NUT MIDLINE CARCINOMA IN THE STATE SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

by

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DECLARATION

I, Antoinette Roets, declare that the coursework Master's Degree mini-dissertation that I herewith submit in a publishable manuscript format for the Master's Degree qualification in Anatomical Pathology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

_____ _____12/05/2020_____

Antoinette Roets **Date**

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TABLE OF CONTENTS

1. Abstract		iv
2. Keywords		v
3. List of abbreviations		vi
4. List of figures		vii
5. List of tables		viii
6. List of appendices		ix
7. Chapter 1		1
7.1 Literature review		1
7.1.1	Background	1
7.1.2	Clinical Presentation	1
7.1.3	Radiology	1
7.1.4	Pathology	2
7.1.5	Molecular Features	8
7.1.6	Treatment	10
7.1.7	Prognosis	11
7.1.8	Rationale Behind Study	12
8. Aim and Objectives		14
9. References		14
10. Chapter 2		19
10.1.1	Authors	20
10.1.2	Abstract	21
10.1.3	Keywords	21
10.1.4	Introduction	22
10.1.5	Materials and methods	23
10.1.6	Results	24
10.1.7	Discussion	26
10.1.8	Conclusion	27
10.1.9	References	28
10.1.10	Appendices	31 - 41

ABSTRACT

Background: NUT midline carcinoma (NMC) is a recently described, rare tumour that can easily be mistaken for a number of other tumours if a NUT immunohistochemical stain is not performed. The tumour is caused by a translocation involving the *NUT* gene and most cases involve *BRD4-NUT* t(15;19) which results in loss of differentiation and uninhibited proliferation. The loss of differentiation is responsible for the monomorphic, primitive morphology of the tumour. The reporting Pathologist should have a high index of suspicion as the tumour shows positively for numerous immunohistochemical markers that vary from case to case. Positivity for CD34, which is unusual in carcinomas, together with positivity for cytokeratins, is a strong diagnostic clue that should prompt testing for the tumour. Previously thought to occur only in midline structures and young patients, recent research has proven the occurrence in a wider age distribution and outside the midline. This tumour is exceptionally aggressive, with only isolated survivors and early identification and aggressive treatment is needed. No research on NMC has been done in South Africa and there is only one case report from the rest of Africa. The incidence of this tumour in South Africa is therefore unknown.

Aim: The aim of this study was to determine the number of cases of NMC seen over a twelve year period by the Department of Anatomical Pathology, University of the Free State and National Health Laboratory Service and to describe the demographic features of any patients identified.

Methods: A retrospective study was performed. All undifferentiated malignant tumours and tumours with evidence of squamous differentiation from the head, neck and thorax seen between 1 January 2005 and 31 December 2016 were included. A NUT immunohistochemical stain was performed on all cases. The stain was regarded as positive if there was speckled nuclear staining in more than 50% of the tumour cells.

Results: Four hundred and ninety eight cases were included in the study of which 424 (85.1%) were male and 74 (14.9%) were female. The mean age was 58.6 years. Only one positive case was identified. The patient was a 30-year-old female with a lung mass and lymph node metastases.

Conclusion: This study confirms the rarity of this entity. Additional research is needed in other provinces of South Africa, including the private sector to provide a comprehensive patient profile of NMC in South Africa.

Keywords:

NUT midline carcinoma, NUT carcinoma, South Africa

LIST OF ABBREVIATIONS

NMC	NUT midline carcinoma
NUT	Nuclear protein in Testis
BDR	Bromodomain containing
BET	Bromodomain and extra-terminal domain
SCC	Squamous cell carcinoma
HPV	Human papilloma virus
PCR	Polymerase chain reaction
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
CT	Computerised tomography
MRI	Magnetic resonance imaging
Brdi	Small molecule bromodomain inhibitors
TAD	Topologically associating domain
DNA	Deoxyribonucleic acid
LncRNA	Long non-coding ribonucleic acid
NCOA	Nuclear receptor coactivator
FDA	Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
WHO	World Health Organization
H&E	Hematoxylin and eosin

LIST OF FIGURES

Chapter 1

Figure 1: NUT Midline Carcinoma. (A) Low power view shows nests, sheets and trabeculae in a desmoplastic stroma with abundant necrosis. 2.5x magnification. (B) Undifferentiated cells with vesicular nuclei and conspicuous nucleoli. Note the abundant mitotic figures (arrows). 10x magnification. (C) Cells with more eosinophilic cytoplasm as evidence of abrupt keratinization (arrow). Note the lymphovascular invasion (star). 20x magnification. (D) Squamous differentiation noted focally. 10x magnification. 2

Figure 2: NUT immunohistochemical stain. Note the diffuse stippled nuclear staining pattern 7

Chapter 2

Figure 1: NUT Midline Carcinoma. (A) Low power view shows nests, sheets and trabeculae in a desmoplastic stroma with abundant necrosis. 2.5x magnification. (B) Undifferentiated cells with vesicular nuclei and conspicuous nucleoli. Note the abundant mitotic figures (arrows). 10x magnification. (C&D) Squamous differentiation noted focally. 10x magnification. (E) Cells with more eosinophilic cytoplasm as evidence of abrupt keratinization (arrow). Note the lymphovascular invasion (star). 20x magnification. (F) Single cell keratinization (arrow). 40x magnification. (G) NUT immunohistochemical stain. Note the diffuse pattern of staining. 40x magnification. (H) Stippled nuclear staining pattern considered positive. 40x magnification. (I) Non-specific staining with NUT. The staining is varied between cells and does not appear stippled. 20x magnification. (J) Negative NUT immunohistochemical stain. 40x magnification. 25

LIST OF TABLES

Chapter 1

Table 1: Differential diagnosis NUT midline carcinoma 3

Chapter 2

Table 1: Number of cases and average age of patients according to diagnosis 24

LIST OF APPENDICES

A. Letter of approval from Health Sciences Research Ethics Committee 31
B. Permission letter from NHLS and Head of School of Pathology 32
C. Permission letter from Head of Department 33
D. Copy of Research Protocol 34
E. Data collection form 39
F. Instructions for authors South African Journal of Oncology 40
G. Summary Report from Turnitin 41

CHAPTER 1

LITERATURE REVIEW

BACKGROUND

NUT midline carcinoma (NMC) is a rare malignant tumour initially described in a case report by Kees et *al.,* in 1991. The patient was an 11 year old girl with a thoracic mass.[1] These tumours are poorly differentiated squamous cell carcinomas and were originally thought to occur only in midline structures, hence the name.[2,3] NMCs are the result of a specific genetic mutation of the *NUT* (nuclear protein in testis) gene, involving a balanced translocation of chromosomes 15 and 19 t(15;19)(q13;p13.1) causing a *NUT*-fusion oncoprotein.[4]

NMC does not arise from a specific organ and no pre-invasive lesion has ever been identified.[5] Initially thought to occur anywhere in the trunk, head or neck, typically in the midline, it is now known to occur in many other locations as well, including bone, salivary gland, kidney, adrenal gland and pancreas.[5,6] As many of them occur outside the midline, the tumour has been given the alternative designation of "NUT carcinoma" by the World Health Organisation (WHO).[7]

CLINICAL PRESENTATION

NMCs were originally described in paediatric patients. However, they can occur in patients of all ages with a range of 0 to 78 years. The median age is 21.9 years, although this may not be accurate as the tumour is often underdiagnosed in older patients.[6,8] Males and females are affected equally.[2,6,8,9]

The symptoms are generally non-specific including weight loss, fever and symptoms related to mass effect.[10,11] Most patients have metastatic disease at the time of diagnosis and the most common sites of metastases include bone, lymph nodes and pleura.[12]

RADIOLOGY

There are no specific features on imaging and NMC can simulate the appearance of other tumours.[13] Features seen on computerised tomography (CT) scan include a hypo-attenuating, heterogeneously enhancing mass with necrosis. Magnetic resonance imaging (MRI) features include a hypointense signal on T1-weighted and hyperintense signal on T2-

weighted images.[14] Initially CT was adequate for preliminary workup and staging, with MRI simply used as an adjunct but MRI has proven superior as the extent of the tumour, lymphovascular and perineural invasion can be evaluated.[11,14]

PATHOLOGY

NMC has the same histological appearance in all the various locations in which it presents. On routine histology, NMC is composed of undifferentiated round blue cells. In some cases, foci of abrupt keratinization are present which would suggest the diagnosis (Figure 1). Isolated cases have also shown chondroid differentiation, of which one such case arose within the parotid gland.[10] However, although the diagnosis may be suspected, it is not possible to diagnose NMC on Haematoxylin and Eosin (H&E) stained sections only.[2,3,6,15] Electron microscopic evaluation confirms squamous differentiation with intermediate junctions, desmosomes and branching tonofilaments.[10]

