7]

According to the percentage of solid growth present, the International Federation of Gynaecology and Obstetrics (FIGO) classifies endometrioid carcinoma into three grades.[2] Grade 1 tumours are composed predominantly of glands with 5% or less solid growth. Grade 2 tumours are composed of 6-50% solid areas. Grade 3 tumours also show more than 50% solid differentiation.[3,7]

1.3.3 Molecular classification

Following the pathogenetic and histological classification, endometrial carcinomas are also classified according to the molecular subtype.[3] A molecular classification provides better understanding of the different cancer pathways, is of prognostic value and contributes notably to the diagnosis and treatment of patients.[1,3] The Cancer Genome Atlas Research (TCGA) Network classified endometrial cancer into four groups according to their underlying molecular aberration: microsatellite instability (hypermutated), POLE-ultra mutated, copy-number high and copy-number low with TP53 mutations.[7,15,23]

POLE-mutated group of tumours are associated with mutations involving *KRAS*, *PTEN*, *PIK3CA*, *PIK2R1*, *ARID1A* and *FBXW*7 genes.[1] The *POLE* gene encodes a part of the DNA polymerase that has a proofreading function during DNA replication.[1] When a mutation occurs within the *POLE* gene, the proofreading function is lost with subsequent mutations during DNA replication, creating a mutator phenotype.[1] POLE-mutated group of tumours show a good prognosis.[1,7] Repeat areas of DNA amplifications or deletions characterize the copy number high

group.[1] The copy number high group of tumours are associated with a poor prognosis and include mutations within *PIK3CA*, *PPP2R1A* and *TP53*, and chromosomal instability.[1,7] The copy number low group of tumours includes all tumours that cannot be categorized as POLE-mutated, copy number high or microsatellite unstable groups, and are associated with an intermediate prognosis.[1]

After the POLE-mutated group, microsatellite instability is the second most common genetic abnormality associated with endometrial carcinoma.[1,3,15] Microsatellite instability is, however, the most common underlying molecular abnormality of endometrioid endometrial carcinomas.[1,3,15] The microsatellite instability (MSI) group are associated with mutations involving *KRAS*, *PTEN*, *PIK3R1*, *PIK3CA*, *ARID1A* and *RPL22*.[1] Microsatellite unstable tumours have shown an intermediate clinical prognosis.[7] MSI is present in up to 90% of Lynch syndrome associated endometrial carcinomas and in as many as 30% of sporadic endometrial carcinomas.[1,15] Sporadic endometrial carcinomas occur secondary to *MLH1* promoter methylation via epigenetic silencing.[3,24]

1.4 Lynch Syndrome and Endometrial Carcinoma:

Lynch syndrome, or Hereditary Nonpolyposis Colorectal Cancer (HNPCC), is an inherited, autosomal dominant syndrome with germline mutation of MMR genes.[19,25] Currently, the incidence of Lynch syndrome differs between populations, ranging from 0-10%.[24]

The genes usually affected are *MSH2, MSH6, PMS2* and *MLH1*.[26] Due to the MMR gene germline mutation, patients with Lynch syndrome show a predisposition to developing multiple cancers.[27] The likelihood of developing a certain type of tumour has been shown to be associated with the specific MMR gene involved, for example, *MSH6* germline mutations are mainly associated with endometrial carcinoma development.[26,27] Many tumours, depending on the specific MMR gene involved, are associated with Lynch syndrome.[27] These include colorectal, endometrial, gastric, pancreas, small bowel, upper urinary tract, sebaceous gland, brain and ovarian tumours.[25,28] Lynch syndrome patients are especially prone to develop colorectal and endometrial carcinoma.[27] The risk of Lynch syndrome patients developing colorectal carcinoma

is 80%, while the risk of developing endometrial carcinoma is 60%.[26] Endometrial carcinoma may be the first tumour identified in an individual and may be the indicator of undiagnosed Lynch syndrome.[26] In Lynch syndrome patients, the time between developing a second tumour after the diagnosis of endometrial carcinoma is on average, 11 years.[26]

Lynch syndrome-associated endometrial carcinomas are usually of endometrioid morphology.[3] Occasionally, this tumour predisposition syndrome is associated with non-endometrioid endometrial carcinomas, including clear cell carcinoma, carcinosarcoma and serous carcinoma.[1,26] The non-endometrioid endometrial carcinomas associated with Lynch syndrome tend to occur at a younger age.[26] Lynch syndrome-associated endometrial carcinomas are prone to arise in the lower uterine segment and are often difficult to differentiate from tumours of primary endocervical origin.[26] It is also well-established that these Lynch syndrome-associated endometrial carcinomas are challenging to subtype as they can have a mixed histological picture and show high-grade morphology.[26] However, some histological features may point towards an underlying microsatellite instability, for example, undifferentiated or dedifferentiated tumours and the identification of a prominent lymphocytic infiltrate within, and surrounding the tumour.[26]

1.5 Multiple Primary Malignant Tumours:

Billroth first reported multiple primary malignant tumours in 1889.[29] Multiple primary malignancies are defined as the development of more than one tumour, synchronous or metachronous, in the same individual fulfilling specific criteria delineated by Warren and Gates.[30,31] The definition, rules and criteria, and the perception of multiple primary malignancies vary and have changed over time.[30] Warren and Gates's criteria include the following: a malignancy must be confirmed histologically, and each tumour should be separate.[31] Multicentric and multifocal tumours, as well as metastases, should not be included as primary tumours.[30]

The two main guidelines currently used are the Surveillance Epidemiology and End Results (SEER) project and the International Association of Cancer Registries and the International Agency for Research on Cancer (IACR/IARC).[30] European cancer registries are more inclined to

use the IACR/IARC guidelines.[30] The SEER guidelines recommend using a two month cut off to differentiate between metachronous and synchronous tumours, whilst the IACR/IARC guidelines recommend that a tumour be registered as synchronous if it occurs within six months of the first tumour, and as metachronous, if it occurs after six months.[30]

Multiple primary tumour incidence differs in literature and is reported to range from 0.5% to 37%.[29,30,32] The risk of developing a second primary malignancy varies according to the site of the initial first malignancy.[30] For example, following the SEER guidelines, the risk of developing a second primary tumour in patients with colon cancer is 19.7%, and the risk in patients with lung cancer is 21%.[30] Second primary tumour development is the most common, whilst developing a third, fourth, or more tumours are reasonably uncommon.[9] Patients are currently surviving longer with primary tumours, providing enough time for such patients to develop second or multiple tumours and is thought to be the result of the significant improvement of cancer diagnostics, screening programmes and treatment modalities.[29,30]

Several lifestyle, host or environmental factors may contribute to an increased risk of developing a second primary tumour.[30] Lifestyle factors include alcohol and tobacco usage, and host factors include genetic predisposition such as tumour syndromes and family history.[30] Environmental factors include exposure to certain pathogens, particular occupations and specific geographical areas.[30] The frequency of multiple primary tumours differs amongst ethnic groups.[32] For example, in contrast with the United States of America (USA), Japan has a low incidence of prostate cancer and a high incidence of gastric cancer.[32]

The incidence of multiple primary tumours in patients specifically diagnosed with endometrial carcinoma is approximately 10% to 23%.[8,9] A large population-based study in the USA undertaken by Uccella *et al*, showed that the incidence of more than one primary tumour in patients with endometrial cancer is approximately 22%.[8] Another USA-based study by Buttin *et al*, assessing the incidence and the different types of synchronous and metachronous tumours in MSI unstable endometrial carcinomas specifically, showed that approximately 20% of microsatellite unstable endometrial carcinomas had associated primary tumours.[33] A study by

Rong *et al* reported that the median age of patients at the time of endometrial carcinoma diagnosis, was 50.3 years.[9]

Several types of cancer are associated with microsatellite instability, most notably colorectal, endometrial (more specifically endometrioid endometrial carcinoma) and gastric cancer.[1,14] Bonneville *et al* found microsatellite instability in 27 out of 39 cancer types and 3.8% of 11 139 cancers evaluated.[14] Cancer types not previously noted to be microsatellite unstable were adrenocortical carcinoma, mesothelioma and cervical cancer.[14] Other cancer types associated with microsatellite instability include breast carcinoma, melanoma, prostate adenocarcinoma, ovarian serous cystadenocarcinoma, head and neck squamous cell carcinoma, oesophageal, hepatocellular and lung carcinoma.[14] A few other cancer types such as uterine carcinosarcoma, thymoma, paediatric neuroblastoma, Wilms tumour, bladder carcinoma, cholangiocarcinoma, chronic lymphocytic leukaemia, glioblastoma, lower-grade gliomas and sarcomas, have shown an association with microsatellite instability.[14] The development of multiple tumours occurring in a patient with endometrial carcinoma, especially in the setting of Lynch syndrome, is well-established.[8,28] These include tumours from the colorectum, small bowel, ovary, pancreaticobiliary tract, stomach, urinary tract, skin and brain.[8,33]

Some studies have shown that the time between the development of the first and second tumour vary from approximately 4 to 10 years.[8,9,32] A previous case series from China reporting the clinicopathological features of multiple primary malignant tumours with endometrial carcinoma, found an interval of 51.27 months between the development of the first and second tumour.[9] Another study by Amer reported an average time to develop a second tumour after the first, was 117 months; but this study was not limited to patients with only endometrial carcinoma and associated tumours, as it included a wide range of tumour types.[32] Uccella *et al* found a median interval of 6 years between the development of endometrial carcinomas and breast carcinomas and a median of 4 years between the diagnosis of endometrial carcinomas and rectal carcinomas.[8]

To the best of our knowledge, a study documenting tumours associated with microsatellite unstable/mismatch repair-deficient endometrial carcinoma in South Africa or other African

countries has not been performed. The burden of these tumours on our population is currently unknown. Assessing tumours in our patient cohort that are associated with microsatellite unstable endometrial cancer and the incidence thereof may facilitate further studies into possible risk factors in our patient profile. Such information may also evaluate the feasibility and cost-effectiveness of the possible implementation of screening programs and germline testing in a developing country.

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2 – SUBMISSIBLE ARTICLE FORMAT

OBSERVATIONAL STUDY OF SYNCHRONOUS/METACHRONOUS TUMOURS IN MICROSATELLITE UNSTABLE/MISMATCH REPAIR DEFICIENT ENDOMETRIAL CARCINOMAS

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2.1 Abstract

Objective

Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma (EEC). Microsatellite unstable endometrial carcinomas may be associated with multiple primary malignancies. We aimed to document the incidence and type of associated tumours in MSI/mismatch repair deficient (MMR-d) endometrial carcinomas at a single institution in South Africa.

Methods

The study assessed EECs for the period 2009-2015 at a Johannesburg hospital and followed on previous research undertaken in our department in which 66/145 MSI/MMR-d EECs were identified using immunohistochemistry and polymerase chain reaction (PCR). After ethical clearance was granted, using the microsatellite unstable/MMR-d endometrial carcinoma case-specific laboratory reference numbers, a retrospective search and cross-reference for associated histologically proven tumours, was performed.

Results

Three patients (4.5%) out of 66 confirmed MSI/MMR-d endometrial carcinoma cases had associated tumours, including an invasive squamous cell carcinoma of the cervix and mucinous breast carcinoma. One patient had two tumours, namely uterine leiomyosarcoma and rectal

adenocarcinoma. The incidence of EEC patients with an associated tumour is significantly lower (p=0.0045) than that documented in the United States of America (USA).

Conclusion

Our study's incidence of associated tumours in MSI/MMR-d endometrial carcinoma patients was significantly lower than studies from the USA. The associated tumours in our study included traditional Lynch syndrome tumours such as colonic adenocarcinoma and demonstrated less commonly associated tumours, namely cervical carcinoma and uterine leiomyosarcoma. These findings highlight the need for further research in our population to assess risk factors and the true incidence of Lynch syndrome-associated tumours.

Keywords: Lynch syndrome, microsatellite instability, endometrial carcinoma, South Africa, metachronous tumours, synchronous tumours.

2.2 Introduction

According to the most recent GLOBOCAN 2020 database release, uterine carcinoma was the 16th most common cancer diagnosed worldwide.[1] In the USA, the American Cancer Society estimates a 7% incidence of uterine carcinoma in 2021.[2] In 2018 the incidence of uterine cancer was 3.2% in sub-Saharan Africa.[3] According to the latest (2016) published National Cancer Registry statistics in South Africa, cancer of the uterine corpus had a similar incidence of 3.77%.[4,5]

Multiple primary malignancies are defined as the development of more than one tumour, synchronous or metachronous, in the same individual fulfilling specific criteria delineated by Warren and Gates.[6,7] These criteria include: a malignancy must be confirmed histologically; and each tumour should be separate and not a metastasis from the other.[7] The International Association of Cancer Registries (IACR) / International Agency for Research on Cancer (IARC) guidelines recommend that a tumour is synchronous if occurring within six months after the first neoplasm, or is metachronous when occurring after six months.[6] The incidence and risk of patients with endometrial carcinoma developing other tumours is approximately 10% to 23%.[8,9]

With improved diagnostic and treatment modalities, patients may now survive longer, thus providing time for such patients to develop second or multiple tumours.[10]

A genomic analysis performed by The Cancer Genome Atlas Research Network (TCGA) classified endometrial cancers into four molecular groups: copy-number low, copy-number high, POLE ultramutated and microsatellite instability hypermutated.[11] Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma and is associated with inherited and sporadic endometrial carcinomas.[12,13] TCGA demonstrated that patients with POLE mutations have the best prognosis of the four categories, while those with MSI and copy-number low mutations have an intermediate prognosis and patients with p53 and copy-number high mutations have the poorest outcomes.[11,14] MSI is present in up to 90% of endometrial carcinomas associated with the heritable Lynch syndrome and in approximately 30% of sporadic endometrial carcinomas.[12] Sporadic endometrial carcinomas are secondary to *MLH1* promoter methylation via epigenetic silencing.[13,15] Lynch syndrome is an autosomal dominant syndrome with germline mutation of DNA mismatch repair (MMR) genes resulting in a predisposition to multiple cancers, especially colorectal and endometrial tumours.[16-18] Endometrial carcinoma may be the first tumour diagnosed and may suggest underlying Lynch syndrome.[19] Endometrial carcinoma is associated with multiple primary malignancies such as cancers of the breast, ovary and most commonly, colorectum.[8,9,13]

To the best of our knowledge, a study documenting tumours associated with microsatellite unstable endometrial carcinoma in South Africa has not been performed. The burden of these tumours on our population is currently unknown. We aimed to assess the incidence of tumours associated with MSI or MMR deficient, endometrial carcinomas at a single institution in South Africa.

