DEMOGRAPHIC AND GENETIC FEATURES OF GESTATIONAL TROPHOBLASTIC DISEASE IN THE PUBLIC SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

by

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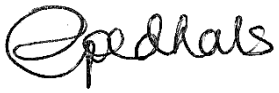
DECLARATION

I, Jacqueline Goedhals, hereby declare that the doctoral research thesis and interrelated publishable manuscripts that I herewith submit for the degree Philosophiae Doctor 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. Six of the patients used in Chapter 4 were previously utilised in a MMed dissertation under my supervision.

I, Jacqueline Goedhals, hereby declare that I am aware that copyright of this doctoral thesis is vested in the University of the Free State.



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Jacqueline Goedhals **Date**

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LIST OF ABBREVIATIONS

CHM	Complete hydatidiform mole
EDTA	Ethylenediaminetetraacetic acid
ETT	Epithelioid trophoblastic tumour
GTD	Gestational trophoblastic disease
GTN	Gestational trophoblastic neoplasia
H&E	Haematoxylin and eosin
hCG	Human chorionic gonadotropin
HIV	Human immunodeficiency virus
hPL	Human placental lactogen
PCR	Polymerase chain reaction
PHM	Partial hydatidiform mole
PLAP	Placental alkaline phosphatase
PSTT	Placental site trophoblastic tumour
SA	South Africa
SSA	Statistics South Africa
STR	Short tandem repeat
WHO	World Health Organization

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SUMMARY

Gestational trophoblastic disease (GTD) is a group of disorders derived from the placenta and includes hydatidiform mole, choriocarcinoma, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). As they are related to pregnancy, they predominantly occur in the reproductive years. These disorders are uncommon and choriocarcinoma, PSTT and ETT are very rare. Choriocarcinoma, PSTT and ETT can arise many years after a previous pregnancy and can therefore be difficult to diagnose. Rapid and accurate diagnosis is important as patients have a very good prognosis if appropriate treatment is provided timeously.

Data from South Africa (SA) are lacking, and the aim of this study was therefore to evaluate the local demographic and genetic features of patients with GTD seen in public health care facilities in the Free State Province to determine whether they conform to the available African and international literature. The demographic features were evaluated by performing a retrospective review of all cases of GTD diagnosed by the Department of Anatomical Pathology, National Health Laboratory Service and University of the Free State over a 10-year period. The department provides Anatomical Pathology services to all public sector health care facilities in the Free State Province of South Africa. In addition, all patients with GTD referred to the Department of Oncology, National District Hospital for clinical management were evaluated to determine whether a human immunodeficiency virus (HIV) positive status is a poor prognostic factor.

Two hundred and twenty-six patients were diagnosed in the 10 year period, 200 with hydatidiform moles (88.5%) and 26 with choriocarcinomas (11.5%). No PSTT or ETT were identified. The age of the patients and the presenting features were similar to that reported in available literature. The incidence of hydatidiform mole and choriocarcinoma was 0.4/1000 deliveries and 0.05/1000 deliveries respectively. This is extremely low and additional studies are required to determine whether this is a true reflection of the incidence or whether it may be due to non-referral of products of conception for histopathological confirmation.

This study confirmed that HIV positive patients with a CD4 count of less than 200 cells/μl have a statistically significantly worse prognosis than HIV positive patients with a CD4 count of more than 200 cells/μl and HIV negative patients ($p=0.03$). This is of clinical significance as the Free State Province has the second highest HIV prevalence in SA and 25.5% of

adults between the ages of 15 and 49 years are HIV positive.

To evaluate the genetic features of GTD, next generation sequencing for *NLRP7* and *KHDC3L* was performed on patients with a history of a previous hydatidiform mole and one or more additional episodes of reproductive wastage. These genes are maternal-effect genes and are associated with recurrent hydatidiform moles. One novel pathogenic *NLRP7* variant and two novel *NLRP7* variants of unknown clinical significance were identified. This is the first report of a pathogenic *NLRP7* variant in a South African patient. In the second part of the study, microsatellite genotyping was performed on 20 patients with choriocarcinoma as was successful in 18 cases. Microsatellite genotyping indicated the majority to be gestational choriocarcinomas (17/18), with only a single case being non-gestational (1/18). Sixteen of the gestational choriocarcinomas were secondary to a prior complete hydatidiform mole (CHM) while one was due to a previous normal pregnancy. Their origin proved to be critical as choriocarcinomas secondary to a prior CHM have the best prognosis and primary choriocarcinoma requires different chemotherapy.

The data obtained from this study will improve patient care for women with GTD and both the molecular techniques will be implemented onto the diagnostic platform after validation.

DEMOGRAPHIC AND GENETIC FEATURES OF GESTATIONAL TROPHOBLASTIC DISEASE IN THE PUBLIC SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Gestational trophoblastic disease (GTD) is composed of a group of disorders arising from placental trophoblastic tissue (Brown *et al.,* 2017). The current World Health Organization (WHO) classification of GTD consists of hydatidiform mole, choriocarcinoma, placental site trophoblastic tumour (PSTT), epithelioid trophoblastic tumour (ETT), miscellaneous trophoblastic lesions and abnormal (non-molar) villous lesions. Hydatidiform moles are further sub-classified as complete, partial and invasive while the miscellaneous trophoblastic lesions consist of exaggerated placental site and placental site nodule and plaque (Kurman *et al.*, 2014).

Placental site nodule and plaque and exaggerated placental site are non-neoplastic while hydatidiform moles have a potential for malignant transformation and choriocarcinoma, PSTT and ETT are classified as neoplasms (Kurman *et al.*, 2014). The malignant lesions are collectively grouped under the term gestational trophoblastic neoplasia (GTN) (Seckl, 2018).

During embryogenesis trophoblast arises from the trophoectoderm (Shih and Kurman, 2002; Sebire and Lindsay, 2010). Trophoblastic tissue can be divided into villous and extravillous trophoblast. Villous trophoblast covers the chorionic villi while all other trophoblast is classified as extravillous. There are three populations of trophoblast namely cytotrophoblast, syncytiotrophoblast and intermediate trophoblast (Shih and Kurman, 2001; Shih and Kurman, 2002; Cheung, 2003). Villous trophoblast is composed predominantly of cytotrophoblast and syncytiotrophoblast while extravillous trophoblast consists of intermediate trophoblast (Shih and Kurman, 2002). Hydatidiform mole and choriocarcinoma arise from villous trophoblast while PSTT and ETT arise from intermediate trophoblast (Shih and Kurman, 2002; Sebire and Lindsay, 2010).

Exaggerated placental site and placental site nodule and plaque are not included in studies

on GTD in the literature. They will therefore not be included in the scope of this dissertation apart from a brief mention later in this chapter.