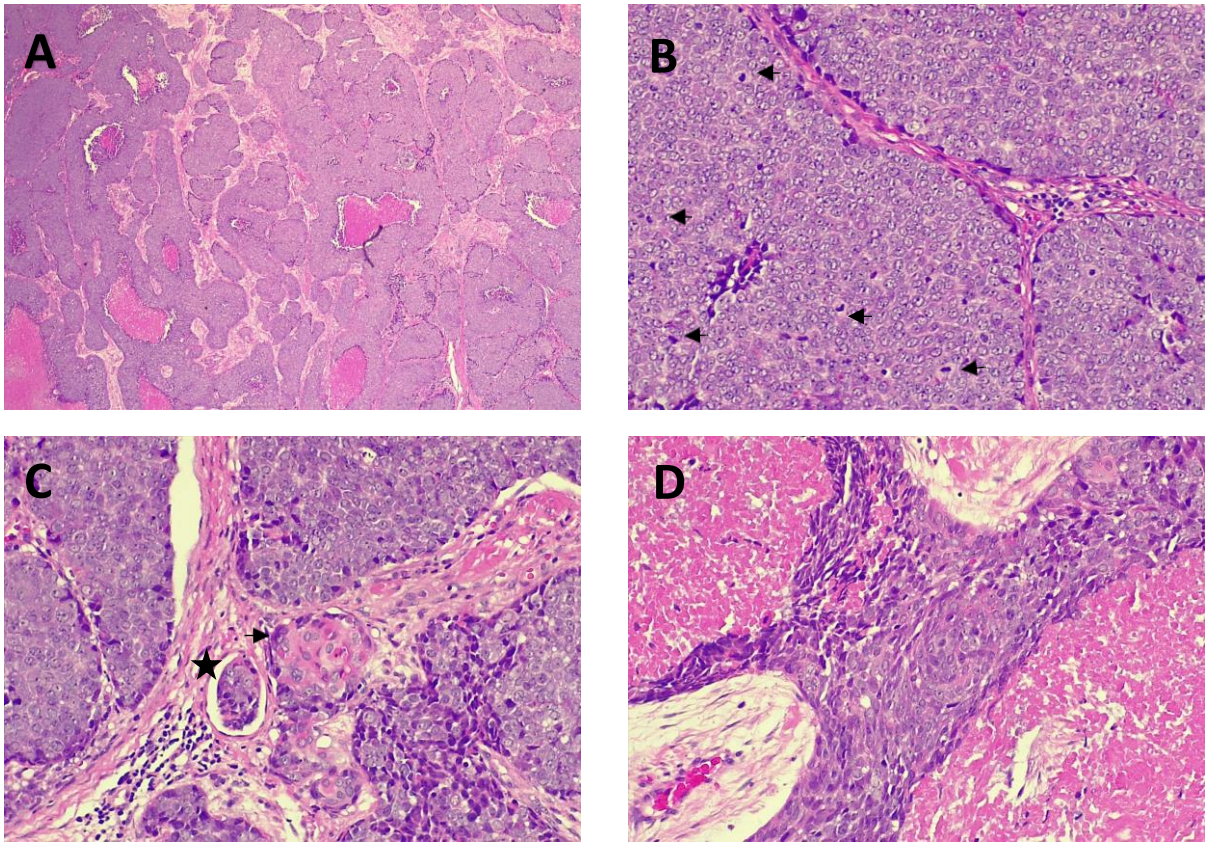

Figure 1. NUT midline carcinoma. (A) Low power view shows nests, sheets and trabeculae in a desmoplastic stroma with abundant necrosis. 2.5x magnification. (B) Undifferentiated cells with vesicular nuclei and conspicuous nucleoli. Note the abundant mitotic figures (arrows). 10x magnification. (C) Cells with more eosinophilic cytoplasm as evidence of abrupt keratinization (arrow). Note the lymphovascular invasion (star). 20x magnification. (D) Squamous differentiation noted focally. 10x magnification.

The differential diagnosis depends on the patients age and the topography of the tumour and includes poorly differentiated squamous cell carcinoma, sinonasal undifferentiated carcinoma, Ewing sarcoma, nasopharyngeal carcinoma, thymic carcinoma, neuroblastoma, small cell neuroendocrine carcinoma, salivary carcinoma, pancreatoblastoma, melanoma and lymphoma.[2,3,6,15]

Table 1. Differential Diagnosis NUT Midline Carcinoma

Tumours	Classic Histology	Clues in Histology	IHC Positive	IHC Negative	Pitfalls	Molecular
NMC	Sheets / Nests Monomorphic Undifferentiated cells Coarse-vesicular chromatin Abrupt keratinization Conspicuous nucleoli	No glandular differentiation Fried egg appearance Neutrophilic infiltrate	NUT Cytokeratins (need cocktail as can be negative some CK) p63/p40 CD34 EMA INI1 retained	EBER-ish TTF-1 S100 Neuroendocrine markers CD99 FLI-1 CD45	Aberrant positivity in numerous markers, especially p16 Heterologous elements	Simple karyotype BRD4-NUT BRD3-NUT NSD3-NUT
HG Lymphoma	Sheets Large undifferentiated cells Irregular nuclear membranes Coarse chromatin Numerous basophilic nucleoli	No keratinization No epithelial differentiation Smearing artifact	Lymphoma markers according to lineage CD45,CD3, CD20, CD30 INI1 retained	NUT p63 Cytokeratins	EMA positivity Epithelioid morphology	Complex karyotype Usually BCL2, BCL6 and MYC alterations
Nasopharyngeal Undifferentiated Carcinoma	Syncytial small nests or single cells Monomorphic Undifferentiated Vesicular chromatin	Single large eosinophilic nucleoli Smooth nuclear membrane Prominent lymphoid infiltrate	Cytokeratins EMA, CEA EBER-ish p63/p40 INI1 retained	NUT CD34 S100 CD45	S100 positive dendritic cells CD30 positivity in 30%	Complex karyotype EBER

Table 1. (continued)

Sinonasal Undifferentiated Carcinoma	Sheets or single cells Large undifferentiated cells Prominent nucleoli	No squamous differentiation No glandular differentiation	IDH1 IDH2 Cytokeratins Focal p63/p40 EMA INI1 retained	NUT EBER-ish CD34 CD45 CD99	Aberrant positivity in synaptophysin, chromogranin, S100	Complex karyotype IDH2 mutation codon R172
SMARCB1 deficient Sinonasal Carcinoma	Sheets or single cells Large undifferentiated cells Prominent nucleoli	Focal rhabdoid / plasmacytoid features No squamous differentiation No glandular differentiation	Cytokeratins Focal p63/p40 EMA	INI1 loss	Aberrant positivity p16, synaptophysin, chromogranin	Complex karyotype Mutation in SMARCB1
Melanoma	Wide variation Sheets / nests / fascicles Epithelioid / spindle / bizarre cells Scant to abundant cytoplasm	Junctional activity helpful Prominent red nucleoli Nuclear pseudoinclusions	SOX10 S100 Melan A HMB45	NUT Cytokeratins p63/p40 Subset loss BAP1	Can be amelanotic Can appear undifferentiated Some myxoid stroma / metaplastic bone Negativity for some melanoma markers	Complex karyotype BRAF RAS NF-2 BAP1 GNAQ / GNA11
Nonkeratinizing Squamous Cell Carcinoma (PD, HPV associated and Basaloid)	Sheets/nests Pleomorphic Squamous differentiation by stratification Minimal/absent keratin	Pushing margin No glandular differenmtiation	CK5/6 p63/p40 Block positivity 16	NUT EBER-ish	HPV associated tumours have minimal pleomorphism Basaloid variant can have abrupt keratinization	Complex karyotype HPV
Large Cell Neuroendocrine Carcinoma	Sheets/nests Large pleomorphic cells Coarse chromatin Visible nucleoli Moderate cytoplasm	Neuroendocrine architecture: organoid / ribbons / palisades	Cytokeratins TTF-1 CD56 Chromogranin Synaptophysin INSM1	NUT CD34 p63/p40	Can have focal p63/p40 positivity	Complex karyotype

Table 1. (continued)

Small Cell Neuroendocrine Carcinoma	Mostly sheets, uncommon neuroendocrine architecture Small hyperchromatic nuclei Fine chromatin Nucleoli inconspicuous Scant cytoplasm	Azzopardi effect Smearing artifact Nuclear molding	Cytokeratins INSM1 TTF-1 CD56 Chromogranin Synaptophysin (dot-like)	NUT CD34 p63/p40	Can focal + p63/p40 Cytokeratins positive in a dot-like fashion (large cell neuroendocrine has cytoplasmic and membrane staining)	Complex karyotype
Ewing sarcoma / PNET	Sheets Monomorphic Undifferentiated Small round cells Hyperchromatic Inconspicuous nucleoli Scant cytoplasm	Neuroendocrine architecture in PNET Can clear cytoplasm (PAS highlights abundant glycogen)	CD99 FLI1 Vimentin	NUT CD45 CD56 SOX10, HMB45 WT1 Desmin myogenin	Aberrant cytokeratin, S100 and neuroendocrine positivity Can form nests and appear carcinomatous	Complex karyotype EWSR1-FLI1 EWSR1-ERG
Olfactory neuroblastoma	Lobules Monomorphic Small cells Round vesicular nuclei Scant cytoplasm Prominent blood vessels	Fibrillary stroma Rosettes Stippled chromatin	NSE Synaptophysin Chromogranin CD56	NUT Cytokeratins CD45 CD99 Desmin HMB45	S100 positivity in sustentacular cells Patchy CK positivity	Complex karyotype

Abbreviations: EBER, Epstein-Barr Virus Encoded RNA; HG, High grade; HPV, Human Papilloma Virus; IHC, Immunohistochemistry; NMC, NUT Midline Carcinoma; PD, Poorly Differentiated