2.3 Material and methods

This study follows previous research undertaken in our department of Anatomical Pathology, on endometrioid endometrial carcinomas (EECs).[20] As microsatellite instability is more frequently seen in association with endometrioid endometrial carcinomas than non-endometrioid subtypes, the study was limited to endometrioid endometrial carcinomas.[20] Previous research by Wadee and Grayson identified a total of 66 mismatch repair deficient and/or microsatellite unstable

EECs using immunohistochemistry and polymerase chain reaction (PCR) tests.[20] Wadee and Grayson considered a case to be mismatch repair deficient (MMR-d) if the tumour showed loss of nuclear staining of one or more of the MMR antibodies (MLH1, PMS2, MSH2 and MSH6).[20] A case was identified as microsatellite unstable by PCR if one or more markers showed a difference in the size of the alleles.[20] A case was classified as MSI-H (microsatellite instability-high) if two or more markers showed dissimilarity in allele size, MSI-L (microsatellite instability-low) if one marker showed difference in allele size, and microsatellite stable (MSS) if none of the markers showed a difference in allele size.[12] The PCR markers used were NR-21, NR-24, NR-27, BAT-25 and BAT-26.[20]

These 66 EECs also underwent *MLH1* promotor methylation analysis in the study by Wadee and Grayson.[20] Of the 66 endometrial carcinomas, 44 were methylated, 17 were not methylated and one did not have enough DNA to be tested. Four cases were not tested for *MLH1* promotor methylation as they did not show loss of MLH1 immunohistochemical staining.[20] (Figure 1)

The EEC cases from 2009-2015 were from the Department of Anatomical Pathology, University of the Witwatersrand/National Health Laboratory Services (NHLS) at Charlotte Maxeke Johannesburg Academic Hospital which provides a pathology service to various hospitals and clinics in the southern Gauteng region.

After ethical clearance (certificate number: M190939) was granted, information was obtained from the LabTrack and DISA database of the NHLS using the microsatellite unstable/mismatch repair-deficient proven endometrial carcinoma case-specific laboratory reference numbers. Information obtained from these reports was used to cross-reference other associated histologically proven primary tumours both before and after the endometrial carcinoma diagnosis. The following information was used to perform a retrospective search and cross-reference for associated histologically proven tumours: hospital number, name and surname, date of birth, laboratory case number and episode number.

When an associated tumour was identified, the pathology report was used to ascertain the type of tumour, site of the associated tumour and patient age at diagnosis of the associated tumour. The date of authorization or sign-out was used as the tumour diagnosis date to establish consistency as the procedure date is often not stated on the clinical request form. No personal identifiers were documented on the data collection sheet, thus ensuring patient anonymity. The slides of the

identified associated tumours were retrieved, reviewed, and the diagnoses confirmed. Multicentric and metastatic tumours were excluded as per criteria delineated by Warren and Gates.[6,7]

To assess the difference in means of both the average age of patients with and without associated tumours and the average age of patients with methylated and unmethylated endometrial carcinomas, a two-tailed t-test was used. To determine any statistically significant differences between the proportion of associated tumours of our study and a previous study, a two-tailed Z-proportion test was used. A significance level of 5% was used.

In accordance with the journal's guidelines, we will provide our data for the reproducibility of this study in other centers if such is required.

2.4 Results

Three patients (4.5%) out of 66 confirmed microsatellite unstable/mismatch repair-deficient endometrioid endometrial carcinoma cases, had associated tumours. These tumours included an invasive, moderately to poorly differentiated, squamous cell carcinoma of the uterine cervix, uterine leiomyosarcoma, a mucin-producing rectal adenocarcinoma and a mucinous carcinoma of the breast. (Figure 2) One patient had two associated tumours, namely uterine leiomyosarcoma and rectal adenocarcinoma. Table I shows that of the three endometrial carcinoma patients who had associated tumours, one patient's tumour showed retention of all MMR proteins but had microsatellite instability in one of 5 PCR markers, while one patient's tumour showed retention of all MMR proteins but had microsatellite instability in two of the 5 PCR markers; whereas the third patient's tumour had loss of both MLH1 and PMS2 immunohistochemical markers and had microsatellite instability in one of the 5 PCR markers used.[20] Thus, only one patient had an MSI-High EEC while 2 patients had MSI-Low tumours, which are considered more similar to the MSS status than to the MSI-high status.

One patient developed a metachronous cervical squamous cell carcinoma, which was diagnosed on a cervical punch biopsy 12 months after the patient underwent a subtotal hysterectomy for endometrial carcinoma. The exact reason for a subtotal hysterectomy having been performed was not stated. The surgery was conducted by doctors who were not gynaecologic oncologists, as this was undertaken at a small district hospital. At the time of diagnosis of another patient's

endometrial carcinoma, an associated (synchronous) uterine leiomyosarcoma was simultaneously diagnosed. This patient was also diagnosed with a metachronous rectal adenocarcinoma 9 months after the diagnosis of EEC. The third patient in our cohort who had developed an associated tumour, was diagnosed with a synchronous mucinous breast carcinoma 1 month after her diagnosis of EEC. (Table I)

Table II shows that the average time between the diagnosis of endometrial carcinoma and other tumours was 7.33 months, with a median of 9 months. There was no significant statistical difference between the average age of those with, and those without a second/associated tumour. (p=0.244). The average age of patients without an associated tumour, at the time of diagnosis of their endometrial carcinoma was 66 years, while patients with endometrial carcinomas who developed associated tumours had an average age of 59 years at the time of their endometrial carcinoma diagnosis.

Table III shows that the average age of patients with methylated endometrial carcinomas, but no associated tumour (n=43) was 65 years, while a single patient who had a methylated EEC and an associated tumour was 55 years of age when the endometrial carcinoma was diagnosed. Patients who had unmethylated endometrial carcinomas and no associated tumours (n=16), had an average age of 67 years, whilst a single patient with an associated tumour and an unmethylated EEC was diagnosed with the EEC at 70 years of age. There was no significant statistical difference between the average age of those with methylated endometrial carcinomas and the average age of those with unmethylated tumours (p=0.331). Of the three endometrial carcinoma patients who had associated primary tumours, one patient had *MLH1* promotor methylation of the endometrial tumour while one patient's EEC was unmethylated. The third patient's tumour, unfortunately, did not have enough DNA available to undergo methylation analysis.

Based on earlier research performed in our department by Wadee and Grayson, 41 out of 145 cases had mismatch repair protein deficiency and 46 out of 145, had microsatellite instability detected by PCR.[20] There was MMR-d/MSI discordance identified in 25 cases.[20] Of the 41 cases shown to have MMR protein deficiency, 20 cases showed both MLH1 and PMS2 loss, 2 cases had both MSH2 and MSH6 deficiency, 16 cases demonstrated isolated MLH1 loss, 2 had isolated MSH2 deficiency and a single case showed loss of 3 markers (MLH1, MSH6 and PMS2).[20]

We compared our current results in addition to results from the previous study in our department by Wadee and Grayson, to a study by Buttin *et al*.[20,21] We found a statistically significant (p=0.0045) difference in the incidence of associated tumours between the present study and that of Buttin *et al*.[21] (Table IV). Furthermore, Table IV shows a statistically significant difference (p=0.0000) in our patient cohort when comparing the proportion of *MLH1* methylated endometrial carcinoma cases to *MLH1* unmethylated cases. There was no significant statistical difference between the proportion of methylated and unmethylated cases in our patient cohort compared to Buttin *et al*.[21]

Table V shows additional clinical and pathological details of one of the patients who had undergone an excision of an associated tumour. Unfortunately, the resection specimens for the other two patients could not be traced despite an extensive search on our laboratory computer database. This may be as a result of the patients not having undergone surgical excisions at our hospital complex or could be due to patients seeking medical attention at other hospitals which we do not provide a pathology service to, or having been lost to follow-up, or the patients may have died of their disease.

2.5 Discussion

Our present study showed a statistically lower incidence of associated tumours when compared to a study from a western country.[21] We also demonstrated associated tumours that are different from those traditionally associated with Lynch syndrome.

Of the confirmed microsatellite unstable or mismatch repair-deficient endometrial carcinoma patients in our study, 3 cases (4.5%) had associated tumours. These associated tumours included the well-documented colorectal carcinoma and tumours less commonly associated with Lynch syndrome, including mucinous breast carcinoma, uterine leiomyosarcoma and cervical squamous cell carcinoma.[19] The rectal adenocarcinoma and cervical squamous cell carcinoma both occurred metachronously. Synchronous tumours included mucinous breast carcinoma and uterine leiomyosarcoma, the latter having been diagnosed at the same time as the endometrial carcinoma on the excision hysterectomy specimen.

The incidence of associated primary tumours in patients with endometrial carcinoma differs in the literature. In the USA, early research from the 1960's and 1980's showed an incidence of

approximately 10%, whilst more recent studies, including that of Uccella *et al*, and Buttin *et al* show an incidence of 20-22%.[8,21] The initial studies were smaller in number and hospital-based compared to recent studies that included a larger number of patients, regions and better follow-up of patients.[8] Uccella *et* al assessed the clinicopathological features of patients (n=1028) with endometrial carcinoma, which included endometrioid and non-endometrioid subtypes, in addition to the associated tumours they developed; and evaluated the risk of patients developing an associated tumour.[8] The study by Buttin *et al*, assessed the incidence and the different types of synchronous and metachronous tumours in MSI unstable endometrial carcinoma patients (n=94) which also included endometrioid and non-endometrioid subtypes.[21]

Our study showed an incidence of 4.5%, which is far lower than that of the American studies (20-22%).[8,21] Our study and the study by Buttin *et al* assessed the incidence and types of associated tumours of microsatellite unstable endometrial carcinomas.*[21]* A comparison of the incidence of associated tumours between our study (4.5%) and that of Buttin *et al* (20.2%) showed a statistically significant difference in proportions. (Table IV)

Whilst our sample size was small, both polymerase chain reaction (PCR) and immunohistochemistry were conducted during the previous study in our department by Wadee and Grayson, to detect microsatellite unstable/MMR deficient endometrial carcinoma cases, thus ensuring a more accurate identification of cases.[20] A study by McConechy *et al* showed a high level of concordance (93.3%) between MMR protein immunohistochemistry and MSI PCR analysis, and as such, IHC may be used instead of PCR.[22] However, a recent publication by Wadee and Grayson, showed that 5% of microsatellite unstable endometrial carcinoma cases would not have been identified if PCR was not used, as these cases did not show loss of immunohistochemical staining.[20] Similarly, Buttin *et al* also used both PCR and immunohistochemistry.[21]

The low incidence in our study (4.5%) compared with American studies may be explained by the smaller study number (66 patients) and that our cohort was limited to microsatellite unstable/MMR-d, *endometrioid* endometrial carcinomas, which contrasts to both that of Uccella *et al* and Buttin *et al.*[8,21]

Other aspects that may have limited our patient sample size include the possibility of patients having been lost to follow up or having been registered on the laboratory system with different

names and surnames which would not have been traceable on our laboratory computer database. In our setting of a developing country, loss to follow up of patients is a common occurrence as we provide a service to state/public sector patients. The patients may not have easy access to transportation to get to hospitals, or the financial means to use transport or may not be able to take time off from work to come to the hospital. In addition, patients may decide to access health care facilities closer to their homes which may be in areas that we do not provide a pathology service to. Furthermore, the possibility of patients having died exists, and such knowledge may not be conveyed to the patients' treating physicians and this may then not be communicated to pathologists.

Buttin *et al* further subdivided the MSI positive group into *MLH1* methylated and *MLH1* unmethylated, categorizing tumours into sporadic and possible germline tumours respectively.[21] Comparisons between MLH1 methylated and unmethylated cases in the studies by Wadee and Grayson and Buttin *et al* showed no statistically significant differences (p=0.7991) (Table IV).[20,21]

The statistically significant difference in proportions when comparing the *MLH1* methylated cases with *MLH1* unmethylated cases in our present study suggests that the proportion of possible germline tumours is significantly lower than the proportion of sporadic tumours in our cohort. Germline mutations in MMR genes have been documented in 2-6% of endometrial carcinomas.[19] Currently, the incidence of Lynch syndrome differs between populations, ranging from 0-10%.[15] In South Africa, Wadee and Grayson's study showed that 6.9% of patients with EEC may have underlying Lynch syndrome, which is slightly higher than the expected 2-6%.[15] Unfortunately, confirmatory germline testing is currently not available in the public sector of Gauteng, South Africa.[15] Another South African study by Vergouwe *et al* showed that in contrast to Western studies, there is a higher rate of MMR deficiency in colorectal carcinoma suggesting an increased proportion of inherited disease in regions of South Africa where the incidence of colorectal carcinoma is relatively low.[23] As such, the incidence of possible Lynch syndrome associated tumours in South Africa was expected to be higher than the 4.5% identified in the present study.