1.1.1 Hydatidiform mole

A hydatidiform mole is an abnormal pregnancy characterized by poor or no fetal development, trophoblast proliferation and hydropic villi. The term hydatid, which means drop-like, was first used by Aetius of Amida, a physician in Justinian's court in the sixth century. Additional case reports were recorded in the following centuries including that of Margaret, Countess of Henneberg who delivered what appeared to be a hydatidiform mole in 1276 (Ober, 1959).

Hydatidiform moles can be divided into partial, complete and invasive moles. Partial hydatidiform mole (PHM) and complete hydatidiform mole (CHM) have different genetic origins as highlighted in Figure 1 (cf. Appendix D). PHM has a triploid chromosome constitution due to an extra haploid set of chromosomes. More than 90% of cases are due to fertilization of an ovum by two sperm. The rest of the cases are due to one haploid sperm fertilizing an ovum with reduplication of the paternal chromosomes or fertilization by one sperm which is diploid due to failure of meiosis I or II. Approximately 70% of PHM are 69,XXY while 27% are 69,XXX and the remainder are 69,XYY (Hui *et al.*, 2017). In contrast, the vast majority of CHM are androgenetic in origin and all 46 chromosomes are paternal. This may occur due to fertilization of an empty ovum by two separate sperms or by a sperm which undergoes division after penetration (Li *et al.*, 2002). A few patients with familial recurrent CHM have a biparental diploid karyotype with mutations in the *NLRP7*, *KHDC3L* or *PADI6* genes (Froeling and Seckl, 2014). In invasive mole the villi infiltrate the myometrium, blood vessels or extra-uterine tissue (Cheung, 2003).

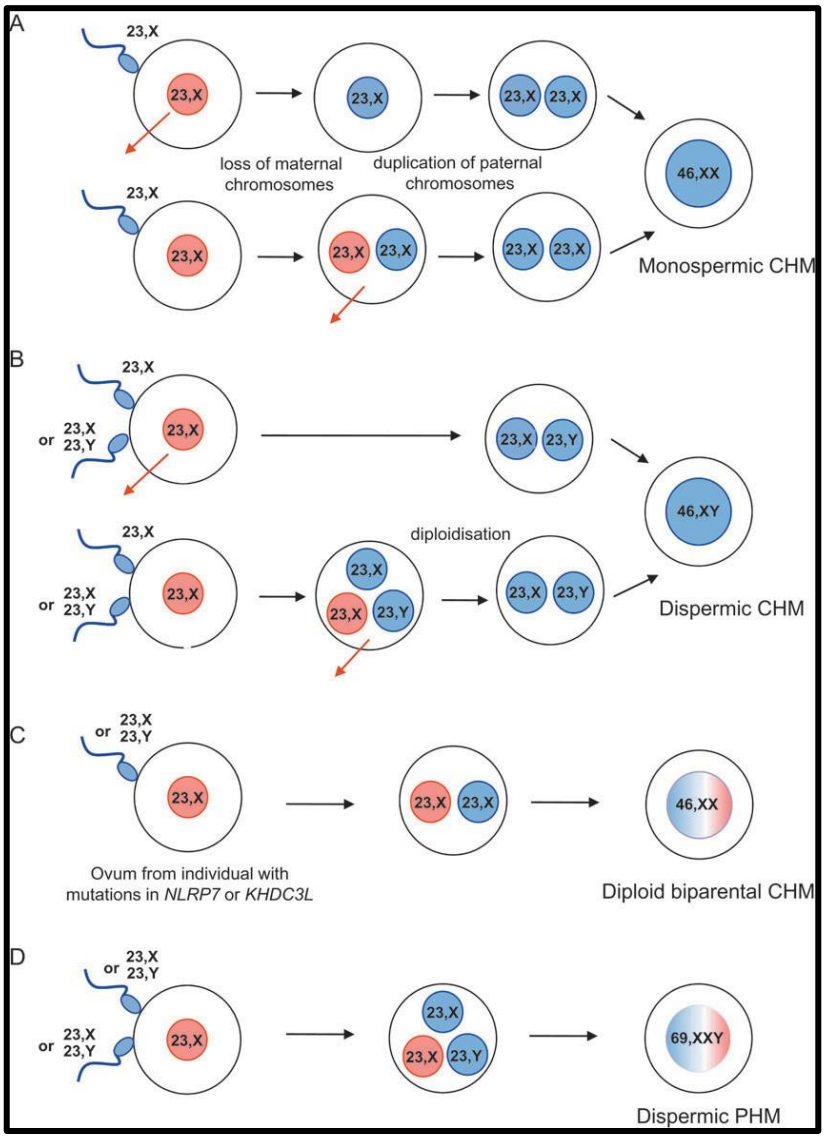

Figure 1.1: Genetic origins of molar pregnancies

A. Monospermic CHMs arise as a result of pre- or post-fertilisation loss of the maternal nuclear genome and duplication of the paternal genome. These androgenetic diploids are 46,XX, 46,YY conceptuses being presumed non-viable.

B. Dispermic CHMs arise as a result of two sperm fertilising an ovum from which the maternal nuclear genome is lost. These androgenetic diploid conceptuses may be 46,XX or 46,XY.

C. Biparental CHMs occur in females who are homozygous, or a compound heterozygote, for variants in *NLRP7* or *KHDC3L.* These biparental conceptuses are phenotypically CHM and may be 46,XX or 46,XY.

D. Dispermic PHMs arise as a result of fertilisation of a single ovum by two sperms. These diandric triploid conceptions may be 69,XXX, 69,XXY or 69,XYY. (Seckl *et al.*, 2013. Permission from Oxford University Press attached in Appendix D).

The evaluation of the incidence of GTD has been complicated by the use of different denominators including live births, pregnancies and deliveries. In addition, the incidence appears to increase when histology and molecular genotyping are used to diagnose these disorders (Bracken, 1987; Smith, 2003). Despite these limiting factors, hydatidiform moles appear to have the highest rates in South East Asia with 12/1000 pregnancies in Indonesia, India and Turkey. North America, Europe and Australia have rates of 0.5 to 1/1000 pregnancies. Data from South America and Africa are lacking (Smith, 2003; Steigrad, 2003). Moodley *et al.* (2003a), reviewed 112 cases of GTD seen at the King Edward Hospital in Durban and found an incidence of 1.2/1000 deliveries. In a later study, Moodley and Marishane (2005), reported an incidence of 1.16/1000 deliveries. Seven studies in Nigeria have reported incidences of between 0.8 and 6/1000 deliveries (Agboola, 1979; Agboola and Abudu, 1984; Egwuatu and Ozumba, 1989; Mayun *et al.*, 2008; Audu *et al.*, 2009; Mbarara *et al.*, 2012; Kolawole *et al.*, 2016). Two studies from Uganda reported incidences of 1.03 and 3.42/1000 deliveries respectively, whereas for Morocco it was 4.3/1000 deliveries (Leighton, 1973; Kaye, 2002; Boufettal *et al.*, 2011).