Although Lund-Iversen *et al.,* deemed testing for NMCs in patients with primary pulmonary carcinomas unnecessary, Sholl *et al*., found 9 positive cases.[16,17] Their study showed that primary lung NMCs have distinctive imaging and histological features. All the NMC-positive patients had a central primary lung mass, mostly in the right lower lobe. The tumours were large (5 to 11cm), ill-defined and had areas of necrosis. In addition, the adjacent hilar and mediastinal lymph nodes were matted and ipsilateral pleural effusions were noted in every case. The opposite lung was never involved. The histology showed the same picture of uniform nests and sheets of medium sized cells that appeared round to epithelioid. The cells had scant light pink cytoplasm, vesicular chromatin and some had distinct nucleoli. The tumours were positive for cytokeratin and p63 or p40.[17]

Bishop *et al*., showed that NMCs of the sinonasal tract had a high proliferation rate including numerous mitotic figures and areas of apoptosis and necrosis. The rest of the histology appeared similar to those found elsewhere.[18] Solomon *et al*., described one case located in the sinonasal area in which the malignant cells seemed to collect around blood vessels, with a desmoplastic background stroma and a noticeable smearing artefact. In addition to the usual features seen, some of the areas appeared discohesive mimicking a lymphoma.[10,15] Gökmen-Polar *et al*., identified two definite cell populations, including better differentiated squamous cells in addition to the undifferentiated cells in all their patients with NMC of the thymic region.[19]

Initially, NMC could only be diagnosed using fluorescence *in situ* hybridization (FISH) or reverse transcriptase-polymerase chain reaction (RT-PCR) as there was diffuse reactivity for numerous other immunohistochemical markers.[20] NMC is often positive for pancytokeratins, confirming epithelial differentiation. In addition, some unanticipated markers may also be positive, including NSE, TTF-1, CD56, CD138, S100, vimentin, CD99, FLI1, CD45, CD34, p16, CD117 and PLAP. The positivity of these unanticipated markers may result in an incorrect diagnosis being made.[15] As germ cells of the ovary and testis are generally the only cells that express NUT, a monoclonal antibody to NUT was developed in 2009 and is the only immunohistochemical stain which can confirm the diagnosis of NMC.[4,21] The antibody has a positive predictive value of 100% and a negative predictive value of 99%. A FISH would only be necessary if a false negative result is suspected due to a high clinical suspicion. The stain is considered positive if there is diffuse nuclear staining in more than 50% of the tumour cells, with a characteristic speckled pattern.[21]

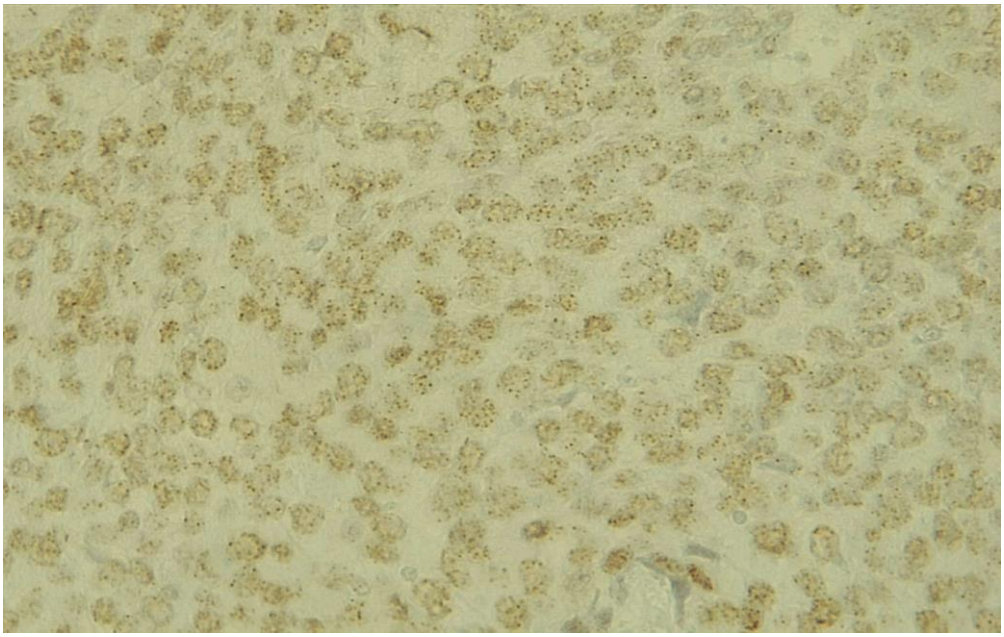

Figure 2. NUT immunohistochemical stain. Note the diffuse stippled nuclear staining pattern.

A diagnostic dilemma is the positivity of NUT in germ cell tumours. This can however be solved by evaluating the morphologic and immunophenotypic features. NMC typically shows abrupt keratinization that is not usually seen in germ cell tumours. In addition, even though certain NMCs can rarely show reactivity for germ cell markers, the staining pattern with the NUT immunohistochemical stain is different. Germ cell tumours stain only focally (<5%) with NUT and with a smooth nuclear pattern of staining. [21]

Another diagnostic dilemma is knowing when to stain for NMC. Screening for NMC is recommended for all poorly differentiated carcinomas without glandular differentiation, especially in the region of the head, neck and torso. Squamous differentiation is not a prerequisite to screen for NMC and screening is deemed unnecessary in tumours with an established aetiology such as in cases which are Epstein-Barr virus and human papilloma virus positive.[11]

The non-specific appearance of the carcinoma together with the disease still being relatively unknown to many pathologists, and the limited availability of the specific immunohistochemical stain for NUT, results in the tumour being misdiagnosed or underdiagnosed. Thus the true incidence of NMC is therefore still unknown.[3,8,22,23]

The international NMC registry (www.nmcregistry.org) was established in 2010 to aid in awareness of the tumour and serve as a database for NMC cases. It provides information on the pathology of NMC, the most recent updates regarding NMC and possible treatment options.[5]

MOLECULAR FEATURES

Most carcinomas develop as a consequence of multiple sequential mutations over a period of time that transform somatic cells into malignant cells. These mutations involve the genes that govern the normal cell proliferation mechanisms; the cells then become autonomous in growth and insensitive to anti-proliferative signals. In addition, the mutated cells gain the ability to evade apoptosis, induce angiogenesis and attain stem cell-like replicative capacity. These changes were coined the 'Hallmarks of Cancer'. The malignant cells eventually overcome the basement membrane barrier and epithelial-mesenchymal transition is evidenced as invasion and metastases.[24] Tumour-promoting inflammation, avoidance of immune destruction and deregulation of cellular energetics (Warburg effect) together with a few or all of the hallmarks of cancer enables the cell to survive in spite of an increase in the number of mutations. This leads to genomic instability and a mutator phenotype, resulting in a malignant cell.[24]

Usual squamous cell carcinomas have numerous mutations secondary to constant exposure to carcinogens.[25] In contrast to the majority of carcinomas, NMC has a simple karyotype and does not have to acquire the hallmarks of cancer or genomic instability to become malignant. In most cases of NMC, the *NUT* rearrangement is the only genetic abnormality present, similar to many leukaemias, lymphomas and sarcomas [26]. This karyotype, in addition to the fact that no in-situ precursor lesion has been detected, reinforces the belief that NMC arises from a stem cell [25].

In over two-thirds of cases, the *NUT* gene (present on chromosome 15) is fused to the *BRD4* gene (on chromosome 19), resulting in a *BRD4-NUT* oncogene.[1,2,4,6,26] In the remaining cases, the majority of the *NUT* gene is fused to *BRD3*, a close homologue of *BRD4*, or *NSD3*, although isolated other genes have been identified.[26,27] The reciprocal transcripts of *NUT-BRD4* are, however, not demonstrable.[4] Even though the precise function of *NUT* remains unknown, *BRD4* is responsible for the transcription of genes encoding BET proteins that are regulators of chromatin or transcription.[26,28] The *BRD-NUT*

fusion proteins inhibit both epithelial differentiation as well as cell cycle arrest, resulting in malignant change.[3,22,26]

BRD4 encodes a long and short isoform, the short is completely included in the *BRD4-NUT* fusion, whereas the long isoform is not. *BRD3* or *BRD4* bind to acetylated histones throughout all phases of the cell cycle and are essential to bind NUT to chromatin. In normal cells, the NUT moves between the cytoplasm and the nucleus whereas in NMC, *NUT* remains only in the nucleus.[10,26] Normally *BRD4* acts as an epigenetic reader by recognizing acetylated histones on chromatin. It influences the transcription of genes, determining which cells will enter into the cell cycle and proliferate. In NMC*, BRD4* is hyperphosphorylated, which leads to abnormal oncogene expression and malignant transformation. The hyperphosphorylation is caused mainly by kinase CDK9, that can be activated by the histone acetyltransferase (HAT) action of p300.[29]

The NUT protein is usually only expressed in spermatids. It contains two acidic potential protein binding domains, one of which binds and activates the histone acetyltransferase p300 during spermatogenesis.[5,28] The *NUT* portion of the *BRD4-NUT* fusion protein binds to acetylated chromatin via the *BRD4* bromodomains. The *NUT* then recruits and activates p300 creating a feed forward loop which results in hyperacetylated chromatin that is transcriptionally inactive and sequesters p300. The feed forward loop created results in p300 that is repeatedly recruited, with further acetylation and additional BRD-NUT fusion proteins. CBP/p300 plays a critical role in the transcription of p53 (a tumour suppressor gene) and if it is sequestered into *BRD4-NUT* it will cause p53 inactivation.[28]