The development of multiple tumours occurring in a patient with endometrial carcinoma, especially in the setting of Lynch syndrome, is well-established.[8,24] These include tumours from

the colorectum, ovary, small bowel, stomach, pancreaticobiliary tract, skin, brain and the urinary tract.[17,24,25] Endometrial and colorectal carcinomas are the most commonly described Lynch syndrome-associated tumours and the association between the two carcinomas is well documented.[17,21] Only one patient in our study had an associated colorectal carcinoma. Although gastrointestinal tumours were the first neoplasms described in Lynch syndrome, endometrial carcinoma is the sentinel tumour in up to 50% of patients.[19,24]

One patient with endometrial carcinoma in our study had an associated mucinous breast carcinoma. The incidence of Lynch syndrome-associated breast carcinomas is marginally increasing, but the association between breast carcinoma and Lynch syndrome varies in the literature and is controversial.[17,26] Stoll *et al.* showed that women with Lynch syndrome do not have an increased risk of breast cancer compared to the general population, while Win *et al.* showed that patients with MMR mutations were at a risk of developing breast carcinoma that was at least four times greater than that of the general population.[26,27]

Furthermore, we identified one patient who had endometrial carcinoma and an associated uterine leiomyosarcoma. Generally, sarcomas are not commonly associated with Lynch syndrome. Sporadic sarcomas are rarely associated with microsatellite instability. However, a Brazilian study recently indicated that sarcomas, although rare, are linked to the Lynch syndrome spectrum of tumours.[28,29] An increased lifetime incidence is reported; but the exact risk for sarcoma development has not been fully elucidated.[28]

We identified one patient with an endometrial carcinoma who had an associated cervical squamous cell carcinoma, which is not considered a traditional Lynch syndrome-associated tumour.[30] However, several case studies have documented cervical carcinoma in association with germline mutations of the MMR genes.[30] Antill *et al* indicated that cervical carcinoma, including squamous cell carcinoma, could be considered part of the extracolonic manifestations of Lynch syndrome.[30] Antill *et al* state that it is plausible that the tumour pathway of cervical carcinoma in MMR gene mutation patients may be secondary to the known carcinogenic effect of human papillomavirus (HPV) exposure, but microsatellite instability should be investigated as a possible underlying molecular pathway.[30] In our study, microsatellite instability as an underlying molecular pathway has been confirmed for the endometrial carcinoma. While it is possible that this is the underlying pathway of the associated cervical carcinoma, such testing was not

performed on any of the associated tumours, including the cervical tumour, as this was a retrospective, observational study and we did not have ethical clearance to perform any new additional studies/investigations.

The average time to development of an associated tumour was 7.33 months. A previous case series from China reporting the clinicopathological features of multiple primary malignant tumours with endometrial carcinoma, found an interval of 51.27 months between the development of the first and second tumour.[9] Another study by Amer reported an average time to develop a second tumour after the first, was 117 months; but this study was not limited to patients with only endometrial carcinoma and associated tumours as it included a wide range of tumour types.[31] Only one month passed between the development of the breast carcinoma and the endometrial carcinoma in our study, compared to Uccella *et al's* median of 6 years.[8] In addition, the diagnosis of the rectal adenocarcinoma in our study was made 9 months after that of the endometrial carcinoma, compared to Uccella *et al's* median time interval of 4 years between diagnosis of the two tumours.[8] It is clear that the time interval between the diagnosis of an associated tumour and the primary tumour in our study is far less than those of other studies. The reason for this is unknown and although our cohort is too small to draw substantial conclusions, further studies investigating possible risk factors contributing to the reduction of the time interval between multiple tumour diagnoses are needed. Furthermore, this finding may suggest that closer follow up of patients with MSI unstable tumours is indicated.

Only 1 patient had an MSI-High EEC while 2 patients had MSI-Low tumours, which have been considered more akin to the MSS status than to the MSI-high status.[22] Methylated tumours point toward a sporadic occurrence whereas unmethylated tumours that have MMR-d may indicate an underlying germline mutation.[20] Hereditary tumours tend to present at a younger age.[32] Our study, however, illustrated that the patient with a *methylated* EEC and synchronous breast carcinoma was 55 years of age at the time of her EEC diagnosis, whereas the patient with an *unmethylated* EEC and metachronous cervical carcinoma was 70 years of age at the time of EEC diagnosis. As such, despite the patient's age (70 years) the occurrence of an *unmethylated* tumour and a metachronous tumour may still suggest an underlying germline mutation and would warrant further investigation. The third patient in our cohort who had EEC and associated neoplasms had a total of 3 malignancies (metachronous and synchronous). Despite there not

being sufficient DNA to perform methylation assessment, the occurrence of 3 neoplasms in this patient, of which one neoplasm was an MSI-high endometrial tumour, suggests that further investigations for possible underlying Lynch syndrome are also warranted.

The incidence of Lynch syndrome in the rest of Africa is not well recorded.[33] A 2019 population-based study from Zimbabwe was the first to study hereditary colorectal carcinomas extensively and found a 3.3% prevalence of Lynch syndrome.[34] A study in Libya assessing the incidence of synchronous and metachronous tumours, reported an incidence of 1.14%, with breast carcinoma being both the most common primary, as well as the second identifiable tumour.[35] To the best of our knowledge no other study from the African continent has investigated the presence or absence of tumours associated with *microsatellite unstable/MMR-d endometrial carcinoma*s.

A Zambian case series reported HIV as a risk factor for the development of multiple primary malignancies.[36] South Africa currently has the highest number of people living with HIV in the world.[37] The influence that HIV has on Lynch syndrome-associated tumours is not well known. Microsatellite alteration has been described in AIDS-associated and non-AIDS associated tumours, for example, lung carcinoma, as microsatellite alterations are present at a higher frequency in HIV associated lung carcinomas compared to sporadic lung carcinomas.[38] A South African study assessing cervical biopsies showed that HPV alone may induce MSI; however, co-infection with HIV may exacerbate disease progression.[39] Our study did not assess microsatellite instability/MMR-d in the associated tumours and did not document the HIV status (as this is not always provided with the patient's clinical information), which is a limitation of our study as the role of HIV in the development of multiple tumours is currently unknown.

2.6 Conclusion

To the best of our knowledge, our study is the first to document the incidence and type of associated tumours in *microsatellite unstable/mismatch repair deficient (MMR-d)* endometrial carcinomas in Africa. The results from our study showed a statistically lower incidence of associated tumours when compared to a western country. We have demonstrated associated tumours that are different from those traditionally associated with Lynch syndrome. The precise reasons for this are currently unknown. Further studies, including assessing the role of HIV, the

incidence of multiple primary tumours and identifying possible risk factors in our population, are needed. Such information may also evaluate the feasibility and cost-effectiveness of the possible implementation of screening programs and germline testing in a developing country. In addition, this study will contribute to global data and provide a greater knowledge base from which gynaecologic oncologists, pathologists, medical oncologists, and radiation oncologists may draw information, with the ultimate aim of optimal patient care.

2.7 Ethics approval and consent to participate

Ethical clearance (certificate number M190939) was obtained through the Human Research Ethics Committee (Medical) of the University of Witwatersrand. There is currently blanket ethics approval (M150884) for use of archived departmental material. Informed consent is not required as this is a retrospective study with no additional cost to the patient or hospital. Dr Wadee had obtained ethical clearance for use of patient tissue samples (M151051) for research towards a PhD.

2.8 Author contributions

SZ contributed to the conception, design, drafting of the manuscript and critically reviewed the manuscript for relevant intellectual content. RW contributed to the conception and the supervision of the study and reviewed the intellectual content of the write-up. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

2.9 Conflict of interest:

The authors declare no competing interests.

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Tables:

Table I: Clinicopathological characteristics of patients with associated synchronous or metachronous tumours

Patient's study assigned case number	**Age of patient at diagnosis of endometrial carcinoma (years)**	**Age of patient at diagnosis of associated tumour (years)**	**Time between diagnosis of endometrial carcinoma and associated tumour (months)**	**Histopathological type of synchronous or metachronous tumour**	***MLH1* methylation status of endometrioid endometrial carcinoma**	**MMR protein expression and MSI status of endometrioid endometrial carcinoma**
11	70	71	12	Invasive, moderately to poorly differentiated squamous cell carcinoma of the cervix **(metachronous tumour)**	Unmethylated	All 4 MMR markers retained MSI-Low for 1/5 PCR markers
22	53	54 53	9 0	Rectal adenocarcinoma **(metachronous tumour)** Uterine leiomyosarcoma **(synchronous tumour)**	Not enough DNA to be tested	All 4 MMR markers retained MSI-High for 2/5 PCR markers
50	55	55	1	Mucinous breast carcinoma **(synchronous tumour)**	Hyper-methylated	Mismatch repair deficient for both MLH1 and PMS2 MSI-Low for 1/5 PCR markers

Table II: Age of patients with endometrial carcinoma and metachronous or synchronous tumours

	Metachronous or synchronous tumours		**p-value**
	Identified (n=3/66)	Not identified (n=63/66)	
Age at diagnosis of endometrial cancer (years)			
Mean ±SD	59.33 (±9.292)	65.75 (±9.225)	0.244
Time to development of metachronous or synchronous tumour (months)			
Mean ±SD	7.33 (±5.686)		
Median (IQR)	9 (1-12)		

Table III: Age and methylation status of endometrial carcinomas in our study.

Factor	Associated tumour		No associated tumour		Total	%	p-value
	n	%	n	%	n	%	
	n=3	4.55%	n=63	95.45%	n=66	100	
Methylated endometrial carcinomas.	1	33.33	43	68.25	44	66.67	
Age at diagnosis of endometrial carcinoma (years) Mean ±SD	55		65.23 (±9.486)				0.331
Median			63				
Unmethylated endometrial carcinomas	1	33.33	16	25.40	17	25.76	
Age at diagnosis of endometrial carcinoma (years) Mean ±SD	70		67.44 (±8.794)				
Median			67				
Cases which did not undergo methylation assessment.	1	33.33	4	6.35	5	7.58	
Reason	Insufficient DNA		Not tested due to intact MLH1 immunohistochemistry				

Table IV: Comparison between studies undertaken at our institution and those of Buttin *et al* [20,21] .

Parameter	**Data sources**	**Number of cases n (%)**	**p-value**
Incidence of associated tumours	Present study Buttin *et al* [21]	3 (4.5) 19 (20.2)	0.0045
Number of *MLH1* methylated cases	Previous study from our department (Wadee and Grayson [20]) Buttin *et al* [21]	44 (72.1) 66 (70.2)	0.7991
Number of *MLH1* unmethylated cases	Previous study from our department (Wadee and Grayson [20]) Buttin *et al* [21]	17 (27.9) 28 (29.8)	0.7991
Present study's cases	*MLH1* methylated cases (Present study) *MLH1* unmethylated cases (Present study)	44 (72.1) 17 (27.9)	0.0000

Table V: Clinical and pathological details of the associated tumour resection specimen.

Parameter	**Leiomyosarcoma/Endometrial carcinoma resection specimen**
Age (years)	53
Tumour size (mm): Leiomyosarcoma Endometrial carcinoma	25x22x19 55x24x10
Lymph node involvement	No lymph nodes submitted for pathological examination
Pathological TNM stage	pT1a Nx Mx, Stage IA

3. APPENDICES

3.1 Figure 1: Flow chart illustrating the number of cases that underwent MSI/MMR-d and *MLH1* promotor methylation analysis.