Risk factors for the development of hydatidiform mole include maternal age, a previous molar pregnancy and ethnicity. Teenagers and women over 35 years of age have a higher risk, with the risk peaking at five-times higher for women over 40. The relative risk of a woman with a previous molar pregnancy is up to 40 times that of the rest of the population (Steigrad, 2003). The rates of molar pregnancy also differ depending on ethnicity, with Indians currently having the highest risk (Steigrad, 2003).

In the past, patients often presented with symptoms such as hyperemesis, anaemia, pre-eclampsia, hyperthyroidism and respiratory distress (Froeling and Seckl, 2014). Presently many patients present with abnormal vaginal bleeding early in pregnancy and are diagnosed with a molar pregnancy on ultrasound. The classic sonar features are those of a snowstorm appearance, but this is only seen in the second trimester. The characteristic features are less specific during the first trimester (Froeling and Seckl, 2014). Macroscopically CHM is characterized by numerous vesicular structures representing extremely hydropic chorionic villi giving the tissue a so called 'bunch of grapes' appearance (cf. Figure 1.2). There are no fetal structures present. In contrast, a fetus or fetal parts are often present in cases of PHM and there are fewer and less well-developed vesicular structures (Cheung, 2003).

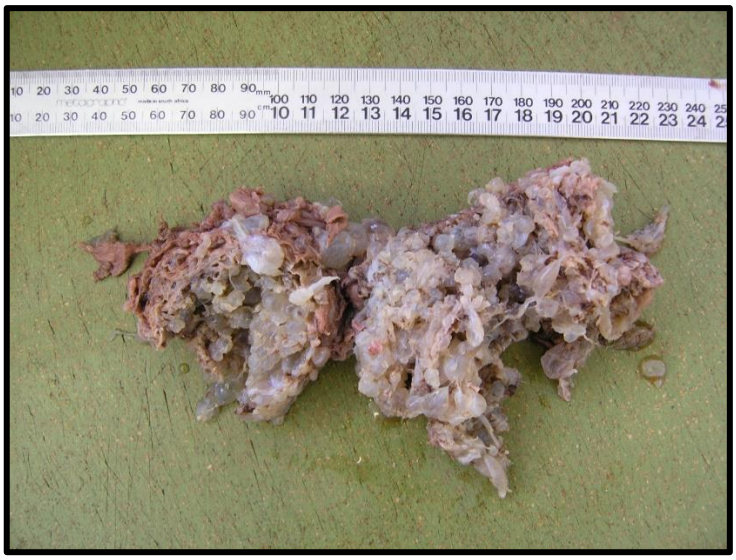

Figure 1.2: Macroscopic appearance of a complete hydatidiform mole demonstrating numerous vesicular structures representing hydropic chorionic villi

Histologically CHM is characterised by large, hydropic villi with non-polar trophoblast proliferation and cistern formation (cf. Figure 1.3a). Early CHM presenting at less than 12 weeks gestation has smaller, abnormally shaped villi which are not markedly hydropic. The villous stroma is hypercellular with karyorrhectic debris (Buza and Hui, 2012). PHM has a dual population with large hydropic villi and normal-sized villi which may be fibrotic (Shih and Kurman, 2002) (cf. Figure 1.3b). The large hydropic villi have an irregular scalloped appearance. Fetal vessels and red blood cells are often seen (Buza and Hui, 2012).

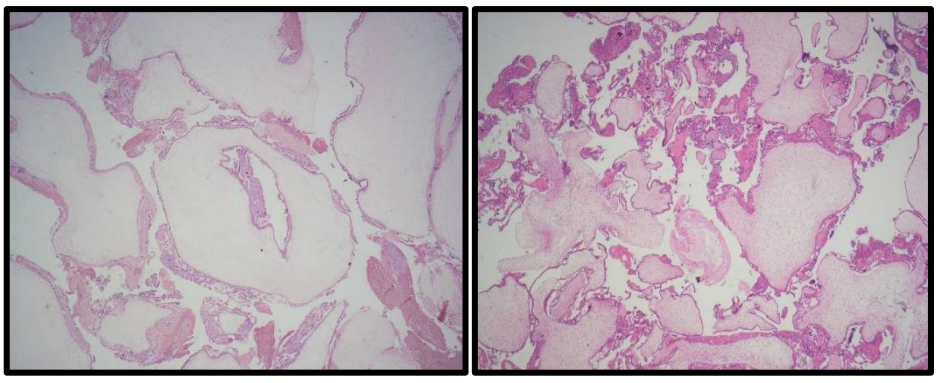

Figure 1.3: (a) Complete hydatidiform mole with markedly hydropic villi with non-polar trophoblast proliferation; (b) Partial hydatidiform mole with a dual population of villi with large hydropic villi and smaller fibrotic villi

It is important to distinguish between PHM and CHM as CHM has a 15% to 20% risk of developing persistent GTD while PHM has a 0,2% to 4% chance (Shih and Kurman, 2002; Vang *et al.*, 2012). It is also important to distinguish between non-molar pregnancies and PHM as PHM requires follow-up with serum β-human chorionic gonadotropin (β-hCG) levels (Li *et al.,* 2002). Numerous studies have demonstrated that there is marked inter-observer

variability in the diagnosis of hydatidiform moles when based only on haematoxylin and eosin (H&E) stained sections (Vang *et al.,* 2012). A number of ancillary techniques, including immunohistochemistry for p57, fluorescence *in situ* hybridization (FISH), flow cytometry and molecular genotyping, are available to improve diagnostic accuracy. p57 immunohistochemistry can be used to distinguish CHM from PHM and non-molar pregnancies as CHM lacks p57 expression while it is retained in PHM and non-molar pregnancies (McConnell *et al.*, 2009) (cf. Figure 1.4). Unfortunately, it cannot be used to differentiate between non-molar pregnancies and PHM.

Flow cytometry can be used to determine ploidy while FISH can be used to determine sex chromosome and copy number. Neither can distinguish between maternal and paternal origin (Nguyen *et al.*, 2018). Molecular genetic analysis can be used to identify androgenetic diploidy, diandric triploidy and biparental diploidy to diagnose CHM, PHM and non-molar pregnancies respectively (McConnell *et al.*, 2009). However, it is expensive and not economically viable to perform it on every specimen. McConnell *et al.* (2009)*,* developed a diagnostic algorithm for the evaluation of products of conception having any features suggestive of a hydatidiform mole. Evaluation of H&E slides is followed by p57 immunohistochemistry. If the morphological features are suggestive of a CHM and the p57 is negative, a diagnosis of CHM can be made. Cases with features suggestive of a mole and having a positive or equivocal p57 result should be referred for molecular genotyping although this is not an option in many centres.