The feed forward loop generates large expanses of contiguous active chromatin termed "megadomains" in the genome (100 to 200 hyperacetylated areas), containing abundant H3K27ac (modified histone 3). These "megadomains" can completely fill topologically associating domains (TADs), and are limited by them, which is a unique feature of *BRD4-NUT*, increasing its malignant potential. These newly formed "megadomains" result in increased transcription of the genes within this domain. It is this feature of the *BRD4-NUT* fusion that enhances the highly aggressive nature of NMC's, as some of these domains contain pro-growth genes, such as *MYC,* together with its' enhancers and long non-coding Ribonucleic acids (lncRNAs). This aberrant *MYC*, as well as p63 and MED24 can be transcribed from the underlying deoxyribonucleic acid (DNA) of these "megadomains".[30]

Aberrant p63 can further compromise the function of p53 by acting as a dominant-negative protein.[30] The loss of differentiation in NMC is exacerbated by overexpression of *MYC.*[31,32]

In addition to *BRD4* and *MYC*, loss of differentiation can also be as a result of the formation of the "megadomains". The sequestered HAT within the "megadomains" create a surplus histone deacetylase (HDAC) in the remaining chromatin. The consequential hypoacetylation in the rest of the genome causes decreased transcription of genes needed for differentiation.[33]

Further morphoproteomic studies showed that in addition to *MYC* (in particular *c-MYC*) overexpression and global hypoacetylation, overexpression of *Sirt1* and *EZH2* also contribute to loss of differentiation in NMC. This is mainly through constitutive activation of *IGF-1R/mTORc2/Akt* pathway, and could potentially be utilized in targeted therapies.[34] The defective DNA-repair mechanisms of NMC are attributed to a repetitive mutation of the *RECQL5* gene, a DNA helicase, as proved by next-generation sequencing. Mutated *RECQL5* results in genomic instability by being unable to repair crosslinks formed within the DNA.[35]

The vast numbers of deregulated genes without DNA repair is the most likely method by which NMC sidesteps the normal stages in tumorigenesis. It is hypothesized that the reason NMC manifests as a poorly differentiated squamous cell carcinoma is that only certain cells, precursor squamous cell in particular, can survive the *BRD4-NUT* translocation due to their specific chromatin configuration.[25,30] A larger DNA pool from patients with NMC is needed to ultimately establish the contribution of other germline mutations involved in the pathogenesis of the tumour.[35]

TREATMENT

There is currently extensive research and a number of clinical trials in progress to try to develop targeted therapies.[36–39] One of the promising targets is *BRD4*, as repression of *BRD4-NUT* leads to squamous differentiation with a stop in the cell cycle.[36] A small molecule, JQ1, has been developed that is highly selective for bromodomains of the BET family of proteins. JQ1 competitively binds to *BRD4* and displaces it from *NUT*.[36] Another possible targeted therapy, Brdi, was developed that also inhibits bromodomains. It acts by preventing the attachment of acetylated histones to *BRD4* and *BRD3*, resulting in terminal differentiation of the tumour cell.[33]

The latest developments in BET inhibitors are OTX015, TEN-010 (closely related to JQ1) and GSK525762, all used in different clinical trials. It was found from these trials that only 30% of the patients responded and all of the patients had tumour recurrence. Although BET inhibitors had adverse effects including gastrointestinal complications and thrombocytopenia, these factors were not considered to be problematic, as they were considered safe owing to their reversibility. However, they can result in treatment discontinuation and tumour relapse. Stathis *et al.*, recommend combining BET inhibitors with other treatments in future trials.[38] Further, patients with abnormal nuclear receptor co-activator *(NCOA3*) respond poorly to bromodomain inhibitors and it may therefore be of value to establish this prior to starting treatment with bromodomain inhibitors.[35]

Recent clinical trials have shown BET inhibitors in combination with other targeted therapies are more effective than single agent therapy and future patient trials will most likely follow.[38]

In 2011, a histone deacetylase inhibitor (HDACi) was developed that increases the action of HAT to restore chromatin acetylation outside the "megadomains", allowing the transcription of pro-differentiation genes. Currently there are two FDA approved reagents available, Vorinostat and Romidepsin. Refinement of these agents are needed as they have an increased side effect profile with continued use.[33,37]

CUDC-907 is an inhibitor of both HDAC and PI3K that has been developed and proved to be more effective than single target HDAC inhibitors, PI3K inhibitors or BET inhibitors. It reduces MYC levels and has a more favourable side effect profile, but still further studies are needed for widespread use.[37]

In conclusion, although numerous targeted therapies have been and are continuously being developed, none of them have been successful in providing a cure for NMC and additional trials are needed.

PROGNOSIS

The prognosis of NMC is very poor, the survival ranging from 4.7 months to 9.7 months, with only a slight improvement in survival with aggressive chemotherapy.[6,8,12] Overall survival is mostly influenced by age, gender, tumour dimensions, surgical margins, lymph

node positivity and the molecular variant of NMC.[8,9] Patients presenting with NMC of the thorax have the worst prognosis of all NMC patients, regardless of the molecular variant.[40]

Only isolated cases have been reported with complete remission. One such case was that of a 10-year-old boy who presented with NMC of the iliac bone in 1991 and was still tumour-free in 2006. This tumour was unusual as it originated in bone and had no epithelial differentiation.[41]

Another case was that of a 13-year-old boy, diagnosed in 2010 with an undifferentiated sarcoma of the epiglottis, who was treated with chemotherapy and radiation therapy but relapsed in 2012 when the diagnosis of NMC was made. Aggressive management in the form of a supraglottic hemilaryngectomy, post-operative radiation as well as chemotherapy was given. Five years post treatment he was still in complete remission with no recurrent disease.[42]

A third case was reported in 2017 of a nine-year-old boy who presented with enlarged cervical lymph nodes and an enlarged right sublingual gland. The patient received radical surgery, aggressive chemotherapy and radiotherapy and had remained tumour-free six years after treatment at the time of write up of the article.[43] The assumption is that NMC is partially sensitive to chemotherapy and radiotherapy and an aggressive multimodal therapeutic strategy may be the preference for patients with NMC.[6,13,42–45]

The last case was that of a 20-year-old female from China who presented with NMC of the larynx in 2016 and was treated only with radiotherapy with adjuvant Aidi and Kushen injections and not radical resection. Aidi and Kushen are traditional Chinese medicines used to enhance the effectiveness of chemotherapy. The patient was still disease free 26 months later at the time of write up of the case. The authors acknowledged that part of the success should be attributed to node negativity and early presentation and treatment.[46]

RATIONALE BEHIND THE STUDY

After a thorough literature search for NMC cases in Africa, only one case study of a patient could be identified in which one of the authors was from the National Cancer Institute, Cairo University in Egypt.[47] This case reported an adolescent female patient with NMC who initially responded well to treatment. The patient presented with widespread undifferentiated carcinoma and multiple skeletal metastases that caused

unmanageable pain, for which she received radiation therapy to the lower spine and sacrum. The pathology of her carcinoma was reviewed at the MD Anderson Cancer Center and the diagnosis of NMC was made.

She was first treated with a conventional chemotherapy regime for a duration of four cycles. The tumour appeared to respond with the first cycles of chemotherapy, but with continuation of the treatment the tumour progressed. Her treatment was then changed to Vorinostat, a histone deacetylase inhibitor, with concomitant radiation therapy to which she responded very well. The dose was decreased after 4 weeks but due to the critical thrombocytopenia she developed from the treatment, treatment was stopped. The patient died a month later, 10 months after initial diagnosis. From this study it was recommended that intermittent treatment with the drug rather than continuous use is preferable to decrease the likelihood of possible side effects. Furthermore, experimentation with the drug alone or in combination with radiation therapy is needed.[47] No other publications on NMC were available from Africa and South Africa.

Our study was prompted by one positive case identified in 2016 at our department of Anatomical Pathology at the University of the Free State. A 30-year-old female, who was previously healthy, presented with a short history of dysphagia, stridor, right middle lobe lung collapse and superior vena cava syndrome. Rapidly enlarging painless lymphadenopathy of the right cervical triangle and mediastinum were identified. The cervical lymph node biopsy showed an undifferentiated carcinoma within a desmoplastic stroma. The cells varied from basaloid in appearance to cells with moderate amount of pale eosinophilic cytoplasm, vesicular nuclei and prominent nucleoli with focal keratinization. Immunohistochemical stains were performed and results showed positivity for p63 and CK5/6 and negativity for CD5, CD117, EBER-ISH and CD34. The diagnosis of a metastatic poorly differentiated squamous cell carcinoma was suggested, with the possibility of NMC. As our laboratory did not have the NUT immunohistochemical stain, the case was referred to Prof J Hornick at Harvard Medical School for a second opinion and staining. Prof Hornick performed the NUT stain, which showed positivity as granular nuclear pattern of staining in more than 50% of the tumour cells and he agreed with the diagnosis of NMC.

As NMC can often be misdiagnosed as a number of other malignancies we were concerned that other cases of NMC were being missed by our department as the NUT

immunohistochemical stain is not currently available in the National Health Laboratory Service.