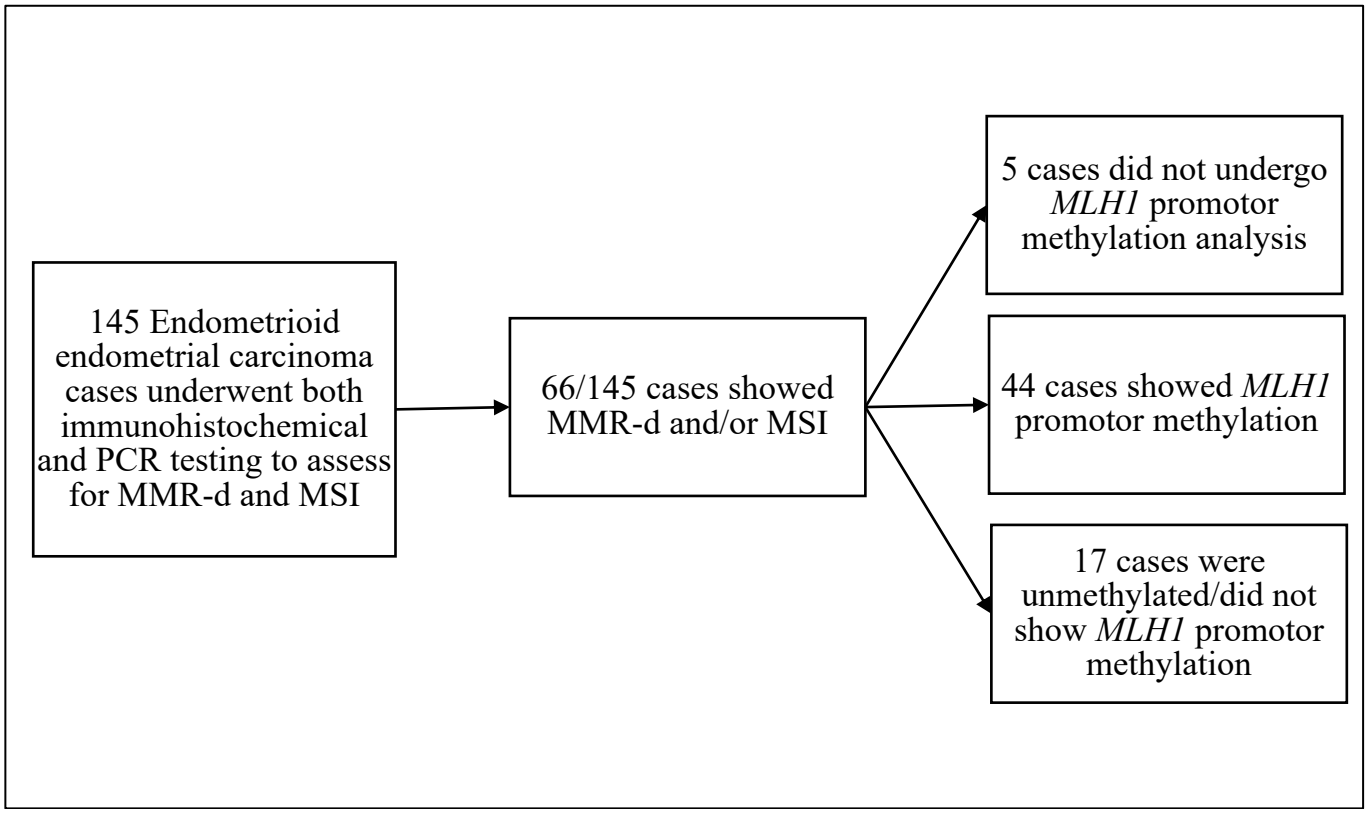


Figure 1: Flow chart illustrating the number of cases that underwent MSI/MMR-d and *MLH1* promotor methylation analysis.[20]

3.2 Figure 2: Various images demonstrating the associated tumours and endometrial carcinoma with relevant immunohistochemical stains.

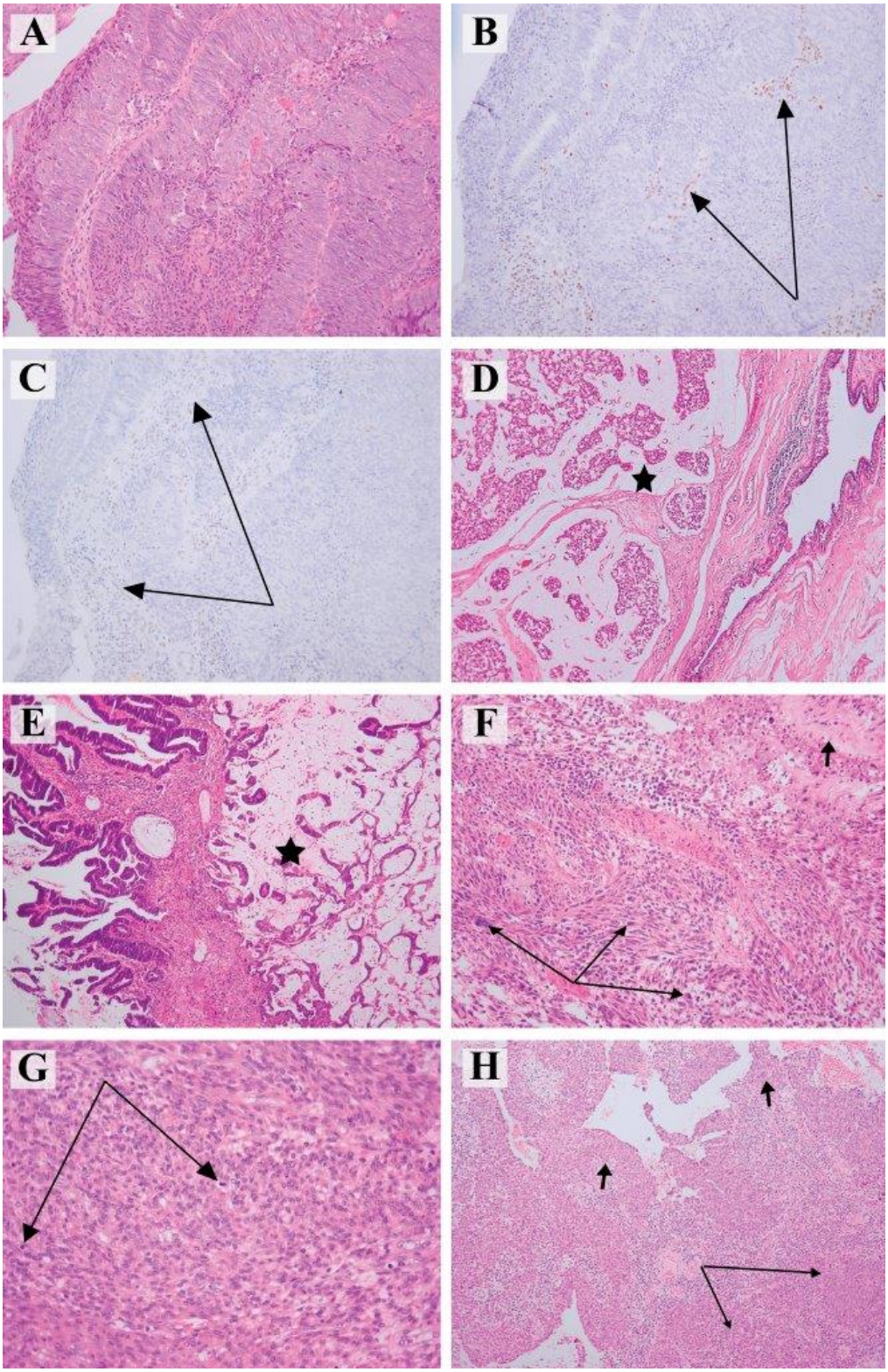


Figure 2: 2A demonstrates a Haematoxylin and Eosin stained endometrioid endometrial carcinoma. 2B shows an MLH1 immunohistochemical stain on the endometrioid endometrial carcinoma, in which lymphocytes and stromal cells have retained staining (two long thin arrows) while the tumour cells show loss of staining. 2C demonstrates loss of PMS2 staining in tumour cells from the endometrial carcinoma with retention of staining of stromal cells and lymphocytes (two long thin arrows). 2D demonstrates a mucinous breast carcinoma with tumour cells in pools of mucin (star). 2E shows the rectal adenocarcinoma with mucin seen at the star. 2F and 2G show the uterine leiomyosarcoma. An area of necrosis is identified (block arrow) and marked cellular atypia is shown (three long thin arrows) in 2F. Two mitotic figures are seen (two long thin arrows) in 2G. 2H demonstrates an invasive moderately to poorly differentiated squamous cell carcinoma of the cervix. Surface dysplasia is noted (block arrows) and islands of tumour cells within the stroma are seen (two long, thin arrows). Original magnification: A, B, C, F x200, D, E, H x100 and G x400.

3.3 Research Protocol

AN OBSERVATIONAL STUDY OF SYNCHRONOUS/METACHRONOUS TUMOURS IN MICROSATELLITE UNSTABLE ENDOMETRIAL CARCINOMAS (2009-2015)

STUDENT

Dr Suzanne Kotze

MBBCh (UFS)

Student number: 1445177

In partial fulfillment of the requirements of the degree, Master of Medicine (MMed), Anatomical Pathology

Registrar

Department of Anatomical Pathology

Faculty of Health Sciences

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National Health Laboratory Services (NHLS)

SUPERVISOR

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1. INTRODUCTION AND BACKGROUND

Microsatellites or short tandem repeats (STR), are defined as small sections of DNA, approximately 10 to 60 base pairs in length, that contain repeat sequences of ≥1-6 base pairs.(1,2) Microsatellites are present widely in the human genome, in coding as well as non-coding areas.(2,3) The length of these microsatellites are usually constant in individuals.(4) Microsatellites are especially prone to DNA replication errors during the cell cycle.(5) However, these replication errors are usually repaired by one of the DNA repair mechanisms of the body, known as the mismatch repair (MMR) system.(2) The MMR system repairs "base-base mismatches" and "insertion-deletion" loops.(6) There are several genes that encode a group of proteins that form part of the MMR system, including mutL homologue 1 (MLH1), mutL homologue 3 (MLH3), mutS homologue 2 (MSH2), mutS homologue 3 (MSH3), mutS homologue 6 (MSH6) and postmeiotic segregation increase 1 (PMS1) and postmeiotic segregation increase 2 (PMS2).(1,6) The MMR system has both major and minor genes, depending on their function.(6) The major genes are MLH1 and MSH2 and these genes are responsible for protein expression.(6) The minor genes are MSH3, MSH6 and PMS2, which support the major genes in their function.(6) Both the major and minor genes are required for effective repair of genetic errors.(6) These genes with their corresponding proteins have a "proof-reading" function ensuring DNA integrity and accurate replication before cell division.(4)

Replication errors arise when there is loss of function of one or more members of the MMR system.(1) The loss of function may be secondary to a genetic mutation, which may be inherited or may occur sporadically; or may be due to epigenetic hypermethylation of the MLH1 gene.(5) The most commonly affected gene is MLH1.(1) Loss of function of mismatch repair genes by themselves are not oncogenic however, their loss does lead to the

accumulation of mutations or errors in other genes, including microsatellites during the normal cell cycle, resulting in a strong mutator phenotype.(4,7) These mutations can, by chance, inactivate tumour suppressor genes or activate proto-oncogenes.(4) When affected cells gain these driver mutations (oncogenes or inactivated tumour suppressor genes), this may result in cancer development.(4) As mentioned above, microsatellite lengths in normal individuals, remain the same.(4) Thus, even if an error occurs during replication affecting the microsatellite number, and therefore the length, the MMR system repairs this aberration, and the microsatellite number generally returns to normal.(3) However, when there is a mismatch repair gene mutation, leading to a dysfunctional MMR mechanism and an error occurs within a microsatellite, the error will not be repaired and the number; and subsequently the length of microsatellites will be affected.(3) The microsatellites are then considered to be genetically unstable and result in either longer or shorter microsatellite lengths.(4) These errors when occurring in coding regions cause a frameshift mutation in important genes involved in cell growth and cancer-related genes.(7) This genetic instability, contributing to cancer development, is termed microsatellite instability.(4,7)

Lynch syndrome is an inherited (germline) mutation of MMR genes and is also known as hereditary nonpolyposis colorectal cancer (HNPCC).(7) Due to the germline mutation of the MMR genes, patients with Lynch syndrome show a predisposition to development of multiple cancers.(8) Many tumours, depending on the specific MMR gene involved, are associated with Lynch syndrome, for example, gastric, brain and ovarian tumours. However, these patients are especially at risk for development of colorectal and endometrial carcinoma.(8) Ninety percent of Lynch syndrome associated endometrial carcinomas show microsatellite instability.(6,13)

In South Africa, uterine malignancies are the 5th most common cancer diagnosed in females, according to the National Cancer Registry report of 2014.(9) Uterine malignancies are only surpassed in number by carcinomas of the breast, cervix, colorectum and carcinomas of unknown primary.(9) In South Africa, a total number of 1256 cases of uterine cancer has been reported in 2014.(9) As of 2014 uterine cancer made up 3.32% of all the cancers diagnosed in South African women.(9) In comparison, in 2019, the estimated percentage of new cases of uterine cancer in the United States of America is 7%.(10) Worldwide, according to the GLOBOCAN September 2018 database release, uterine cancer was listed as the 16th most common cancer diagnosed.(11) Endometrial carcinoma however, is the most common cancer of the female genital tract in the Western world according to the American Cancer Society.(12) Endometrial carcinoma has several subtypes histologically, of which endometrioid adenocarcinoma is the most common.(3) Traditionally, endometrial carcinoma has been classified into type 1 and 2 tumours. Type 1 endometrial carcinoma is usually seen in women who are exposed to unopposed estrogen, the patients are relatively younger peri- or premenopausal women and the tumours are well-differentiated, resembling normal endometrium.(3,12) Type 2 endometrial carcinoma occurs in older, post-menopausal women, is not associated with estrogen exposure and is non-endometrioid. Type 1 endometrial carcinoma generally has a better prognosis.(3,12)

More recently, The Cancer Genome Atlas Research (TCGA) Network classified endometrial cancer into 4 types according to the underlying molecular aberration: POLE ultramutated, MSI hypermutated, copy-number low and copy-number high(3). MSI hypermutated is, after POLE ultramutated, the second most common gene mutation in endometrial carcinoma.(3) Mismatch repair function is lost in approximately 20-30% of endometrial cancers and of these, Lynch syndrome constitute about 25%. The remaining cases are secondary to MLH1

promotor hypermethylation or somatic mutation of the relevant mismatch repair genes.(3) The latter both lead to sporadic cancers, with no inherited component.(3)

The first case of a single individual developing more than one primary tumour was described in the 19th century.(13) The incidence and risk of developing a second tumour or more, either synchronous (occurring at the same time, the Surveillance Epidemiology and End results programme definition is within two months)(14) or metachronous (occurring at a different time, more than six months apart)(14), in patients diagnosed with endometrial carcinoma has been reported, at a rate of approximately 10%.(13) However, these studies are noted to be limited by a small study group or clinical history details.(13) A large population-based study undertaken by Uccella *et al* (13), showed that the incidence of more than one primary tumour in patients with endometrial cancer, is approximately 22%.(13) Endometrial cancers are mainly associated with cancers of the breast, ovary and colorectum.(13)

Several types of cancer are associated with microsatellite instability, most notably colorectal, endometrial (more specifically endometrioid endometrial carcinoma) and gastric cancer.(2,6) Bonneville *et al* (2) assessed the landscape of microsatellite instability across 39 cancer types and found microsatellite instability present in 27 cancer types and in 3.8% of all cancers evaluated.(2) Cancer types not previously noted to be microsatellite unstable were adrenocortical carcinoma, mesothelioma and cervical cancer.(2) Other cancer types associated with microsatellite instability include breast carcinoma, melanoma, prostate adenocarcinoma, ovarian serous cystadenocarcinoma, head and neck squamous cell carcinoma, lung, hepatocellular and oesophageal carcinoma.(2) A few other cancer types such as uterine carcinosarcoma, thymoma, paediatric neuroblastoma, Wilms tumor, bladder

carcinoma, cholangiocarcinoma, chronic lymphocytic leukemia, glioblastoma, lower-grade gliomas and sarcomas have shown an association with microsatellite instability.(2)

To the best of our knowledge, a South African study assessing metachronous and synchronous tumours associated with endometrial carcinoma, specifically microsatellite unstable endometrial carcinomas, has not yet been undertaken. As shown by Bonneville *et al*, a large number of tumours are associated with microsatellite instability (2) and as stated by Kunitomi *et al*(3) several tumours are associated with endometrial carcinoma.(3) Microsatellite unstable tumours not only include Lynch syndrome associated tumours but also sporadic tumours caused by MLH1 promotor hypermethylation.(3) Thus, by assessing tumours associated with microsatellite unstable endometrial carcinomas, these will include inherited as well as sporadic forms of neoplasia.