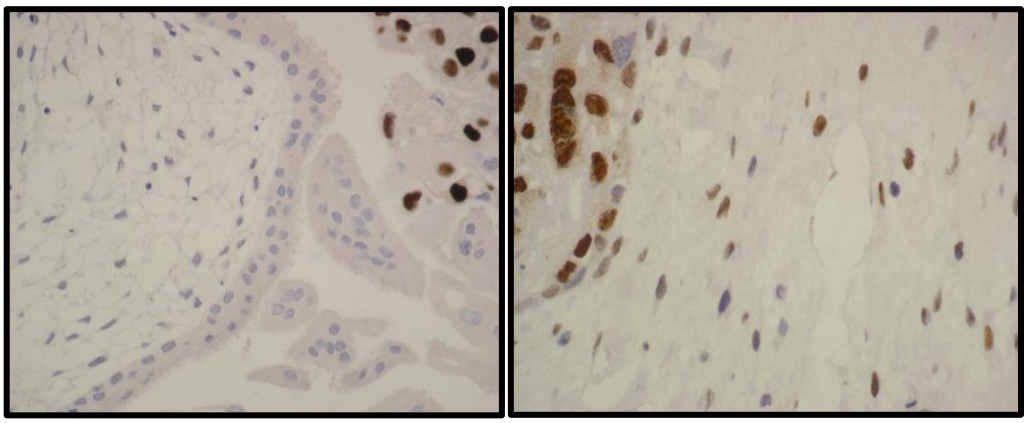

Figure 1.4: (a) CHM with negative p57 staining in the villous stroma. The positivity in the trophoblast serves as an internal control; (b) PHM with positive staining in the villous stroma

1.1.2 *NLRP7* and *KHDC3L* mutations in hydatidiform mole

Pathogenic variants in *NLRP7* and *KHDC3L* (*C6orf221*) genes have been identified as causative for familial recurrent hydatidiform mole (Andreasen *et al.*, 2013). Recurrent

hydatidiform mole is defined as two or more hydatidiform moles in the same patient. This occurs in 1-9% of patients with a previous hydatidiform mole (Qian *et al.*, 2018). *NLRP7* and *KHDC3L* are maternal-effect genes and their expression is required for normal embryo development (Manokhina *et al.*, 2013). In addition, *NLRP7* variants are also associated with recurrent spontaneous abortions, still births and intrauterine growth restriction (Murdoch *et al.,* 2006). *NLRP7* was originally mapped to chromosome 19q13.3-13.4 (Moglabey *et al.*, 1999), and identified by Murdoch *et al.* in 2006. *NLRP7* is a member of a family of genes termed nucleotide-binding, leucine-rich repeat, pyrin domains. The other members of this family are involved in inflammation and innate immunity (Hayward *et al.*, 2009). *NLRP7* consists of an N-terminal pyrine domain, 9-10 leucine-rich repeats, a NACHT-associated domain (NAD) and a NACHT region. It encodes for a protein of 1037 amino acids (Moein-Vaziri *et al.*, 2018). *KHDC3L* or KH domain containing 3-like gene was identified in 2011 and mapped to chromosome 6 (Parry *et al.*, 2011). *KHDC3L* has 3 exons and encodes for a protein consisting of 217 amino acids (Moein-Vaziri *et al.*, 2018). Figure 1.5 (cf. Appendix E), provides a schematic representation of both genes with previously described variants.

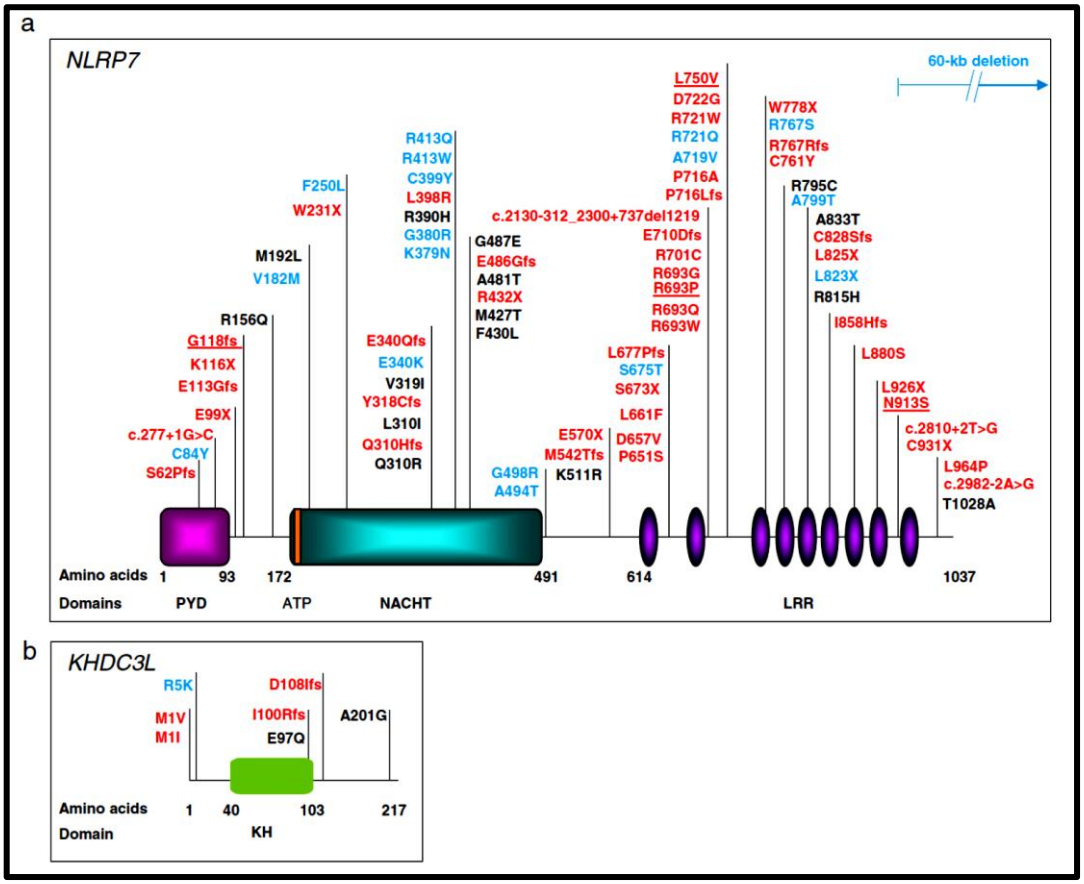

Figure 1.5: Schematic representations of *NLRP7* and *KHDC3L* protein structures with identified mutations and non-synonymous variants in patients with hydatidiform moles and reproductive loss