AIM AND OBJECTIVES

Aim: To determine the number and profile of patients with NMC in the state sector in the Free State Province.

Objectives:

1. To determine the number of cases of NMC seen over a twelve year period from January 2005 to December 2016 by the Department of Anatomical Pathology, University of the Free State and NHLS.
2. To evaluate the demographic features of patients diagnosed with NMC.

Should a significant number of cases be identified, the NUT antibody will be added to the diagnostic platform for route use.

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CHAPTER 2

ARTICLE: NUT MIDLINE CARCINOMA IN THE STATE SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

The article was prepared according to the journal submission guidelines for the *South African Journal of Oncology* (cf. Appendix H).

NUT MIDLINE CARCINOMA IN THE STATE SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

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ABSTRACT

Background: NUT midline carcinoma (NMC) is a recently described, rare tumour that can easily be mistaken for a number of other tumours if a NUT immunohistochemical stain is not performed and the reporting Pathologist does not have a high index of suspicion. This tumour is exceptionally aggressive, with only isolated survivors and early identification and aggressive treatment is needed. No research on NMC has been done in South Africa and there is only one case report from the rest of Africa. The incidence of this tumour in South Africa is therefore unknown.

Aim: The aim of this study was to determine the number of cases of NMC seen over a twelve year period by the Department of Anatomical Pathology, University of the Free State and National Health Laboratory Service and to describe the demographic features of any patients identified.

Methods: A retrospective study was performed. All undifferentiated malignant tumours and tumours with evidence of squamous differentiation from the head, neck and thorax seen between 1 January 2005 and 31 December 2016 were included. A NUT immunohistochemical stain was performed on all cases. The stain was regarded as positive if there was speckled nuclear staining in more than 50% of the tumour cells.

Results: Four hundred and ninety eight cases were included in the study of which 424 (85.1%) were male and 74 (14.9%) were female. The mean age was 58.6 years. Only one positive case was identified. The patient was a 30-year-old female with a lung mass and lymph node metastases.

Conclusion: This study confirms the rarity of this entity. Additional research is needed in other provinces of South Africa, including the private sector to provide a comprehensive patient profile of NMC in South Africa.

Keywords:

NUT midline carcinoma, NUT carcinoma, South Africa

ARTICLE

INTRODUCTION

Nuclear Protein in Testis (NUT) midline carcinoma (NMC) was first described in 1991. It is a rare form of poorly differentiated squamous cell carcinoma caused by a translocation involving the *NUT* gene.[1–4] In most cases it fuses with *BRD4* but other genes such as *BRD3* can also be involved. It was previously thought to occur only in young patients in midline locations. However, research has shown that NMC can occur at any age as well as in locations away from the midline.[2,5–8]

It is an aggressive tumour that has a very poor prognosis with a median survival of 6.7 months.[6,9] Conventional chemo- and radiotherapy have proven unsuccessful in most cases and only isolated survivors have been documented.[10–13] As a result of this, numerous targeted therapies are under development in the hope of finding a cure.[14–18] As this is a translocation-associated tumour with a simple karyotype, these targeted therapies are aimed toward epigenetic components, including histone deacetylase inhibitors, BET-inhibitors or specific components of the cell cycle.[19,20]

Due to the rarity of this entity, a lack of awareness on the part of pathologists and its undifferentiated appearance, NMC has been misdiagnosed in the past as poorly differentiated squamous cell carcinoma or undifferentiated carcinoma.[2,3,6,21] The typical morphological appearance is that of an undifferentiated carcinoma with foci of abrupt keratinization, with varied and sometimes unexpected staining with a number of immunohistochemical markers.[21]

Identification of these patients is important for the provision of counselling for families, surveillance for other metachronous carcinomas and earlier, more aggressive treatment.[7] Limited research has been done on NMC, and most of the identified patients to date are from the United States of America and Europe with limited data from other continents.[6,21–23] Additional research can aid in building the NMC international registry, identifying more patients for clinical trials in the development of novel treatments and in the overall demographic profiling of patients.

To date, no research on NMC is available from South Africa, and only a single case report documenting an Egyptian patient is available from the rest of Africa.[13] The incidence and demographic profile of patients from Africa is unknown. The aim of this study was therefore

to determine the number of cases of NMC seen over a twelve year period by the Department of Anatomical Pathology, University of the Free State (UFS) and National Health Laboratory Service (NHLS) and to describe the demographic features of any patients identified.

METHODS

A retrospective descriptive study was performed. A SNOMED search of the NHLS electronic databases was performed to identify all malignant tumours of the head, neck and chest diagnosed by the Department of Anatomical Pathology, UFS and NHLS over a twelve year period from 1 January 2005 to 31 December 2016. Prior to this there was no electronic laboratory information system. The department provides histology services to all state hospitals and clinics in the Free State Province of South Africa.

Cases selected included all undifferentiated malignant tumours and tumours showing squamous differentiation. Males and females of all ages were included. Seven cases were excluded from the study as insufficient tissue was available in the wax blocks. Furthermore, tumours were excluded if they showed neuroendocrine or glandular differentiation, had specific diagnoses, such as Ewing Sarcoma or lymphoma, or had evidence of a specific aetiology determined by the presence of p16 and EBER-ISH positivity. Once the suitable cases had been identified, the slides were retrieved from the departmental archives. All the cases were reviewed and a representative slide was chosen.

The wax blocks were then retrieved from the departmental archives, and 4µm sections were cut and stained using an anti-NUT rabbit polyclonal antibody (clone abl22649, Abcam Inc., Cambridge, MA). A dilution of 1:500 was used. Slides were stained using a Benchmark XT automated slide preparation system (Ventana Medical Systems Inc., Tucson, AZ). The slides were then counterstained with Mayers haematoxylin, dehydrated and cover slipped. The slides were evaluated by a registrar and a pathologist. NUT was scored as positive when speckled nuclear staining was evident in 50% of more of the tumour cells. Otherwise, it was scored as negative. Cases with non-specific staining were reviewed by an expert pathologist at Brigham and Women's Hospital in Boston. Additional information including the patient's age, sex, topography of the biopsy and the original diagnosis was also recorded.

Approval to perform the study was obtained from the Health Sciences Research Ethics Committee, UFS (UFS-HSD2017/1164). Statistical analysis was performed by the Department of Biostatistics, UFS. Results were expressed as frequencies and percentages (categorical variables) and means, standard deviations or percentiles (numerical variables).

RESULTS

A total of four hundred and ninety-eight cases which met the inclusion criteria were identified in the 12 year study period. Four hundred and twenty-four (85.1%) patients were male and seventy-four (14.9%) were female. The mean age of the patients was 58.6 years with a median of 59 years and an age range of 17 to 89 years. Twenty-five (5%) patients were under the age of 40 years at the time of diagnosis. The most common locations were larynx with 287 cases (57.6%) and lungs with 118 (23.7%) cases (Table 1).

Table 1. Number of cases and average age of patients according to diagnosis.

Topography	Total Cases	Average Age (Std Dev)	Median age	Basaloid squamous cell carcinoma	Degenerate malignant cells	Sarcomatoid carcinoma	Squamous cell carcinoma	Undifferentiated carcinoma	Verrucous carcinoma
Floor of mouth	3	58 (6.2)	56	1 (33.3%)			2 (66.7%)		
Larynx	287	59.2 (9.4)	59	3 (1.0%)			283 (98.6%)	1 (0.3%)	
Lung	118	60.8 (10.9)	60	1 (0.8%)	1 (0.8%)	1 (0.8%)	91 (77.1%)	24 (20.3%)	
Mediastinum	6	59.5 (10.8)	61		1 (16.7%)		5 (83.3%)		
Neck	3	59 (23.3)	63				2 (66.7%)	1 (33.3%)	
Nose	8	55.1 (13.1)	54				7 (87.5%)		1 (12.5%)
Oesophagus	2	54 (9.9)	54	1 (50%)			1 (50%)		
Paranasal sinuses	22	53 (11.3)	56.5				21 (95.5%)	1 (4.5%)	
Pharynx	44	52.4 (17.3)	57.5	1 (2.3%)			30 (68.2%)	13 (29.5%)	
Submandibular lymph node	3	62.7 (10)	62				3 (100%)		
Tongue	2	51 (9.9)	51				2 (100%)		
Average Age (Std Dev)				61 (17.4)	56 (2.8)	61	59.3 (9.7)	50.4 (19.3)	66
Median age				61	56	61	59	57	66
Grand Total	498			7 (1.4%)	2 (0.4%)	1 (0.2%)	447 (89.8%)	40 (8.0%)	1 (0.2%)

The most common diagnosis was that of squamous cell carcinoma with 447 cases (89.8%). This was followed by undifferentiated carcinoma with 40 cases (8%) (Table 1).

Only one case was positive with the anti-NUT antibody. The patient was a 30-year-old female with right middle lobe lung collapse and mediastinal and cervical lymphadenopathy. The histology was that of an undifferentiated carcinoma with focal abrupt keratinisation. The remaining 496 cases were negative. Seven cases showed non-specific staining and were confirmed as negative by and expert pathologist from Brigham and Women's Hospital in Boston (Figure 1).