Assessing tumours in our patient cohort that are associated with microsatellite unstable endometrial cancer and the incidence thereof may facilitate further studies into possible risk factors in our patient profile. The statistics obtained will also assist in guiding the development of screening programs, especially with regards to the cost effectiveness of such programs in a resource limited setting.

2. AIM AND OBJECTIVES

AIM:

To identify possible synchronous and metachronous tumours in patients with microsatellite unstable endometrial carcinomas.

OBJECTIVES:

- To document the different histologically proven metachronous or synchronous tumours, identified in patients with microsatellite unstable endometrial carcinoma patients within the period 2009 to 2015, at Charlotte Maxeke Academic Johannesburg Hospital, as obtained from the NHLS database.
- To ascertain the age at which the different tumours were diagnosed and the time period that has lapsed between the diagnosis of the different tumours.
- To compare the findings of this study with the available data of other studies with a larger population or study sample.
- To assess the demographics of patients diagnosed with such tumours.

3. MATERIAL AND METHODS:

3.1 STUDY DESIGN

This study will be a cross-sectional, retrospective study.

3.2. THE SITE OF THE STUDY

The Department of Anatomical Pathology, University of the Witwatersrand/ National Health Laboratory Services at Charlotte Maxeke Johannesburg Academic Hospital.

3.3 THE STUDY TIME PERIOD

The study will include all female patients diagnosed with microsatellite unstable endometrioid endometrial carcinoma within the period 2009 to 2015.

3.4 STUDY SAMPLE SIZE

Based on PhD work of Dr. R.Wadee, a total of 66 microsatellite unstable endometrioid endometrial carcinomas have been identified using immunohistochemistry and polymerase chain reaction tests.

3.5 INCLUSION AND EXCLUSION CRITERIA:

INCLUSION CRITERIA:

All proven microsatellite unstable endometrioid endometrial carcinoma diagnosed immunohistochemically and by Polymerase Chain Reaction (PCR) in the period 2009 – 2015.

EXCLUSION CRITERIA:

- All cases where the associated tumour was not histologically proven and the information was only obtained from the history.
- Conflicting individual patient information in cases where the patients identifying details differ, for example, different date of birth / surname etc.

3.6 <u>METHODS:</u>

1. Previously diagnosed endometrioid endometrial carcinoma slides have been retrieved and the diagnosis reviewed. The NHLS data base will be searched using the microsatellite unstable proven endometrial carcinoma cases' specific laboratory reference number. Information obtained from these reports will then be used to cross reference other associated histologically proven tumours.

The following information may be used to search the NHLS database:

- "Hospital number"
- "Name and Surname"
- "Date of birth"
- "Laboratory case number"
- "Episode number"
- "Soundex"

2. If associated tumours are found, these individual reports will be used to ascertain the type of associated tumour and age of the patient, and subsequently document this on the data collection sheet. If more than one associated tumour is found, the same process as above will be followed.

3. The individual microsatellite unstable endometrial carcinoma cases have already been previously confirmed as such, as this formed part of the work undertaken by Dr Reubina Wadee for a Doctor in Philosophy degree (PhD).

4. The slides of the relevant metachronous and synchronous tumours identified will be reviewed.

4. DATA ANALYSIS

The majority of this study is a prevalence study and the data will be expressed quantitatively as summary statistics and/or visually as graphs and/or tables. The distribution of data (including percentages, mean, median, standard deviation) will be determined using the statistical programme, STATISTICA. In the instance of skewed data, if possible, the data will be transformed to a normal distribution. When the data is compared to other studies, the parametric t-test will be used (our sample compared to another study sample results). If any difficulties arise in the data analysis process, assistance from the Department of Biostatistics will be obtained.

5. FUNDING

The study will not require funding as it a retrospective study on histology results where a final diagnosis has been reached and no additional investigations will be needed.

6. ETHICS

A project specific application to the Human Research Ethics Committee (Medical) and Faculty of Health sciences of the University of the Witwatersrand, Postgraduate office has been submitted. There is currently blanket ethics approval (M150884) for use of archived departmental material. This retrospective study will incur no additional cost to

the patient or hospital, therefore informed consent is not acquired. Dr Wadee had obtained ethical clearance for use of patient tissue samples (M151051) for research towards a PhD.

Although some personal information (for example name and surname) may be used to cross reference search the NHLS database, the data sheet will be anonymous with only the accession number of the case being used for record purposes. There will be no personal identifying information (such as name or date of birth) on the data sheet. Patient reports will be kept in a locked cupboard in the Department of Anatomical Pathology, School of Pathology, Faculty of Health Sciences, to which only the applicant and supervisor shall have access. The final submission will not have any personal patient information. Patient privacy will be maintained throughout the study.

7. TIMELINE

	July – Aug 2019	Aug – Sept 2019	Oct – Nov 2019	Dec 2019 - Jan 2020	Febr – June 2020	July 2020
Protocol Preparation						
Protocol assessment						
Ethics application						
Data collection						
Data analysis						

Writing of research project						
Final submission						

8. ANTICIPATED DIFFICULTIES:

Histopathology reports, prior to implementation of the Trackcare system might not be easily retrievable or not retrievable at all. Access to the DISA system will also be obtained by the researcher, Suzanne Kotze.

APPENDIX A:

DATA COLLECTION SHEET

PATIENT CASE NUMBER	
Date at which endometrial carcinoma was diagnosed	
Associated tumour 1	
Date at which associated tumour 1 was diagnosed	
Associated tumour 2	
Date at which associated tumour 2 was diagnosed	
Associated tumour 3	
Date at which associated tumour 3 was diagnosed	
Age of patient (at diagnosis of endometrial carcinoma)	
Site of tumour	

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3.4 Ethics certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Suzanne Kotze

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190939

NAME: (Principal Investigator)	Dr Suzanne Kotze
DEPARTMENT:	Anatomical Pathology National Health Laboratory Service (NHLS) Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	An observational study of synchronous/metachronous tumours in microsatellite unstable endometrial carcinomas (2009-2015)
DATE CONSIDERED:	27/09/2019
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Reubina Wadee
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	30/09/2019

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 on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, 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**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **September** and will therefore be due in the month of **September** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 04/10/2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

3.5 Consent for access to NHLS database

NATIONAL HEALTH LABORATORY SERVICE
UNIVERSITY OF THE WITWATERSRAND – JOHANNESBURG

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

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Dr Y Perner MBBCh (Witwatersrand) FCPath (SA); MMed (Witwatersrand)

August 19, 2019

Human Research Ethics Committee (Medical)
University of the Witwatersrand
Johannesburg
2000

Re: Consent for access to NHLS database

This letter serves to confirm that the Department of Anatomical Pathology at the University of the Witwatersrand and NHLS is happy to grant Dr Suzanne Kotze access to patient reports on the DISA and TrakCare systems for her study entitled "Evaluation of synchronous/metachronous tumours in microsatellite unstable endometrial carcinomas (2009-2015)".

Publications emanating from the research will be accredited to the Division of Anatomical Pathology.

Assuring you of the Department of Anatomical Pathology's co-operation in this and future research projects.

With best wishes.

Yours sincerely,

Dr Yvonne Perner
Head: Department of Anatomical Pathology

3.6 Consent for access to case numbers of Dr Wadee

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Wednesday 11th September 2019

Dear Dr. Kotze

This serves to confirm that I will provide you with access to patient laboratory case numbers from the cohort of patients diagnosed with endometrioid endometrial carcinoma that I had used as part of my PhD research. I will gladly release the laboratory case numbers to you upon receipt of ethical clearance for your study.

Yours sincerely



Dr. Reubina Wadee
Reubina Wadee
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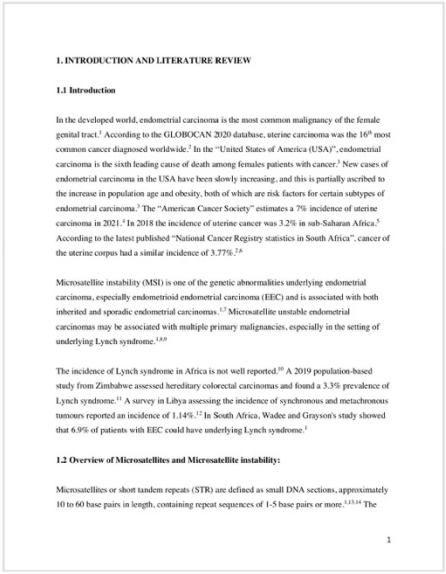
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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In the developed world, endometrial carcinoma is the most common malignancy of the female genital tract.[1] According to the GLOBOCAN 2020 database, uterine carcinoma was the 16th most common cancer diagnosed worldwide.[2] In the "United States of America (USA)", endometrial carcinoma is the sixth leading cause of death among females patients with cancer.[3] New cases of endometrial carcinoma in the USA have been slowly increasing, and this is partially ascribed to the increase in population age and obesity, both of which are risk factors for certain subtypes of endometrial carcinoma.[3] The "American Cancer Society" estimates a 7% incidence of uterine carcinoma in 2021.[4] In 2018 the incidence of uterine cancer was 3.2% in sub-Saharan Africa.[5] According to the latest published "National Cancer Registry statistics in South Africa", cancer of the uterine corpus had a similar incidence of 3.77%.[2,6]

Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma (EEC) and is associated with both inherited and sporadic endometrial carcinomas.[3,7] Microsatellite unstable endometrial carcinomas may be associated with multiple primary malignancies, especially in the setting of underlying Lynch syndrome.[3,8,9]

The incidence of Lynch syndrome in Africa is not well reported.[10] A 2019 population-based study from Zimbabwe assessed hereditary colorectal carcinomas and found a 3.3% prevalence of Lynch syndrome.[11] A survey in Libya assessing the incidence of synchronous and metachronous tumours reported an incidence of 1.14%.[12] In South Africa, Wadee and Grayson's study showed that 6.9% of patients with EEC could have underlying Lynch syndrome.[3]

1.2 Overview of Microsatellites and Microsatellite instability:

Microsatellites or short tandem repeats (STR) are defined as small DNA sections, approximately 10 to 60 base pairs in length, containing repeat sequences of 1-5 base pairs or more.[3,13,14] The

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1. INTRODUCTION AND LITERATURE REVIEW

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1.2 Overview of Microsatellites and Microsatellite instability:

Microsatellites or short tandem repeats (STR) are defined as small DNA sections, approximately 10 to 60 base pairs in length, containing repeat sequences of 1-5 base pairs or more.[1,13,14] The

repeat of the base pair cytosine and adenine (CA), is most frequently seen.[1] Microsatellites are present throughout coding and non-coding areas within the human genome, including telomeres and centromeres.[1,14,15]

Microsatellites are prone to DNA replication errors during the cell cycle.[16] The errors occur secondary to DNA slippage during replication.[17] Errors in replication are repaired by one of the body's DNA repair mechanisms, known as the mismatch repair (MMR) system.[14] which corrects replication errors such as "base-base mismatches" and "insertion-deletion" loops in the microsatellite areas.[1] Several genes encode the group of proteins that form part of the MMR system, including "mutL homologue 1 (MLH1), mutL homologue 3 (MLH3), mutS homologue 2 (MSH2), mutS homologue 3 (MSH3), mutS homologue 6 (MSH6), postmeiotic segregation increased 1 (PMS1) and post-meiotic segregation increased 2 (PMS2)".[1,13] The MMR system has both major and minor genes with different functions.[1] Major genes include *MSH2* and *MLH*, and these genes are responsible for protein expression.[1] The minor genes include *MSH3*, *MSH6* and *PMS2*, which support and stabilize the major genes in their function.[1] For example, *MSH6* and *MSH2* form a complex called "MutSα"which functions to identify base-pair mismatches and can repair insertion-deletion and single base-pair mutations.[1] These genes have a "proofreading" function with their corresponding proteins, ensuring DNA integrity and accurate replication before cell division.[18]