A. *NLRP7* protein structure with its domains. PYD = pyrin domain; NACHT == domain present in NAIP, CIITA, HET-E, and TP1 family proteins; ATP = 5'-triphosphate binding motif; LRR = leucine-rich repeats. The ATP binding domain is a small motif of 8 amino acids and starts at position 178.
B. *KHDC3L* protein structure with identified variants and non-synonymous variants. KH stands for K homology domain. Variant nomenclature is according to the Human Genome Variation Society guidelines (http://www.hgvs.org/mutnomen/recs.html). Variants found in patients with two defective alleles are in red. Non-synonymous variants (NSVs) found only in patients in heterozygous state and not in controls are in blue. NSVs found in patients and in subjects from the general population are in black. Variants found in patients who had at least one live birth are underlined (Nguyen and Slim, 2014; Permission from Springer attached in Appendix E).

Pathogenic variants of *NLRP7* are present in 48-80% of patients with recurrent hydatidiform moles with more than 60 pathogenic variant described thus far. For *KHDC3L*, deleterious variants have been recorded in 10-14% of cases negative for NLRP7, with 6 pathogenic variants described (Parry *et al.,* 2011; Reddy *et al.*, 2013; Reddy *et al.*, 2016; Nguyen *et al.,* 2018)

The majority of mutations described to date are in Caucasian or Asian women. *NLRP7* mutations have been also reported in Tunisian, Senegalese, Egyptian and Moroccan women while *KHDC3L* mutations have been described in women of Tunisian and African American origin (Puechberty *et al.*, 2009; Landolsi *et al.*, 2011; Parry *et al.*, *2011*; Landolsi *et al.*, 2012; Slim *et al.,* 2012; Reddy *et al.*, 2013). No cases of *NLRP7* or *KHDC3L* mutations have been described in South African patients.

A third gene, *PADI6*, has recently been identified. It was originally described as a cause of female infertility characterized by early embryonic arrest (Xu *et al.*, 2016). In 2018, it was linked to hydatidiform mole when biallelic missense variants were noted in a family of Han Chinese origin (Qian *et al.*, 2018). *PADI6* is located on chromosome 1p36.13. It encodes a protein involved in the subcortical maternal complex which is necessary for embryonic progression past the 2-cell stage in mice (Xu *et al.*, 2016).

1.1.3 Choriocarcinoma

Choriocarcinoma is the most common malignant trophoblastic tumour and occurs predominantly in pre-menopausal women with a mean age of 30 years (Kaur and Sebire*,*

2018; Hui, 2019). The first definite report of choriocarcinoma was a series of three cases presented by Hans Chiari in 1877. Felix Marchand (1846-1928) was the first person to recognise that these lesions arose from the placenta although a number of years passed before this was widely accepted by the medical community (Ober, 1959).

Choriocarcinomas can be divided into gestational or non-gestational depending on their origin. The majority of choriocarcinomas develop from pregnancies including molar pregnancies, induced and spontaneous abortions, ectopic pregnancies and term or pre-term deliveries. These are called gestational or secondary choriocarcinoma (Zhao *et al.*, 2009). There is a 1000 times greater risk of developing a choriocarcinoma after a CHM than after a non-molar pregnancy (Hoffner and Surti*,* 2012). A few choriocarcinomas are not related to pregnancy and are called non-gestational or primary choriocarcinoma (Zhao *et al.*, 2009). Primary choriocarcinoma arises from germ cells in the ovaries or in extragonadal midline sites such as the retroperitoneum and mediastinum (Cheung *et al.*, 2009).

Determining and comparing the incidence of choriocarcinoma is difficult as different denominators are used in various studies including pregnancies, deliveries and live births. However, similar to hydatidiform mole, the incidence of choriocarcinoma is highest in India and Indonesia with rates of 19.1 and 15.3/1000 pregnancies and lowest in North America, Europe and Australia with rates of up to 0.7/1000 pregnancies (Steigrad, 2003; Smith, 2003). Studies from Nigeria reported an incidence of between 1 and 5/1000 deliveries while a study from Uganda reported an incidence of 0.3/1000 deliveries (Leighton, 1973; Agboola and Abudu, 1984; Mbarara *et al.*, 2012; Mayun *et al.*, 2012; Kolawole *et al.*, 2016). Moodley *et al.* reported incidences of 0.5/1000 deliveries and 1.07/1000 deliveries in two studies from KwaZulu-Natal, South Africa (Moodley *et al.,* 2003a; Moodley and Marishane*,* 2005).

Patients present with abnormal vaginal bleeding or with symptoms related to metastatic disease including severe haemorrhage in metastatic sites (Cheung, 2003; Froeling and Seckl, 2014). The most common sites of metastases are lung, brain and liver (Cheung, 2003). The serum β-hCG is markedly raised and is almost always more then 10 000 mIU/L (Kaur and Sebire*,* 2018). Most choriocarcinomas are located in the uterus but occasional cases occur in extra-uterine sites such as fallopian tube and ovary (Kaur and Sebire*,* 2018).

On gross examination one or more dark red tumour masses are noted with extensive haemorrhage and areas of necrosis (Kaur and Sebire*,* 2018). Microscopically both forms of choriocarcinoma are characterized by a biphasic pattern with central areas of mononuclear

cytotrophoblast surrounded by multinucleated syncytiotrophoblast (cf. Figure 1.6). The tumour cells are predominantly found at the edge of the lesion with central haemorrhage and necrosis (Cheung, 2003). Intraplacental choriocarcinomas can also occur (Seckl *et al.,* 2010). These usually present as a red nodule in a third trimester placenta which can mimic an infarct or intervillous thrombus. In approximately 60% of cases, the patient will already have metastatic disease and metastases to the fetus can also occur (Sebire and Lindsay*,* 2010). Immunohistochemically choriocarcinomas are positive for AE1/AE3 and hCG is strongly and diffusely positive in the syncytiotrophoblast. The Ki67 index is usually more than 90% (Kurman *et al.*, 2014).