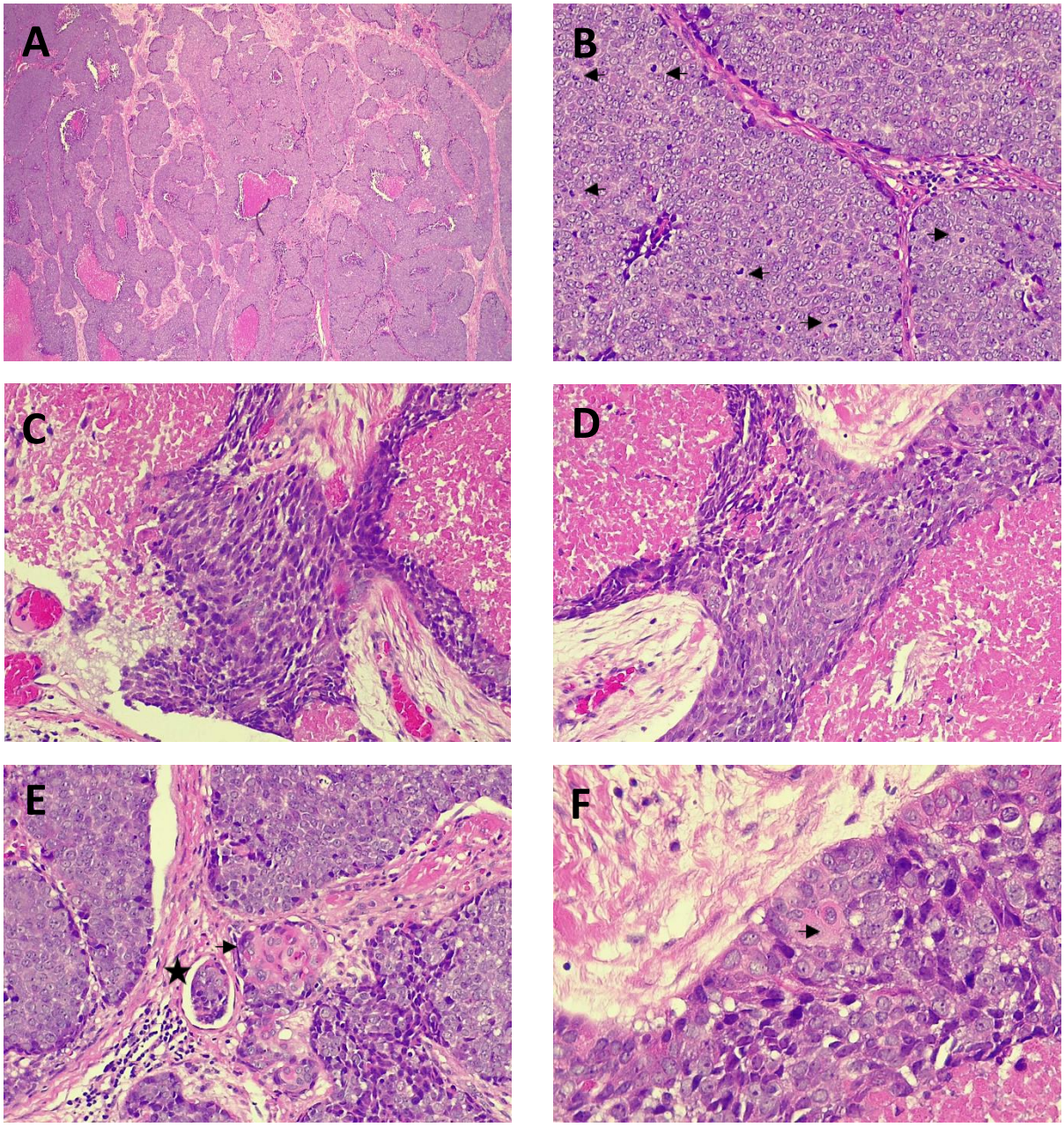

Figure 1. NUT Midline Carcinoma. (A) Low power view shows nests, sheets and trabeculae in a desmoplastic stroma with abundant necrosis. 2.5x magnification. (B) Undifferentiated cells with vesicular nuclei and conspicuous nucleoli. Note the abundant mitotic figures (arrows). 10x magnification. (C&D) Squamous differentiation noted focally. 10x magnification. (E) Cells with more eosinophilic cytoplasm as evidence of abrupt keratinization (arrow). Note the lymphovascular invasion (star). 20x magnification. (F) Single cell keratinization (arrow). 40x magnification.

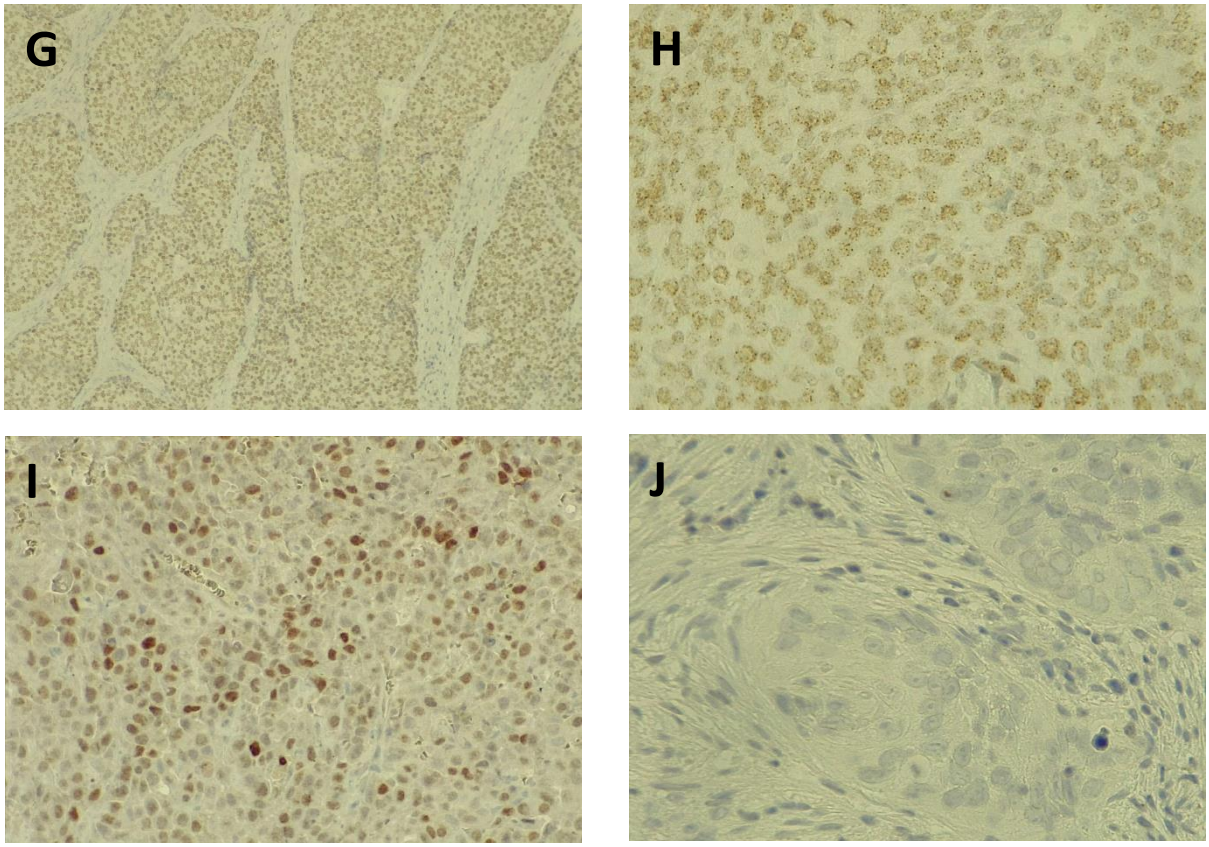

Figure 1. (continued). (G) NUT immunohistochemical stain. Note the diffuse pattern of staining. 40x magnification. (H) Stippled nuclear staining pattern considered positive. 40x magnification. (I) Non-specific staining with NUT. The staining is varied between cells and does not appear stippled. 20x magnification. (J) Negative NUT immunohistochemical stain. 40x magnification.

DISCUSSION

NMC is a recently discovered highly aggressive translocation-associated carcinoma.[1–4,6,7,22] It was initially diagnosed using fluorescent *in situ* hybridisation and an immunohistochemical stain only became available in 2009.[24] The histological features are those of an undifferentiated carcinoma with foci of abrupt keratinisation and the diagnosis can be missed if a high index of suspicion is not maintained with confirmation with NUT immunohistochemistry.[2,3,6,21,24] NMC also shows positivity for p63 and CK5/6 confirming squamous differentiation. Most are CK7 positive and they are often CD34 positive.[25] No in situ component has been identified and the cell of origin is unknown, most probably from a stem cell.[26] The majority of cases occur in midline locations such as the upper aerodigestive tract and mediastinum. However, cases have also been described involving numerous other sites such as pancreas, adrenal gland and bladder.[3,5,7,27] Patients often present with mass-

related symptoms and many have metastases at the time of diagnosis.[9,22,25] The most common metastatic sites are lymph node, bone and pleura.[2,25,28]

Although specific translocations are associated with a number of sarcomas, NMC is one of few translocation-associated carcinoma identified to date. Most carcinomas accumulate numerous mutations with time and have an extremely complex karyotype.[2,4,5,19,29]

Only one case of NMC was identified out of 498 cases evaluated in this study. According to Statistics South Africa (SSA), the Free State Province has a population of 2.8 million people of which 63.5% use public health care facilities which utilise this department for histology services.[30] This confirms the extremely rare nature of this tumour. The case showed the classical histological features with strong positivity on NUT immunohistochemistry and the diagnosis was made at the time of biopsy. The patient also conformed to the classical clinical profile of a young patient with a midline tumour.