Loss of function of one or more MMR system members causes accumulation of mutations within the microsatellites.[13] Loss of MMR gene function may be secondary to a genetic mutation, which may be inherited, may occur sporadically, or may be due to epigenetic hypermethylation of the *MLH1* gene.[15,16] The most commonly affected gene is *MLH1*.[13] Loss of mismatch repair gene function on its own does not directly cause malignant transformation of cells. However, their loss does lead to the accumulation of mutations or errors in other genes, including microsatellites, during the normal cell cycle, resulting in a strong mutator phenotype.[18,19] The range of mutations that follows after the MMR system's loss of function is broad, causing missense mutations, silent mutations or deletions/insertions that may result in frameshift mutations.[1,18] These mutations may then accidentally inactivate tumour suppressor genes or activate proto-oncogenes.[18] When affected cells gain these driver mutations (oncogenes or inactivated tumour suppressor genes),

this may result in cancer development.[18] This genetic instability and accumulation of mutations throughout the genome in microsatellites contributes to cancer development and is termed microsatellite instability.[17–19]

MLH1 promotor hypermethylation is an epigenetic process that leads to gene silencing by adding a methyl group to cytosine which then blocks transcription factors attaching to DNA and thus prevents DNA transcription.[1]

Microsatellite instability and mismatch repair deficiency can be detected predominantly by using polymerase chain reaction (PCR) analysis and/or immunohistochemistry (IHC).[16] A case is identified as microsatellite unstable by PCR if one or more markers show a difference in the size of the alleles.[20] Several markers are used to detect microsatellite instability and different centres choose to use different panels; however, the current gold standard mononucleotide panel includes BAT-25, NR-21, NR-24, BAT-26 and NR-27.[13] A case is classified as MSI-H (microsatellite instability-high) if two or more markers show dissimilarity in allele size, MSI-L (microsatellite instability-low) if one marker shows difference in allele size, and microsatellite stable (MSS) if none of the markers show a difference in allele size.[1] A case is identified as mismatch repair deficient if one or more of the MMR antibodies (PMS2, MLH1, MSH6 and MSH2) show loss of nuclear staining.[20] A study by McConechy *et al* showed a high level of concordance (93.3%) between MMR protein immunohistochemistry and MSI PCR analysis, and as such, IHC may be used instead of PCR.[21]

1.3 Classification of Endometrial Carcinoma:

Endometrial carcinoma can be classified according to pathogenesis, histological appearance or molecular subtype.[1,3] There is, however, significant overlap between the groups, and all three classifications should be used to help predict risk and prognosis more accurately.[1,3] The current recommendation is to use molecular classification in combination with histology for risk stratification and is the best prediction of prognosis.[7]

1.3.1 Pathogenetic classification

Bokhman divided endometrial carcinomas into the traditional oestrogen dependent (Type I) endometrial carcinomas and oestrogen independent (Type II) pathway.[1,22] Sources of oestrogen can be either be exogenous or endogenous. Tamoxifen, used as a treatment for breast carcinoma, and hormonal therapy without opposing progesterone, are both sources of exogenous oestrogen.[1] Tamoxifen is used as a treatment for breast carcinoma as it has an anti-oestrogenic effect on the breast. However, in contrast, tamoxifen has a pro-oestrogenic impact on the endometrium, causing abnormal endometrial proliferation. Oestrogen therapy in the absence of progesterone, also causes abnormal endometrial proliferation. Both give rise to endometrial hyperplasia and thus possible evolution to cancer.[1] Endogenous oestrogen sources include adipose tissue in obese females and ovarian pathology, such as certain ovarian neoplasms and polycystic ovarian syndrome.[1]

Type I tumours (oestrogen dependent) develop in relatively younger peri- or premenopausal women.[1,3,15,17] These tumours may be preceded by endometrial hyperplasia as a precursor lesion.[17] Type II tumours (non-oestrogen dependent) are also called non-endometrioid endometrial carcinoma (NEEC) and occur in older, non-obese, post-menopausal women.[1,3,15,17] The background non-neoplastic endometrium of Type II tumours tends to be atrophic.[17] As these tumours are not oestrogen-dependent, the tumour is thought to arise from intraepithelial carcinoma.[17] Type II tumours generally show a more aggressive clinical behaviour than Type I tumours.[7,15,17]

1.3.2 Histological classification

The "World Health Organization (WHO)" Blue book of Female Genital Tumours currently classifies endometrial carcinomas according to the light microscopic features, dividing them into endometrioid, serous, clear cell, undifferentiated/dedifferentiated, mixed and other.[3,7] A small subset of endometrial carcinomas shows mesenchymal differentiation, and these are termed carcinosarcomas.[3] Amongst newly diagnosed cases of endometrial cancer, the endometrioid subtype is the most common, accounting for approximately 85% of tumours.[3]

Endometrioid carcinoma derives its name from the fact that the neoplastic glands closely resemble normal proliferative endometrium.[3] Endometrioid carcinoma can show different histological patterns depending on the type of cellular differentiation present.[3] Typically, the this subtype is characterized by neoplastic gland formation with a complex architecture showing branching and papillary structures.[3] A smooth luminal border is maintained.[3] The neoplastic cells lining the glands show pseudostratified, elongated to round nuclei with a variable degree of atypia.[3,7] Other histological patterns associated with endometrioid carcinoma, include squamous, mucinous, and secretory patterns.[3,7]

According to the percentage of solid growth present, the "International Federation of Gynaecology and Obstetrics (FIGO)" classifies endometrioid carcinoma into three grades.[2] Grade 1 tumours are composed predominantly of glands with 5% or less solid growth. Grade 2 tumours are composed of 6-50% solid areas. Grade 3 tumours also show more than 50% solid differentiation.[3,7]

1.3.3 Molecular classification

Following the pathogenetic and histological classification, endometrial carcinomas are also classified according to the molecular subtype.[3] A molecular classification provides better understanding of the different cancer pathways, is of prognostic value and contributes notably to the diagnosis and treatment of patients.[1,3] "The Cancer Genome Atlas Research (TCGA) Network" classified endometrial cancer into four groups according to their underlying molecular aberration: microsatellite instability (hypermutated), POLE-ultra mutated, copy-number high and copy-number low with TP53 mutations.[7,15,23]

POLE-mutated group of tumours are associated with mutations involving *KRAS*, *PTEN*, *PIK3CA*, *PIK2R1*, *ARID1A* and *FBXW7* genes.[1] The *POLE* gene encodes a part of the DNA polymerase that has a proofreading function during DNA replication.[1] When a mutation occurs within the *POLE* gene, the proofreading function is lost with subsequent mutations during DNA replication, creating a mutator phenotype.[1] POLE-mutated group of tumours show a good prognosis.[1,7] Repeat areas of DNA amplifications or deletions characterize the copy number high

group.[1] The copy number high group of tumours are associated with a poor prognosis and include mutations within *PIK3CA*, *PPP2R1A* and *TP53*, and chromosomal instability.[1,7] The copy number low group of tumours includes all tumours that cannot be categorized as POLE-mutated, copy number high or microsatellite unstable groups, and are associated with an intermediate prognosis.[1]

After the POLE-mutated group, microsatellite instability is the second most common genetic abnormality associated with endometrial carcinoma.[1,3,15] Microsatellite instability is, however, the most common underlying molecular abnormality of endometrioid endometrial carcinomas.[1,3,15] The microsatellite instability (MSI) group are associated with mutations involving *KRAS*, *PTEN*, *PIK3R1*, *PIK3CA*, *ARID1A* and *RPL22*.[1] Microsatellite unstable tumours have shown an intermediate clinical prognosis.[7] MSI is present in up to 90% of Lynch syndrome associated endometrial carcinomas and in as many as 30% of sporadic endometrial carcinomas.[1,15] Sporadic endometrial carcinomas occur secondary to *MLH1* promoter methylation via epigenetic silencing.[3,24]

1.4 Lynch Syndrome and Endometrial Carcinoma:

Lynch syndrome, or Hereditary Nonpolyposis Colorectal Cancer (HNPCC), is an inherited, autosomal dominant syndrome with germline mutation of MMR genes.[19,25] Currently, the incidence of Lynch syndrome differs between populations, ranging from 0-10%.[24]

The genes usually affected are *MSH2, MSH6, PMS2* and *MLH1*.[26] Due to the MMR gene germline mutation, patients with Lynch syndrome show a predisposition to developing multiple cancers.[27] The likelihood of developing a certain type of tumour has been shown to be associated with the specific MMR gene involved, for example, *MSH6* germline mutations are mainly associated with endometrial carcinoma development.[26,27] Many tumours, depending on the specific MMR gene involved, are associated with Lynch syndrome.[27] These include colorectal, endometrial, gastric, pancreas, small bowel, upper urinary tract, sebaceous gland, brain and ovarian tumours.[25,28] Lynch syndrome patients are especially prone to develop colorectal and endometrial carcinoma.[27] The risk of Lynch syndrome patients developing colorectal carcinoma

is 80%, while the risk of developing endometrial carcinoma is 60%.[26] Endometrial carcinoma may be the first tumour identified in an individual and may be the indicator of undiagnosed Lynch syndrome.[26] In Lynch syndrome patients, the time between developing a second tumour after the diagnosis of endometrial carcinoma is on average, 11 years.[26]

Lynch syndrome-associated endometrial carcinomas are usually of endometrioid morphology.[3] Occasionally, this tumour predisposition syndrome is associated with non-endometrioid endometrial carcinomas, including clear cell carcinoma, carcinosarcoma and serous carcinoma.[1,26] The non-endometrioid endometrial carcinomas associated with Lynch syndrome tend to occur at a younger age.[26] Lynch syndrome-associated endometrial carcinomas are prone to arise in the lower uterine segment and are often difficult to differentiate from tumours of primary endocervical origin.[26] It is also well-established that these Lynch syndrome-associated endometrial carcinomas are challenging to subtype as they can have a mixed histological picture and show high-grade morphology.[26] However, some histological features may point towards an underlying microsatellite instability, for example, undifferentiated or dedifferentiated tumours and the identification of a prominent lymphocytic infiltrate within, and surrounding the tumour.[26]

1.5 Multiple Primary Malignant Tumours:

Billroth first reported multiple primary malignant tumours in 1889.[29] Multiple primary malignancies are defined as the development of more than one tumour, synchronous or metachronous, in the same individual fulfilling specific criteria delineated by Warren and Gates.[30,31] The definition, rules and criteria, and the perception of multiple primary malignancies vary and have changed over time.[30] Warren and Gates's criteria include the following: a malignancy must be confirmed histologically, and each tumour should be separate.[31] Multicentric and multifocal tumours, as well as metastases, should not be included as primary tumours.[30]

The two main guidelines currently used are the "Surveillance Epidemiology and End Results (SEER) project" and the "International Association of Cancer Registries" and the "International Agency for Research on Cancer (IACR/IARC)".[30] European cancer registries are more inclined

to use the IACR/IARC guidelines.[30] The SEER guidelines recommend using a two month cut off to differentiate between metachronous and synchronous tumours, whilst the IACR/IARC guidelines recommend that a tumour be registered as synchronous if it occurs within six months of the first tumour, and as metachronous, if it occurs after six months.[30]

Multiple primary tumour incidence differs in literature and is reported to range from 0.5% to 37%.[29,30,32] The risk of developing a second primary malignancy varies according to the site of the initial first malignancy.[30] For example, following the SEER guidelines, the risk of developing a second primary tumour in patients with colon cancer is 19.7%, and the risk in patients with lung cancer is 21%.[30] Second primary tumour development is the most common, whilst developing a third, fourth, or more tumours are reasonably uncommon.[9] Patients are currently surviving longer with primary tumours, providing enough time for such patients to develop second or multiple tumours and is thought to be the result of the significant improvement of cancer diagnostics, screening programmes and treatment modalities.[29,30]

Several lifestyle, host or environmental factors may contribute to an increased risk of developing a second primary tumour.[30] Lifestyle factors include alcohol and tobacco usage, and host factors include genetic predisposition such as tumour syndromes and family history.[30] Environmental factors include exposure to certain pathogens, particular occupations and specific geographical areas.[30] The frequency of multiple primary tumours differs amongst ethnic groups.[32] For example, in contrast with the United States of America (USA), Japan has a low incidence of prostate cancer and a high incidence of gastric cancer.[32]

The incidence of multiple primary tumours in patients specifically diagnosed with endometrial carcinoma is approximately 10% to 23%.[8,9] A large population-based study in the USA undertaken by Uccella *et al*, showed that the incidence of more than one primary tumour in patients with endometrial cancer is approximately 22%.[8] Another USA-based study by Buttin *et al*, assessing the incidence and the different types of synchronous and metachronous tumours in MSI unstable endometrial carcinomas specifically, showed that approximately 20% of microsatellite unstable endometrial carcinomas had associated primary tumours.[33] A study by

Rong *et al* reported that the median age of patients at the time of endometrial carcinoma diagnosis, was 50.3 years.[9]

Several types of cancer are associated with microsatellite instability, most notably colorectal, endometrial (more specifically endometrioid endometrial carcinoma) and gastric cancer.[1,14] Bonneville *et al* found microsatellite instability in 27 cancer types and 3.8% of all cancers evaluated.[14] Cancer types not previously noted to be microsatellite unstable were adrenocortical carcinoma, mesothelioma and cervical cancer.[14] Other cancer types associated with microsatellite instability include breast carcinoma, melanoma, prostate adenocarcinoma, ovarian serous cystadenocarcinoma, head and neck squamous cell carcinoma, oesophageal, hepatocellular and lung carcinoma.[14] A few other cancer types such as uterine carcinosarcoma, thymoma, paediatric neuroblastoma, Wilms tumour, bladder carcinoma, cholangiocarcinoma, chronic lymphocytic leukaemia, glioblastoma, lower-grade gliomas and sarcomas, have shown an association with microsatellite instability.[14] The development of multiple tumours occurring in a patient with endometrial carcinoma, especially in the setting of Lynch syndrome, is well-established.[8,28] These include tumours from the colorectum, small bowel, ovary, pancreaticobiliary tract, stomach, urinary tract, skin and brain.[8,33]

Some studies have shown that the time between the development of the first and second tumour vary from approximately 4 to 10 years.[8,9,32] A previous case series from China reporting the clinicopathological features of multiple primary malignant tumours with endometrial carcinoma, found an interval of 51.27 months between the development of the first and second tumour.[9] Another study by Amer reported an average time to develop a second tumour after the first, was 117 months; but this study was not limited to patients with only endometrial carcinoma and associated tumours, as it included a wide range of tumour types.[32] Uccella *et al* found a median interval of 6 years between the development of endometrial carcinomas and breast carcinomas and a median of 4 years between the diagnosis of endometrial carcinomas and rectal carcinomas.[8]

To the best of our knowledge, a study documenting tumours associated with microsatellite unstable/mismatch repair-deficient endometrial carcinoma in South Africa or other African

countries has not been performed. The burden of these tumours on our population is currently unknown. Assessing tumours in our patient cohort that are associated with microsatellite unstable endometrial cancer and the incidence thereof may facilitate further studies into possible risk factors in our patient profile. Such information may also evaluate the feasibility and cost-effectiveness of the possible implementation of screening programs and germline testing in a developing country.