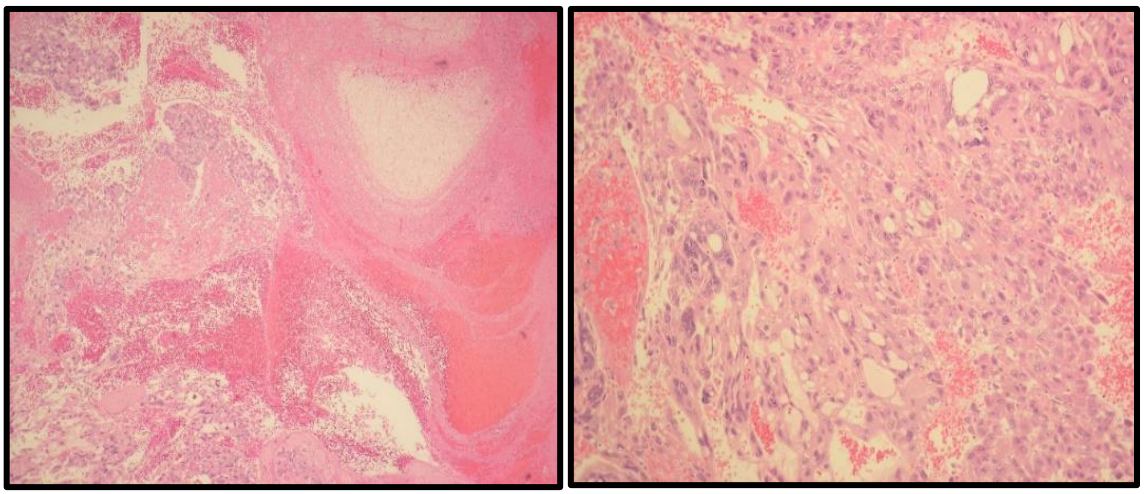

Figure 1.6: H&E sections of a choriocarcinoma demonstrating the mixture of cytotrophoblast and syncytiotrophoblast with large areas of haemorrhage and necrosis

Gestational and non-gestational choriocarcinomas have different genetic origins and they also differ with regards to sensitivity to chemotherapy and prognosis. Identification of these two types is therefore important in order to select the correct therapy and to evaluate the prognosis (Fisher *et al.,* 2007; Zhao *et al.,* 2009). In cases of gestational choriocarcinoma, it is important to determine the nature of the original pregnancy as the prognosis of choriocarcinoma following a molar pregnancy is better than that following a non-molar pregnancy (Zhao *et al.*, 2009). It has also been shown that the pregnancy immediately prior to the development of the choriocarcinoma may not be the causative pregnancy in some cases (Zhao *et al.*, 2009).

The karyotype of gestational trophoblastic tumours should reflect that of the pregnancy from which they originated. After a live birth or spontaneous abortion both maternal and paternal DNA should be present while after a CHM only paternal DNA will be noted. A triploid karyotype is indicative of a previous PHM. The absence of paternal DNA in the tumour is

characteristic of non-gestational tumours (Arima *et al.,* 1995).

Until recently the clinical history, clinical presentation and histological features were used to categorize choriocarcinoma as gestational or non-gestational (Cankovic *et al.*, 2006). However, these findings do not always enable the correct classification. Microsatellite (short tandem repeat [STR]) profiling can be used in genotyping choriocarcinomas to distinguish non-gestational from gestational tumours and to identify the causative pregnancy of gestational choriocarcinoma so that the correct treatment can be implemented (Cankovic *et al.*, 2006; Fisher *et al.,* 2007). STR markers are polymorphic DNA loci that contain a repeated nucleotide sequence. The STR repeat unit can be from two to seven nucleotides in length. The number of nucleotides per repeat unit is the same for a majority of repeats within an STR locus. The number of repeat units at an STR locus may differ, so alleles of many different lengths are possible. These STR markers are unique to an individual and are stably inherited. They are routinely used for forensic human identification and paternity testing (Cankovic *et al.*, 2006).

Although choriocarcinomas are extremely aggressive malignant tumours, gestational choriocarcinomas respond well to chemotherapy and the overall cure rate is more than 90% in cases where the appropriate treatment is given (Kaur and Sebire*,* 2018).

1.1.4 Placental site trophoblastic tumour

PSTT is a rare tumour arising from implantation site intermediate trophoblast (Shih and Kurman, 2001; Santoro *et al.*, 2017). It was originally described in 1976 under the name trophoblastic pseudotumour (Kurman *et al.*, 1976). It usually occurs in women of reproductive age and most patients present with amenorrhoea or abnormal bleeding. The majority of cases are associated with a preceding normal pregnancy or a miscarriage and β-hCG levels are usually low (Shih and Kurman, 2001; Zhao *et al.,* 2016). The average time between the preceding pregnancy and the development of the PSTT is between 18 and 36 months (Sebire and Lindsay, 2010).

Macroscopically PSTT can present as a poorly circumscribed mass or a well circumscribed nodule in the myometrium which may protrude into the endometrial cavity (Cheung, 2003). Microscopically they are composed of mononuclear trophoblastic cells with variable nuclear atypia interspersed by occasional multinucleate cells. The cells can be polygonal, round or spindle-shaped with an invasive growth pattern. There is usually prominent extracellular

eosinophilic fibrinoid material present between the tumour cells (Shih and Kurman, 2001; Sebire and Lindsay, 2010; Horowitz *et al.*, 2017). The ki67 index varies between 10 and 20% (Santoro *et al.*, 2017). PSTT is positive for cytokeratin, inhibin-α, human placental lactogen (hPL) and Mel-CAM (CD146) but is usually negative or only focally positive for β-hCG (Shih and Kurman, 2001).

The differential diagnosis includes other forms of GTD and non-trophoblastic tumours such as epithelioid smooth muscle tumours, poorly differentiated carcinomas and melanomas (Shih and Kurman, 2001). Shih and Kurman (2001) used a panel of markers including cytokeratin 18, HLA-G, hPL, hCG, p63 and Ki67 to distinguish between PSTT, ETT, choriocarcinoma, exaggerated placental site and placental site nodule. Cytokeratin 18 and HLA-G are used to confirm the trophoblastic nature of the tumour. This is then followed by stains for hPL, p63, hCG and ki67. If the p63 is positive in the cytotrophoblast and the hCG is positive in syncytiotrophoblast then a diagnosis of choriocarcinoma can be made. If the p63 is diffusely positive and the hPL is only focally positive, then the lesion is either an ETT or a placental site nodule. A Ki67 of more than 10% is compatible with an ETT while a Ki67 of less than 10% is indicative of a placental site nodule. If the p63 is negative and the hPL is diffusely positive, then the lesion is either a PSTT or an exaggerated placental site. A Ki67 of more than 1% confirms a diagnosis of PSTT while a Ki67 of less than 1% confirms a diagnosis of exaggerated placental site (Shih and Kurman, 2004). Smooth muscle tumours will be positive for actin, desmin and h-caldesmon and lack the fibrinoid material seen in PSTT. Carcinomas will be negative for hPL and inhibin-α while melanomas will be positive for S100, HMB45 and melan-A (Shih and Kurman, 2001).