The other 497 cases included in this study were all negative. This finding is reassuring as cases are not being misdiagnosed as squamous cell carcinoma or undifferentiated carcinoma. In addition, other tumours such as Ewing sarcoma, rhabomyosarcoma, rhabdoid tumour, desmoplastic small round cell tumour, olfactory neuroblastoma, melanoma, lymphoma, synovial sarcoma, undifferentiated nasopharyngeal carcinoma, thymic carcinoma or sinonasal undifferentiated carcinoma (SNUC) can also enter the differential diagnosis depending on the clinical setting.[2,3,6,21] It is also important to note that the NUT antibody can be positive in germ cell tumours, but the pattern of staining differs. Germ cell tumours stain only focally and in a diffuse nuclear manner, whereas NMC stains diffusely and in a granular manner.[24]

Seven of the cases showed non-specific staining with the antibody used in this study. Although this may result in incorrect classification as a NMC, careful evaluation allows for accurate interpretation as the specked pattern of staining is not evident.

CONCLUSION

NMC is a rare and highly aggressive carcinoma which can be misdiagnosed as squamous cell carcinoma or undifferentiated carcinoma if a high index of suspicion is not maintained. In the state sector of the Free State Province, NMC is not

underdiagnosed as only one case was identified. Our results corroborate the rarity of this tumour. In addition, our patient corresponds to initial studies profiling NMC patients as young patients with tumours occurring in the midline. Further studies are needed to establish the frequency in which NMC occurs in other provinces of South Africa and in the rest of Africa.

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APPENDIX A

Letter of approval from the Health Sciences Research Ethics committee

UFS·UV

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

30 August 2017

ANTOINETTE E ROETS
DEPT OF ANATOMICAL PATHOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Antoinette E Roets

HSREC 116/2017 (UFS-HSD2017/1164)
PRINCIPAL INVESTIGATOR: ANTOINETTE E ROETS
PROJECT TITLE: NUT MIDLINE CARCINOMA IN THE STATE SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

MODIFICATIONS REQUIRED

1. You are hereby kindly informed that, at the meeting held on 29 August 2017, the Health Sciences Research Ethics Committee (HSREC) reviewed the above research project. A decision could not be reached as there are modifications required to the protocol / outstanding requests from the HSREC. Please see below for details:

   1.1. Ethics clearance application:
   1.1.1. Data collection incorrectly stated to start 1 Jan 2005. This should just be changed to the period stipulated in the protocol.
   1.2. Documents checklist:
   1.2.1. The Head of Department is the supervisor, thus will need somebody else to give permission from the Department for research to be conducted.
   1.2.2. Uploaded certificate of MGPV7900 instead of GCP, although not necessary in this case.
   1.2.3. CV of co-supervisor not uploaded. Please submit.
   1.2.4. Proof of HPCSA registration of supervisor and co-supervisor not uploaded. Please submit.
   1.2.5. Multiple typing errors in protocol. Please review and resubmit.

Dr D Goedhals recused herself from the meeting for the duration of the discussion and decision of this project.

PLEASE NOTE: Upon receipt of the updated documentation/other request(s) from the HSREC in RIMS, the project will be re-considered.

Please highlight all changes made before resubmitting on RIMS.

If anything is unclear, please contact HSREC Administration.

2. Kindly use the **HSREC NR** as reference in correspondence to HSREC Administration.

3. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 46] (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite); Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines; Constitution of the HSREC of the Faculty of Health Sciences.

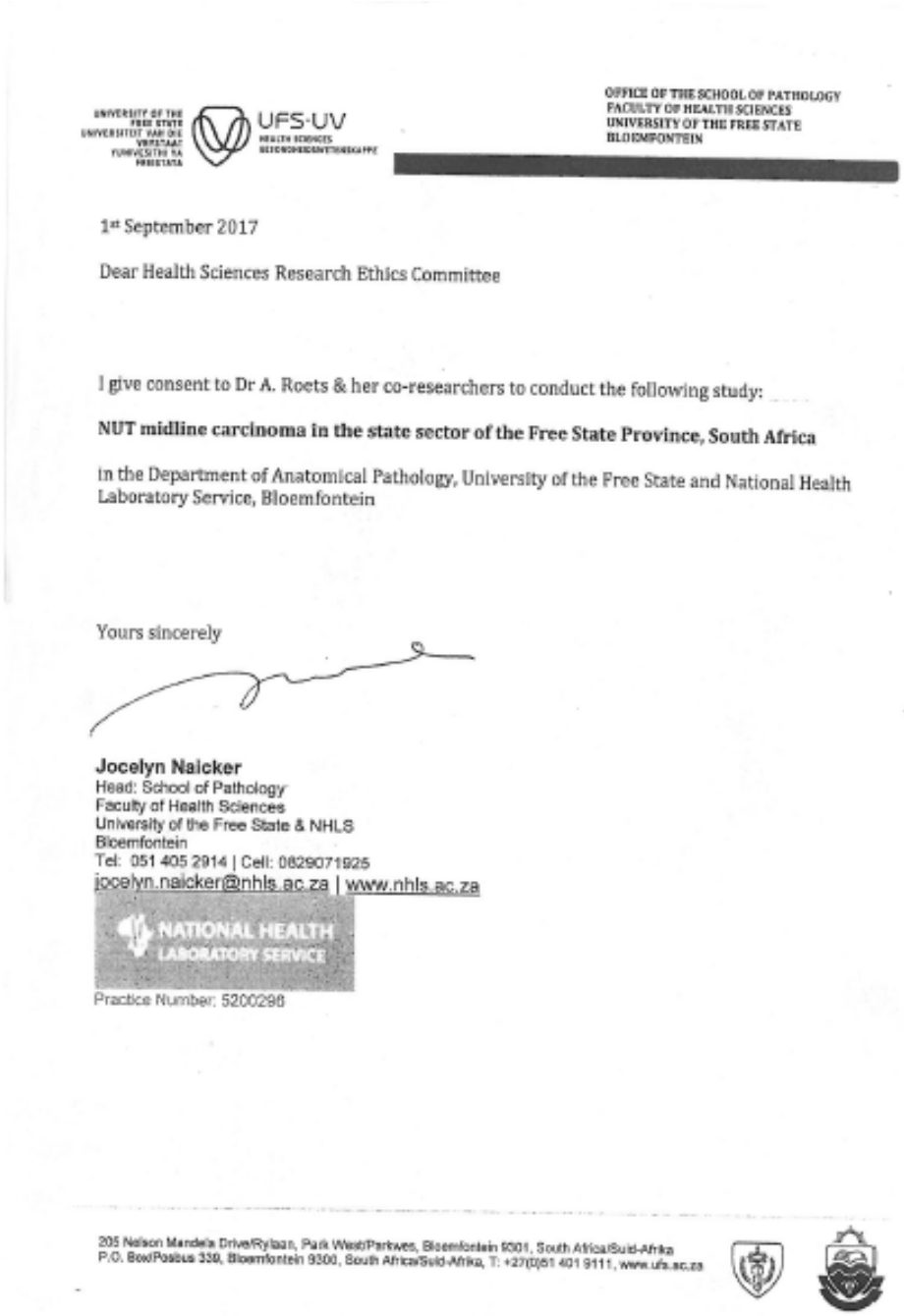
Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

APPENDIX B

Permission letter from the NHLS and Head of School of Pathology



UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES

OFFICE OF THE SCHOOL OF PATHOLOGY
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF THE FREE STATE
BLOEMFONTEIN

1st September 2017

Dear Health Sciences Research Ethics Committee

I give consent to Dr A. Roets & her co-researchers to conduct the following study:

NUT midline carcinoma in the state sector of the Free State Province, South Africa

in the Department of Anatomical Pathology, University of the Free State and National Health Laboratory Service, Bloemfontein

Yours sincerely

Jocelyn Naicker
Head: School of Pathology
Faculty of Health Sciences
University of the Free State & NHLS
Bloemfontein
Tel: 051 405 2914 | Cell: 0829071925
jocelyn.naicker@nhls.ac.za | www.nhls.ac.za

NATIONAL HEALTH LABORATORY SERVICE

Practice Number: 5200296

APPENDIX C

Permission letter from HOD

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES

27 July 2017

The Chairperson
Ethics Committee
Faculty of Health Sciences
University of the Free State

Dear Dr Le Grange

STUDY: **NUT midline carcinoma in the state sector of the Free State Province, South Africa**

This is to certify that Dr A Roets and co-workers have my permission to carry out the above mentioned study in this department.