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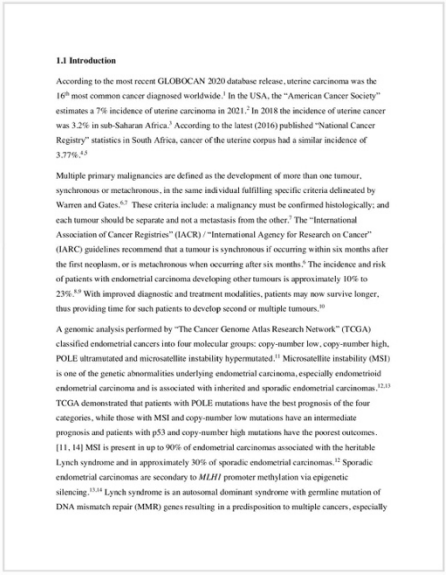
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Multiple primary malignancies are defined as the development of more than one tumour, synchronous or metachronous, in the same individual fulfilling specific criteria delineated by Warren and Gates.[6,7] These criteria include: a malignancy must be confirmed histologically; and each tumour should be separate and not a metastasis from the other.[7] The “International Association of Cancer Registries” (IACR) / “International Agency for Research on Cancer” (IARC) guidelines recommend that a tumour is synchronous if occurring within six months after the first neoplasm, or is metachronous when occurring after six months.[8] The incidence and risk of patients with endometrial carcinoma developing other tumours is approximately 10% to 23%.[8,9] With improved diagnostic and treatment modalities, patients may now survive longer, thus providing time for such patients to develop second or multiple tumours.[10]

A genomic analysis performed by “The Cancer Genome Atlas Research Network” (TCGA) classified endometrial cancers into four molecular groups: copy-number low, copy-number high, POLE ultramutated and microsatellite instability hypermutated.[11] Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma and is associated with inherited and sporadic endometrial carcinomas.[12,13] TCGA demonstrated that patients with POLE mutations have the best prognosis of the four categories, while those with MSI and copy-number low mutations have an intermediate prognosis and patients with p53 and copy-number high mutations have the poorest outcomes. [11, 14] MSI is present in up to 90% of endometrial carcinomas associated with the heritable Lynch syndrome and in approximately 30% of sporadic endometrial carcinomas.[12] Sporadic endometrial carcinomas are secondary to *MLH1* promoter methylation via epigenetic silencing.[13,14] Lynch syndrome is an autosomal dominant syndrome with germline mutation of DNA mismatch repair (MMR) genes resulting in a predisposition to multiple cancers, especially

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1.1 Introduction

According to the most recent GLOBOCAN 2020 database release, uterine carcinoma was the 16th most common cancer diagnosed worldwide.[1] In the USA, the "American Cancer Society" estimates a 7% incidence of uterine carcinoma in 2021.[2] In 2018 the incidence of uterine cancer was 3.2% in sub-Saharan Africa.[3] According to the latest (2016) published "National Cancer Registry" statistics in South Africa, cancer of the uterine corpus had a similar incidence of 3.77%.[4,5]

Multiple primary malignancies are defined as the development of more than one tumour, synchronous or metachronous, in the same individual fulfilling specific criteria delineated by Warren and Gates.[6,7] These criteria include: a malignancy must be confirmed histologically; and each tumour should be separate and not a metastasis from the other.[7] The "International Association of Cancer Registries" (IACR) / "International Agency for Research on Cancer" (IARC) guidelines recommend that a tumour is synchronous if occurring within six months after the first neoplasm, or is metachronous when occurring after six months.[6] The incidence and risk of patients with endometrial carcinoma developing other tumours is approximately 10% to 23%.[8,9] With improved diagnostic and treatment modalities, patients may now survive longer, thus providing time for such patients to develop second or multiple tumours.[10]

A genomic analysis performed by "The Cancer Genome Atlas Research Network" (TCGA) classified endometrial cancers into four molecular groups: copy-number low, copy-number high, POLE ultramutated and microsatellite instability hypermutated.[11] Microsatellite instability (MSI) is one of the genetic abnormalities underlying endometrial carcinoma, especially endometrioid endometrial carcinoma and is associated with inherited and sporadic endometrial carcinomas.[12,13] TCGA demonstrated that patients with POLE mutations have the best prognosis of the four categories, while those with MSI and copy-number low mutations have an intermediate prognosis and patients with p53 and copy-number high mutations have the poorest outcomes. [11, 14] MSI is present in up to 90% of endometrial carcinomas associated with the heritable Lynch syndrome and in approximately 30% of sporadic endometrial carcinomas.[12] Sporadic endometrial carcinomas are secondary to *MLH1* promoter methylation via epigenetic silencing.[13,14] Lynch syndrome is an autosomal dominant syndrome with germline mutation of DNA mismatch repair (MMR) genes resulting in a predisposition to multiple cancers, especially

colorectal and endometrial tumours.[15–17] Endometrial carcinoma may be the first tumour diagnosed and may suggest underlying Lynch syndrome.[18] Endometrial carcinoma is associated with multiple primary malignancies such as cancers of the breast, ovary and most commonly, colorectum.[8,9,13]

To the best of our knowledge, a study documenting tumours associated with microsatellite unstable endometrial carcinoma in South Africa has not been performed. The burden of these tumours on our population is currently unknown. We aimed to assess the incidence of tumours associated with MSI or MMR deficient, endometrial carcinomas at a single institution in South Africa.

1.2 Material and methods

This study follows previous research undertaken in our department of Anatomical Pathology, on endometrioid endometrial carcinomas (EECs).[19] As microsatellite instability is more frequently seen in association with endometrioid endometrial carcinomas than non-endometrioid subtypes, the study was limited to endometrioid endometrial carcinomas.[19] Previous research by Wadee and Grayson identified a total of 66 mismatch repair deficient and/or microsatellite unstable EECs using immunohistochemistry and polymerase chain reaction (PCR) tests.[19] Wadee and Grayson considered a case to be mismatch repair deficient (MMR-d) if the tumour showed loss of nuclear staining of one or more of the MMR antibodies (MLH1, PMS2, MSH2 and MSH6).[19] A case was identified as microsatellite unstable by PCR if one or more markers showed a difference in the size of the alleles.[19] A case was classified as MSI-H (microsatellite instability-high) if two or more markers showed dissimilarity in allele size, MSI-L (microsatellite instability-low) if one marker showed difference in allele size, and microsatellite stable (MSS) if none of the markers showed a difference in allele size.[12] The PCR markers used were NR-21, NR-24, NR-27, BAT-25 and BAT-26.[19]

These 66 EECs also underwent *MLH1* promotor methylation analysis in the study by Wadee and Grayson.[19] Of the 66 endometrial carcinomas, 44 were methylated, 17 were not methylated and one did not have enough DNA to be tested. Four cases were not tested for *MLH1* promotor methylation as they did not show loss of MLH1 immunohistochemical staining.[19] (Figure 1)

The EEC cases from 2009-2015 were from the Department of Anatomical Pathology, University of the Witwatersrand/National Health Laboratory Services (NHLS) at Charlotte Maxeke Johannesburg Academic Hospital which provides a pathology service to various hospitals and clinics in the southern Gauteng region.

After ethical clearance (certificate number: M190939) was granted, information was obtained from the LabTrack and DISA database of the NHLS using the microsatellite unstable/mismatch repair-deficient proven endometrial carcinoma case-specific laboratory reference numbers. Information obtained from these reports was used to cross-reference other associated histologically proven primary tumours both before and after the endometrial carcinoma diagnosis. The following information was used to perform a retrospective search and cross-reference for associated histologically proven tumours: hospital number, name and surname, date of birth, laboratory case number and episode number.

When an associated tumour was identified, the pathology report was used to ascertain the type of tumour, site of the associated tumour and patient age at diagnosis of the associated tumour. The date of authorization or sign-out was used as the tumour diagnosis date to establish consistency as the procedure date is often not stated on the clinical request form. No personal identifiers were documented on the data collection sheet, thus ensuring patient anonymity. The slides of the identified associated tumours were retrieved, reviewed, and the diagnoses confirmed. Multicentric and metastatic tumours were excluded as per criteria delineated by Warren and Gates.[6,7]

To assess the difference in means of both the average age of patients with and without associated tumours and the average age of patients with methylated and unmethylated endometrial carcinomas, a two-tailed t-test was used. To determine any statistically significant differences between the proportion of associated tumours of our study and a previous study, a two-tailed Z-proportion test was used. A significance level of 5% was used.

In accordance with the journal's guidelines, we will provide our data for the reproducibility of this study in other centers if such is required.

1.3 Results

Three patients (4.5%) out of 66 confirmed microsatellite unstable/mismatch repair-deficient endometrioid endometrial carcinoma cases, had associated tumours. These tumours included an invasive, moderately to poorly differentiated, squamous cell carcinoma of the uterine cervix, uterine leiomyosarcoma, a mucin-producing rectal adenocarcinoma and a mucinous carcinoma of the breast. (Figure 2) One patient had two associated tumours, namely uterine leiomyosarcoma and rectal adenocarcinoma. Table I shows that of the three endometrial carcinoma patients who had associated tumours, one patient's tumour showed retention of all MMR proteins but had microsatellite instability in one of 5 PCR markers, while one patient's tumour showed retention of all MMR proteins but had microsatellite instability in two of the 5 PCR markers; whereas the third patient's tumour had loss of both MLH1 and PMS2 immunohistochemical markers and had microsatellite instability in one of the 5 PCR markers used.[19] Thus, only one patient had an MSI-High EEC while 2 patients had MSI-Low tumours, which are considered more similar to the MSS status than to the MSI-high status.

One patient developed a metachronous cervical squamous cell carcinoma, which was diagnosed on a cervical punch biopsy 12 months after the patient underwent a subtotal hysterectomy for endometrial carcinoma. The exact reason for a subtotal hysterectomy having been performed was not stated. The surgery was conducted by doctors who were not gynaecologic oncologists, as this was undertaken at a small district hospital. At the time of diagnosis of another patient's endometrial carcinoma, an associated (synchronous) uterine leiomyosarcoma was simultaneously diagnosed. This patient was also diagnosed with a metachronous rectal adenocarcinoma 9 months after the diagnosis of EEC. The third patient in our cohort who had developed an associated tumour, was diagnosed with a synchronous mucinous breast carcinoma 1 month after her diagnosis of EEC. (Table I)

Table II shows that the average time between the diagnosis of endometrial carcinoma and other tumours was 7.33 months, with a median of 9 months. There was no significant statistical difference between the average age of those with, and those without a second/associated tumour. (p=0.244). The average age of patients without an associated tumour, at the time of diagnosis of their endometrial carcinoma was 66 years, while patients with endometrial carcinomas who developed associated tumours had an average age of 59 years at the time of their endometrial carcinoma diagnosis.

Table III shows that the average age of patients with methylated endometrial carcinomas, but no associated tumour (n=43) was 65 years, while a single patient who had a methylated EEC and an associated tumour was 55 years of age when the endometrial carcinoma was diagnosed. Patients who had unmethylated endometrial carcinomas and no associated tumours (n=16), had an average age of 67 years, whilst a single patient with an associated tumour and an unmethylated EEC was diagnosed with the EEC at 70 years of age. There was no significant statistical difference between the average age of those with methylated endometrial carcinomas and the average age of those with unmethylated tumours (p=0.331). Of the three endometrial carcinoma patients who had associated primary tumours, one patient had *MLH1* promotor methylation of the endometrial tumour while one patient's EEC was unmethylated. The third patient's tumour, unfortunately, did not have enough DNA available to undergo methylation analysis.