Between 15 and 20% of PSTTs develop either local recurrence or metastasis (Sebire and Lindsay, 2010). Poor prognostic factors include metastatic disease and an interval of more than four years between the preceding pregnancy and the development of the tumour (Hassadia *et al.*, 2005). Patients age of more than 35 years, deep myometrial invasion, tumours with high grade histological features and high β-hCG levels have also been proposed as poor prognostic factors (Santoro *et al.*, 2017).

1.1.5 Epithelioid trophoblastic tumour

The term ETT was originally suggested by Shih and Kurman in 1998 (Shih and Kurman, 1998). This tumour arises from chorionic-type intermediate trophoblast and occurs mainly in women of reproductive age (Shih and Kurman, 2001). In the majority of cases it follows

a previous term pregnancy but can also occur following a miscarriage or molar pregnancy (Horowitz *et al.*, 2017). The ETT may arise up to 18 years after the antecedent pregnancy. Most patients present with vaginal bleeding and a raised β-hCG although the β-hCG levels are much lower than those found in cases of choriocarcinoma (Shih and Kurman, 2001).

ETT presents as a nodule in the uterine wall, lower segment or endocervix and can measure up to 5 cm in diameter. Microscopically they are composed of relatively monomorphic intermediate trophoblastic cells arranged in nests, cords and solid sheets with intervening hyaline-like matrix and necrosis. The number of mitoses ranges from 0 to 9 per 10 high power fields and the Ki67 index ranges from 10 to 25% (Shih and Kurman, 1998; Shih and Kurman, 2001). ETT shows diffuse positivity for inhibin-α, cytokeratin and placental alkaline phosphatase (PLAP) and weak focal staining for hCG and hPL (Sebire and Lindsay, 2010).

ETT must be distinguished from cervical squamous cell carcinoma as the hyaline-like matrix may be misdiagnosed as keratin. A lack of intercellular bridges and the presence of decidualised stroma favour a diagnosis of ETT. Immunohistochemical stains for cytokeratin 18 and inhibin-α can be helpful as ETT is positive for both markers while cervical squamous cell carcinoma is negative (Shih and Kurman, 2001, Allison *et al.*, 2006). In addition, it can be confused with other forms of GTD including placental site nodule, PSTT and choriocarcinoma.

The prognosis of ETT is similar to that of PSTT. Poor prognostic factors include extrauterine disease and an interval of more than four years between the antecedent pregnancy and the development of the tumour (Davis *et al.*, 2015).

1.1.6 Exaggerated placental site

Exaggerated placental site was originally termed syncytial endometritis and consists of extensive infiltration of the myometrium by implantation site intermediate trophoblast. The cut off between a normal placental site and an exaggerated placental site is not clearly defined. It can occur with a normal pregnancy, abortion or hydatidiform mole (Cheung, 2003; Sebire and Lindsay, 2010).

Microscopically it consists of extensive infiltration of the endometrium and myometrium by single cells and small groups of intermediate trophoblast. However, the placental bed architecture is maintained. There is no necrosis and the Ki67 index is less than 1% (Shih

and Kurman, 2001). Exaggerated placental site is non-neoplastic and is important to recognise as it can be confused with PSTT (Shih and Kurman, 2001).

1.1.7 Placental site nodule and plaque

Placental site nodules are usually incidental findings in women of reproductive age in endometrial and cervix biopsies as well as hysterectomy specimens. They can occur in the endometrium, endocervix and even in the fallopian tube (Shih and Kurman, 2001).

Placental site nodules present as a well-circumscribed nodule composed of mononuclear intermediate trophoblastic cells in a hyaline-like or fibrinoid matrix. No infiltration of the endometrium, myometrium or blood vessels is present (Shih *et al.*, 1999). Immunohistochemical stains for cytokeratin, inhibin-α and PLAP are positive and the Ki67 index is less than 5% (Sebire and Lindsay, 2010).

These lesions are non-neoplastic but are important as they may be misdiagnosed as a PSTT, ETT or cervical squamous cell carcinoma (Shih and Kurman, 2001).

1.1.8 GTD and human immunodeficiency virus infection

Limited data are available with regards to the effect of human immunodeficiency virus (HIV) status on the outcome of GTD. In 1992, Ojwang *et al.* published three cases of gestational trophoblastic disease in HIV positive patients with an aggressive clinical course and they proposed that HIV infection be regarded as a poor prognostic risk factor (Ojwang *et al.*, 1992). These cases together with seven additional case reports are listed in Table 1.1.

Table 1.1: Published case reports of GTD in patients with HIV

	Age	Type of GTD	CD4 count	Outcome
Ojwang *et al.,* 1992	24 years	Choriocarcinoma	Unknown	Responding to treatment
	35 years	Choriocarcinoma	Unknown	Lost to follow up after 9 months
	26 years	Choriocarcinoma	Unknown	Lost to follow up after 1 month
Tangtrakul *et al.*, 1998	24 years	Choriocarcinoma	404	Complete remission
Moodley and Moodley, 2001	20 years	Choriocarcinoma	799	Complete remission
Ashley, 2002	26 years	Choriocarcinoma	173	Died
Moodley and Moodley, 2003b	24 years	Persistent molar pregnancy	156	Complete remission
Moodley, 2007	27 years	PSTT	238	Complete remission
Barnardt and Relling, 2015	33 years	Choriocarcinoma	290	Died
	20 years	Choriocarcinoma	200	Died

In 2003, Moodley and Moodley performed a retrospective analysis of 41 patients with choriocarcinoma of which 12 were HIV positive. They found that none of the HIV infected patients who received chemotherapy died due to the choriocarcinoma while two patients who did not receive chemotherapy due to low CD4 counts both died. They proposed that HIV infected patients with a CD4 count of >200 cells/µl should receive standard treatment (Moodley *et al.*, 2003c).

A second series of 78 patients with GTD of which 23 had choriocarcinoma was published in 2009. Twenty four of the 78 patients were HIV positive, of which eight had a CD4 count of <200 cells/µl and seven died. They suggested that HIV positivity with a low CD4 count should be included as a poor prognostic factor in patients with GTD (Moodley *et al.,* 2009).

In 2011, a series of 76 patients was published of which 14 were HIV positive. Forty-four of the patients had a hydatidiform mole and 21 had choriocarcinomas. Of the 13 patients who died due to GTD, five were HIV positive. The overall five-year survival for HIV positive patients was 64,3% versus 85% for the HIV negative and HIV unknown groups. This was not statistically significant (p=0.141) (Tayib *et al.,* 2011).

A recent article reviewing 63 patients with trophoblastic disease found that HIV positive patients presented at a higher stage than HIV negative patients (p=0.023). However, all the HIV positive patients had a CD4 count of ≥200 cells/µl and there was no significant difference in the survival (Makhathini *et al.*, 2019).