PROF J GOEDHALS
HEAD: DEPT OF ANATOMICAL PATHOLOGY

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APPENDIX D

Copy of research protocol

NUT MIDLINE CARCINOMA IN THE STATE SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

RESEARCHERS:

Dr A Roets[1]
Prof G Joubert[2]
Prof J Goedhals[1]

[1]Department of Anatomical Pathology, University of the Free State and National Health Laboratory Service
[2]Department of Biostatistics, University of the Free State

INTRODUCTION

NUT midline carcinoma (NMC) is a rare malignant tumour originally described in 1991 (Kees et al, 1991). They are undifferentiated or poorly differentiated squamous cell carcinomas which occur in midline structures (French et al, 2008). These tumours are the result of a specific genetic mutation of the *NUT* (nuclear protein in testis) gene, involving a balanced translocation of chromosomes 15 and 19 t(15;19)(q13;p13.1) (French et al, 2008). In most cases *NUT* (present on chromosome 15) is fused to *BRD4* (on chromosome 19). In other cases, the *NUT* gene is fused to *BRD3*, a close homologue of *BRD4*, or an unknown gene (Parikh et al, 2013). Even though the precise function of *NUT* remains unknown, *BRD4* is responsible for the transcription of specific genes. The BRD-NUT fusion proteins inhibit both epithelial differentiation and cell cycle arrest, resulting in malignant change (French et al, 2008).

NMC does not arise from a specific organ and can occur anywhere in the trunk or head and neck typically in the midline. These tumours do not have a characteristic morphology and are often misdiagnosed as squamous cell carcinomas. They occur in both males and females of all ages although they were originally described in paediatric patients (French, 2014). In contrast to the majority of carcinomas, NMC has a simple karyotype and in most cases the NUT rearrangement is the only genetic abnormality present, similar to leukaemias, lymphomas and sarcomas (French, et al, 2008). Usual squamous cell carcinomas have numerous mutations secondary to constant exposure to carcinogens (French, 2014). These factors, in addition to the disease still being relatively unknown to many pathologists, and the limited availability of the specific immunohistochemical stain for NUT, results in the tumour being misdiagnosed or underdiagnosed. Thus the true incidence of NMC is therefore still unknown (French, 2010).

On routine histology NMC is composed of undifferentiated round blue cells and the differential diagnosis depending on the patients age and the topography of the tumour

includes poorly differentiated squamous cell carcinoma, sinonasal undifferentiated carcinoma, Ewing sarcoma, nasopharyngeal carcinoma, thymic carcinoma, neuroblastoma, small cell neuroendocrine carcinoma, salivary gland carcinoma, pancreatoblastoma, melanoma or lymphoma. In some cases foci of abrupt keratinization are present which would suggest the diagnosis. However, it is impossible to diagnose NMC on Haematoxylin and Eosin stained sections only (Solomon et al, 2015).

NMC is often positive for cytokeratins which are markers for epithelial differentiation. In addition, some unanticipated markers may also be positive, including NSE, TTF-1, CD56, CD138, S100, vimentin, CD99, FLI1, CD45, CD34, p16, CD117 and PLAP. The positivity of these unanticipated markers may result in an incorrect diagnosis being made (Solomon et al, 2015). The immunohistochemical stain for NUT is the only stain which can confirm the diagnosis of NMC with certainty with a characteristic speckled pattern of staining (French, 2013).

The prognosis of NMC is very poor with a median survival of 6.7months. Although chemotherapy has proved ineffective, early extensive surgery and radiation might offer increased survival rates (Bauer et al, 2012). There is currently extensive research and a number of clinical trials in progress to try to develop targeted therapy including a molecule inhibitor, targeting the BDR4-NUT fusion oncogene (Filippakopoulus et al, 2010).

Screening for NMC should be performed in all poorly differentiated carcinomas without glandular differentiation, especially in the region of the head, neck and torso. Squamous differentiation is not a prerequisite to screen for NMC and screening is deemed unnecessary in tumours with an established aetiology such as in cases which are Epstein-Barr virus and human papilloma virus positive. The diagnosis only requires positive nuclear staining in more than 50% of the tumour cells with the immunohistochemical stain for NUT (Bauer et al, 2012).

There is no published research regarding NUT midline carcinoma in South Africa. At present the NUT immunohistochemical stain required to diagnose NMC is not available in South Africa and suspected cases have to be sent to the United States of America for staining. This option only become available in 2016 and we could therefore not diagnose NMC before this.

AIM

The aim of the study is to determine the number and profile of patients with NMC in the state sector in the Free State Province.

Objectives:

3. To determine the number of cases of NMC seen over a twelve year period from January 2005 to December 2016 by the Department of Anatomical Pathology, University of the Free State and NHLS.
4. To evaluate the demographic features of patients diagnosed with NMC.

METHODS

A retrospective descriptive study will be performed. A SNOMED search of the NHLS electronic databases will be used to identify all malignant tumours of the head, neck and chest sent to the Department of Anatomical Pathology at the University of the Free State and NHLS over a twelve year period from 1 January 2005 to 31 December 2016. The department provides histology services to all state hospitals and clinics in the Free State Province. The DISA system will be searched for the years 2005 to 2014 and the Labtrak system will be searched for the years 2014 to 2016.

Inclusion criteria are as follows:

1. Undifferentiated malignant tumours
2. Tumours showing squamous differentiation
3. All ages
4. Males and females
5. Sufficient tissue available in archived wax blocks

Exclusion criteria:

1. Tumours with a specific diagnosis such as Ewing sarcoma, lymphoma etc
2. Tumours with neuroendocrine differentiation
3. Tumours with glandular differentiation
4. p16 positive tumours
5. EBER-ISH positive tumours

Once the cases have been identified, the slides and wax blocks will be retrieved from the departmental archives and the slides will be reviewed by Dr Roets together with Dr van der Westhuizen. If the cases meet the inclusion criteria then a representative wax block will be chosen. The wax blocks will be sectioned at 4µm and the sections placed on glass slides. The slides will be stained using an anti-NUT rabbit polyclonal antibody from Abcam. Slides will be stained using a Benchmark XT automated slide stainer. All reagents are pre-diluted, and ready to use. The slides will be counterstained with Mayers Haematoxylin, dehydrated and cover slipped. The slides will then be evaluated by Dr Roets together with Prof Goedhals to determine whether they are positive or negative. A stain is regarded as positive if there is speckled nuclear staining in more than 50% of the tumour cells. In addition the patient's age, sex, race, topography of the biopsy, results of other immunohistochemical stains and clinical presentation will be noted which will be obtained from the pathology report. We estimate that approximately 400 cases will meet the inclusion criteria. The data will then be captured in an Excel spreadsheet for statistical analysis (appendix 1).

TIME FRAME

The study should take approximately 15 months to complete once approval has been obtained from the Health Sciences Research Ethics Committee.

Submission for Ethics approval: August 2017
October – December 2017: Identification of cases

January – June 2018: Laboratory work
June – December 2018: Analysis and write up

STATISTICS

Statistical analysis will be performed by Prof G Joubert from the Department of Biostatistics, University of the Free State. Results will be summarized by frequencies and percentages (categorical variables) and means, standard deviations or percentiles (numerical variables).

FINANCIAL IMPLICATIONS

Anti-NUT antibody kit:	R8432.25 x 2
Total:	R16864.59

Funding for the anti-NUT antibody will be taken from Prof J Goedhals's research entity. Other reagents, slides and cover slips left over from a previous project will be used and therefore no additional funding is necessary.

ETHICAL ASPECTS

Each case will be allocated a unique study number by Prof J Goedhals for anonymity. None of the other investigators will have access to identifiers. Please see the signed letter from Prof Goedhals confirming that she will not release the identifiers to any other parties. Immunohistochemical stains, data collection and data analysis will be performed using only the unique study number in order to protect the participant's identities. Permission has been obtained from the National Health Laboratory Service. As only data from the NHLS pathology reports will be used it will not be necessary to obtain permission from the Free State Department of Health.

PROPOSED OUTCOME

The proposed project will be completed in fulfilment of the requirements for an MMed degree for Dr A Roets under the supervision of Prof J Goedhals. The results will be submitted for publication in a peer reviewed journal.

Should cases of NMC be identified then the NUT stain will be added to the diagnostic platform for future use.

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APPENDIX E

Data collection form

study number	Diagnosis	Age	Sex	Topography	IHC	Clinical presentation

APPENDIX F

Instructions for authors South African Journal of Oncology

Overview

The author guidelines include information about the types of articles received for publication and preparing a manuscript for submission. Other relevant information about the journal's policies and the reviewing process can be found under the about section. The **compulsory cover letter** forms part of a submission and must be submitted together with all the required **forms.** All forms need to be completed in English.

Original Research Article

An original article provides an overview of innovative research in a particular field within or related to the focus and scope of the journal, presented according to a clear and well-structured format. Systematic reviews should follow the same basic structure as other original research articles. The aim and objectives should focus on a clinical question that will be addressed in the review. The methods section should describe in detail the search strategy, criteria used to select or reject articles, attempts made to obtain all important and relevant studies and deal with publication bias (including grey and unpublished literature), how the quality of included studies was appraised, the methodology used to extract and/or analyse data. Results should describe the homogeneity of the different findings, clearly present the overall results and any meta-analysis.

Word limit	3500-4000 words (excluding the structured abstract and references)
Structured abstract	250 words to include a Background, Aim, Setting, Methods, Results and Conclusion
References	60 or less
Tables/Figures	no more than 7 Tables/Figure
Ethical statement	should be included in the manuscript
Compulsory supplementary file	ethical clearance letter/certificate

APPENDIX G

Summary from Turnitin