Based on earlier research performed in our department by Wadee and Grayson, 41 out of 145 cases had mismatch repair protein deficiency and 46 out of 145, had microsatellite instability detected by PCR.[19] There was MMR-d/MSI discordance identified in 25 cases.[19] Of the 41 cases shown to have MMR protein deficiency, 20 cases showed both MLH1 and PMS2 loss, 2 cases had both MSH2 and MSH6 deficiency, 16 cases demonstrated isolated MLH1 loss, 2 had isolated MSH2 deficiency and a single case showed loss of 3 markers (MLH1, MSH6 and PMS2).[19]

We compared our current results in addition to results from the previous study in our department by Wadee and Grayson, to a study by Buttin *et al*.[19,20] We found a statistically significant (p=0.0045) difference in the incidence of associated tumours between the present study and that of Buttin *et al*.[20] (Table IV). Furthermore, Table IV shows a statistically significant difference (p=0.0000) in our patient cohort when comparing the proportion of *MLH1* methylated endometrial carcinoma cases to *MLH1* unmethylated cases. There was no significant statistical difference between the proportion of methylated and unmethylated cases in our patient cohort compared to Buttin *et al*.[20]

Table V shows additional clinical and pathological details of one of the patients who had undergone an excision of an associated tumour. Unfortunately, the resection specimens for the other two patients could not be traced despite an extensive search on our laboratory computer database. This may be as a result of the patients not having undergone surgical excisions at our

hospital complex or could be due to patients seeking medical attention at other hospitals which we do not provide a pathology service to, or having been lost to follow-up, or the patients may have died of their disease.

1.4 Discussion

Our present study showed a statistically lower incidence of associated tumours when compared to a study from a western country. We also demonstrated associated tumours that are different from those traditionally associated with Lynch syndrome.

Of the confirmed microsatellite unstable or mismatch repair-deficient endometrial carcinoma patients in our study, 3 cases (4.5%) had associated tumours. These associated tumours included the well-documented colorectal carcinoma and tumours less commonly associated with Lynch syndrome, including mucinous breast carcinoma, uterine leiomyosarcoma and cervical squamous cell carcinoma.[18] The rectal adenocarcinoma and cervical squamous cell carcinoma both occurred metachronously. Synchronous tumours included mucinous breast carcinoma and uterine leiomyosarcoma, the latter having been diagnosed at the same time as the endometrial carcinoma on the excision hysterectomy specimen.

The incidence of associated primary tumours in patients with endometrial carcinoma differs in the literature. In the USA, early research from the 1960's and 1980's showed an incidence of approximately 10%, whilst more recent studies, including that of Uccella *et al*, and Buttin *et al* show an incidence of 20-22%.[8,20] The initial studies were smaller in number and hospital-based compared to recent studies that included a larger number of patients, regions and better follow-up of patients.[8] Uccella *et* al assessed the clinicopathological features of patients (n=1028) with endometrial carcinoma, which included endometrioid and non-endometrioid subtypes, in addition to the associated tumours they developed; and evaluated the risk of patients developing an associated tumour.[8] The study by Buttin *et al*, assessed the incidence and the different types of synchronous and metachronous tumours in MSI unstable endometrial carcinoma patients (n=94) which also included endometrioid and non-endometrioid subtypes.[20]

Our study showed an incidence of 4.5%, which is far lower than that of the American studies (20-22%).[8,20] Our study and the study by Buttin *et al* assessed the incidence and types of associated tumours of microsatellite unstable endometrial carcinomas.[20] A comparison of the

incidence of associated tumours between our study (4.5%) and that of Buttin *et al* (20.2%) showed a statistically significant difference in proportions. (Table IV)

Whilst our sample size was small, both polymerase chain reaction (PCR) and immunohistochemistry were conducted during the previous study in our department by Wadee and Grayson, to detect microsatellite unstable/MMR deficient endometrial carcinoma cases, thus ensuring a more accurate identification of cases.[19] A study by McConechy *et al* showed a high level of concordance (93.3%) between MMR protein immunohistochemistry and MSI PCR analysis, and as such, IHC may be used instead of PCR.[21] However, a recent publication by Wadee and Grayson, showed that 5% of microsatellite unstable endometrial carcinoma cases would not have been identified if PCR was not used, as these cases did not show loss of immunohistochemical staining.[19] Similarly, Buttin *et al* also used both PCR and immunohistochemistry.[20]

The low incidence in our study (4.5%) compared with American studies may be explained by the smaller study number (66 patients) and that our cohort was limited to microsatellite unstable/MMR-d, *endometrioid* endometrial carcinomas, which contrasts to both that of Uccella *et al* and Buttin *et al*.[8,21].

Other aspects that may have limited our patient sample size include the possibility of patients having been lost to follow up or having been registered on the laboratory system with different names and surnames which would not have been traceable on our laboratory computer database. In our setting of a developing country, loss to follow up of patients is a common occurrence as we provide a service to state/public sector patients. The patients may not have easy access to transportation to get to hospitals, or the financial means to use transport or may not be able to take time off from work to come to the hospital. In addition, patients may decide to access health care facilities closer to their homes which may be in areas that we do not provide a pathology service to. Furthermore, the possibility of patients having died exists, and such knowledge may not be conveyed to the patients' treating physicians and this may then not be communicated to pathologists.

Buttin *et al* further subdivided the MSI positive group into *MLH1* methylated and *MLH1* unmethylated, categorizing tumours into sporadic and possible germline tumours respectively.[20] Comparisons between MLH1 methylated and unmethylated cases in the studies by Wadee and

Grayson and Buttin *et al* showed no statistically significant differences (p=0.7991) (Table IV).[19,20]

The statistically significant difference in proportions when comparing the *MLH1* methylated cases with *MLH1* unmethylated cases in our present study suggests that the proportion of possible germline tumours is significantly lower than the proportion of sporadic tumours in our cohort. Germline mutations in MMR genes have been documented in 2-6% of endometrial carcinomas.[18] Currently, the incidence of Lynch syndrome differs between populations, ranging from 0-10%.[14] In South Africa, Wadee and Grayson's study showed that 6.9% of patients with EEC may have underlying Lynch syndrome, which is slightly higher than the expected 2-6%.[14] Unfortunately, confirmatory germline testing is currently not available in the public sector of Gauteng, South Africa. [14] Another South African study by Vergouwe *et al* showed that in contrast to Western studies, there is a higher rate of MMR deficiency in colorectal carcinoma suggesting an increased proportion of inherited disease in regions of South Africa where the incidence of colorectal carcinoma is relatively low.[22] As such, the incidence of possible Lynch syndrome associated tumours in South Africa was expected to be higher than the 4.5% identified in the present study.

The development of multiple tumours occurring in a patient with endometrial carcinoma, especially in the setting of Lynch syndrome, is well-established.[8,23] These include tumours from the colorectum, ovary, small bowel, stomach, pancreaticobiliary tract, skin, brain and the urinary tract.[16,23,24] Endometrial and colorectal carcinomas are the most commonly described Lynch syndrome-associated tumours and the association between the two carcinomas is well documented.[16,20] Only one patient in our study had an associated colorectal carcinoma. Although gastrointestinal tumours were the first neoplasms described in Lynch syndrome, endometrial carcinoma is the sentinel tumour in up to 50% of patients.[18,23]

One patient with endometrial carcinoma in our study had an associated mucinous breast carcinoma. The incidence of Lynch syndrome-associated breast carcinomas is marginally increasing, but the association between breast carcinoma and Lynch syndrome varies in the literature and is controversial.[16,25] Stoll *et al*. showed that women with Lynch syndrome do not have an increased risk of breast cancer compared to the general population, while Win *et al*.

showed that patients with MMR mutations were at a risk of developing breast carcinoma that was at least four times greater than that of the general population.[25,26]

Furthermore, we identified one patient who had endometrial carcinoma and an associated uterine leiomyosarcoma. Generally, sarcomas are not commonly associated with Lynch syndrome. Sporadic sarcomas are rarely associated with microsatellite instability. However, a Brazilian study recently indicated that sarcomas, although rare, are linked to the Lynch syndrome spectrum of tumours.[27,28] An increased lifetime incidence is reported; but the exact risk for sarcoma development has not been fully elucidated.[27]

We identified one patient with an endometrial carcinoma who had an associated cervical squamous cell carcinoma, which is not considered a traditional Lynch syndrome-associated tumour.[29] However, several case studies have documented cervical carcinoma in association with germline mutations of the MMR genes.[29] Antill *et al* indicated that cervical carcinoma, including squamous cell carcinoma, could be considered part of the extracolonic manifestations of Lynch syndrome.[29] Antill *et al* state that it is plausible that the tumour pathway of cervical carcinoma in MMR gene mutation patients may be secondary to the known carcinogenic effect of human papillomavirus (HPV) exposure, but microsatellite instability should be investigated as a possible underlying molecular pathway.[29] In our study, microsatellite instability as an underlying molecular pathway has been confirmed for the endometrial carcinoma. While it is possible that this is the underlying pathway of the associated cervical carcinoma, such testing was not performed on any of the associated tumours, including the cervical tumour, as this was a retrospective, observational study and we did not have ethical clearance to perform any new additional studies/investigations.

The average time to development of an associated tumour was 7.33 months. A previous case series from China reporting the clinicopathological features of multiple primary malignant tumours with endometrial carcinoma, found an interval of 51.27 months between the development of the first and second tumour.[9] Another study by Amer reported an average time to develop a second tumour after the first, was 117 months; but this study was not limited to patients with only endometrial carcinoma and associated tumours as it included a wide range of tumour types.[30] Only one month passed between the development of the breast carcinoma and

the endometrial carcinoma in our study, compared to Uccella *et al's* median of 6 years.[8] In addition, the diagnosis of the rectal adenocarcinoma in our study was made 9 months after that of the endometrial carcinoma, compared to Uccella *et al's* median time interval of 4 years between diagnosis of the two tumours.[8] It is clear that the time interval between the diagnosis of an associated tumour and the primary tumour in our study is far less than those of other studies. The reason for this is unknown and although our cohort is too small to draw substantial conclusions, further studies investigating possible risk factors contributing to the reduction of the time interval between multiple tumour diagnoses are needed. Furthermore, this finding may suggest that closer follow up of patients with MSI unstable tumours is indicated.

Only 1 patient had an MSI-High EEC while 2 patients had MSI-Low tumours, which have been considered more akin to the MSS status than to the MSI-high status.[22] Methylated tumours point toward a sporadic occurrence whereas unmethylated tumours that have MMR-d may indicate an underlying germline mutation.[19] Hereditary tumours tend to present at a younger age.[31] Our study, however, illustrated that the patient with a *methylated* EEC and synchronous breast carcinoma was 55 years of age at the time of her EEC diagnosis, whereas the patient with an *unmethylated* EEC and metachronous cervical carcinoma was 70 years of age at the time of EEC diagnosis. As such, despite the patient's age (70 years) the occurrence of an *unmethylated* tumour and a metachronous tumour may still suggest an underlying germline mutation and would warrant further investigation. The third patient in our cohort who had EEC and associated neoplasms had a total of 3 malignancies (metachronous and synchronous). Despite there not being sufficient DNA to perform methylation assessment, the occurrence of 3 neoplasms in this patient, of which one neoplasm was an MSI-high endometrial tumour, suggests that further investigations for possible underlying Lynch syndrome are also warranted.

The incidence of Lynch syndrome in the rest of Africa is not well recorded.[32] A 2019 population-based study from Zimbabwe was the first to study hereditary colorectal carcinomas extensively and found a 3.3% prevalence of Lynch syndrome.[33] A study in Libya assessing the incidence of synchronous and metachronous tumours, reported an incidence of 1.14%, with breast carcinoma being both the most common primary, as well as the second identifiable tumour.[34] To the best of

our knowledge no other study from the African continent has investigated the presence or absence of tumours associated with *microsatellite unstable/MMR-d endometrial carcinomas*.

A Zambian case series reported HIV as a risk factor for the development of multiple primary malignancies.[35] South Africa currently has the highest number of people living with HIV in the world.[36] The influence that HIV has on Lynch syndrome-associated tumours is not well known. Microsatellite alteration has been described in AIDS-associated and non-AIDS associated tumours, for example, lung carcinoma, as microsatellite alterations are present at a higher frequency in HIV associated lung carcinomas compared to sporadic lung carcinomas.[37] A South African study assessing cervical biopsies showed that HPV alone may induce MSI; however, co-infection with HIV may exacerbate disease progression.[38] Our study did not assess microsatellite instability/MMR-d in the associated tumours and did not document the HIV status (as this is not always provided with the patient's clinical information), which is a limitation of our study as the role of HIV in the development of multiple tumours is currently unknown.

1.5 Conclusion

To the best of our knowledge, our study is the first to document the incidence and type of associated tumours in *microsatellite unstable/mismatch repair deficient (MMR-d)* endometrial carcinomas in Africa. The results from our study showed a statistically lower incidence of associated tumours when compared to a western country. We have demonstrated associated tumours that are different from those traditionally associated with Lynch syndrome. The precise reasons for this are currently unknown. Further studies, including assessing the role of HIV, the incidence of multiple primary tumours and identifying possible risk factors in our population, are needed. Such information may also evaluate the feasibility and cost-effectiveness of the possible implementation of screening programs and germline testing in a developing country. In addition, this study will contribute to global data and provide a greater knowledge base from which gynaecologic oncologists, pathologists, medical oncologists, and radiation oncologists may draw information, with the ultimate aim of optimal patient care.

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2	"Precision Molecular Pathology of Uterine Cancer", Springer Science and Business Media LLC, 2017 Publication	1%
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3.11 Plagiarism declaration



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SENATE PLAGIARISM POLICY: APPENDIX ONE

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