1.1.9 Staging, stratification and treatment of patients with GTD

Hydatidiform moles produce β-hCG and this can be used in the management of the disorder. Following the diagnosis of a molar pregnancy a suction curettage is the initial treatment of choice in most cases. After this, the patient's β-hCG level should be monitored and a plateau or rising level is indicative of malignant change termed persistent gestational trophoblastic disease (Seckl *et al.*, 2010). All patients with persistent GTD and choriocarcinoma should be staged using the International Federation of Gynaecology and Obstetrics (FIGO)/WHO scoring system (Ngan *et al.*, 2012; Seckl *et al.*, 2013). This system predicts the possibility for the development of resistance to single agent chemotherapy using either methotrexate or actinomycin D. A score of 0-6 is indicative of low risk disease while a score of ≥7 is indicative of high risk disease (cf. Table 1.2). In cases of low risk disease single agent chemotherapy is the treatment of choice while in cases of high-risk disease multi-agent chemotherapy regimens are required (Seckl *et al.*, 2013).

Table 1.2: FIGO/WHO scoring system based on prognostic factors

FIGO/WHO risk factor scoring with FIGO staging	0	1	2	4
Age	<40	>40	-	-
Antecedent pregnancy	Mole	Abortion	Term	
Interval from index pregnancy, months	<4	4-6	7-12	>12
Pretreatment hCG/mL	$<10^3$	$>10^3$-10^4	$>10^4$-10^5	$>10^5$
Largest tumour size including uterus, cm	-	3-4	≥5	-
Site of metastases identified	Lung	Spleen, kidney	Gastrointestinal tract	Brain, liver
Number of metastases identified	-	1-4	5-8	>8
Previous failed chemotherapy	-	-	Single drug	Two or more drugs

The risk of relapse after chemotherapy is approximately 3% and is highest in the first year of follow-up. Patients should use a contraceptive to avoid falling pregnant for at least one year after treatment (Seckl *et al.*, 2013). After β-hCG levels have returned to normal, the serum β-hCG levels should be monitored monthly until the levels have remained normal for one year (Snyman, 2009).

The FIGO/WHO scoring system does not apply to ETT and PSTT. These tumours are staged as follows: Stage 1, disease confined to the uterus; Stage 2, extends into the pelvis; Stage 3, spread to the lungs and/or vagina; Stage 4, all other metastatic sites including liver, kidney, spleen and brain (Seckl *et al.*, 2013). Patients with ETT and PSTT are treated primarily with surgery and undergo a hysterectomy and lymph node dissection. Adjuvant chemotherapy is given in cases with metastatic disease as well as in those with adverse risk factors such as a mitotic rate of more than six per 10 high power fields, a time period of more than 2 years from the previous pregnancy, tumour necrosis, deep myometrial invasion or inadequate resection margins (Goldstein and Berkowitz, 2012).

1.2 RATIONALE BEHIND THE STUDY

CHM has a 15-20% risk of developing persistent GTD while PHM has a 0,2%-4% chance. Incorrect diagnosis can result in under estimation of the risk of persistent GTD and improper clinical management and follow up. In addition, although choriocarcinomas are extremely aggressive malignant tumours, gestational choriocarcinomas respond well to chemotherapy with an overall cure rate of more than 90% in cases where the correct treatment is given. Identification of possible cases with rapid, accurate pathological diagnosis is therefore important and this is aided by a high index of suspicion. Very little data are available regarding GTD in South Africa. Moodley, together with various co-workers, has published a number of articles on GTD in KwaZulu-Natal concentrating predominantly on hydatidiform

moles and choriocarcinomas but has also described three cases of PSTT. In two separate studies the incidence of hydatidiform mole was found to be 1.2/1000 deliveries and 1.16/1000 deliveries, while that of choriocarcinoma was 0.5/1000 deliveries and 1.07/1000 deliveries. Isolated articles are available from the Western Cape Province and Limpopo Province, but there are no data from the Free State Province, therefore the incidence in the Free State Province is unknown. Accurate data will allow for better planning and allocation of health care resources.

The presence of HIV infection with a CD4 count of less than 200 cells/µl has been postulated to be a poor prognostic factor in patients with GTD. However, additional evidence is required for confirmation. HIV status has also been found to have a statistically significant influence on FIGO staging. The number of people living with HIV in sub-Saharan Africa is increasing and in South Africa one fifth of women between the ages of 15 and 49 years are HIV positive. The Free State Province has the second highest HIV prevalence in South Africa after KwaZulu-Natal and 25.5% of adults between the ages of 15 and 49 years are HIV positive. It is therefore important to confirm that HIV with a low CD4 count is a poor prognostic factor as this will affect patient management.

In recent years, progress has been made in understanding the genetics of underlying GTD. In 1999 the *NLRP7* gene was first identified which was linked to cases of familial recurrent hydatidiform mole. This was followed by the identification of *KHDC3L* in 2011 and *PADI6* in 2018. Since then over 60 pathogenic *NLRP7* variants and six pathogenic *KHDC3L* variants have been identified. Although variants have been described in patients from North Africa, there are no documented cases from Southern Africa. Patients with pathogenic variants in these genes present with recurrent hydatidiform moles and most require assisted reproductive technology and oocyte donation. Therefore, these patients need to be identified so that they can be sent for counseling and treatment.

Another development in the genetics of GTD is molecular genotyping, which has been used to more accurately classify hydatidiform moles into partial and complete moles and has also been used to classify choriocarcinomas as gestational or non-gestational. This has implications for treatment and prognosis as they require different chemotherapy regimes and non-gestational tumours have a poorer prognosis. Although the technique is available in South Africa and is currently used for paternity testing, it has not yet been applied to cases of GTD. The data on GTD in South African patients is therefore severely lacking and additional data is required to assist with and improve patient care.

1.3 AIMS AND OBJECTIVES

Aim 1: To evaluate the demographic characteristics of patients with GTD in the public sector of the Free State Province over a 10-year period from January 2006 to December 2015.

Objectives:

i. To determine the number and patient demographics of all cases of GTD from the public sector of the Free State Province as referred to the Department of Anatomical Pathology, Universitas Academic Laboratories, National Health Laboratory Service (NHLS).
ii. To ascertain whether an HIV positive status is a poor prognostic factor in patients with GTD. This will be performed by evaluating all patients referred to the Department of Oncology, National District Hospital during the study period. Demographic data, treatment regime, follow up data, cause of death and HIV status will be assessed.

Aim 2: To evaluate the genetic aetiology of patients with hydatidiform mole and choriocarcinoma in the public sector of the Free State Province.

Objectives:

i. To determine the presence of *NLRP7* and *KHDC3L* variants in patients in the public sector of the Free State Province, as referred to the Department of Anatomical Pathology, with hydatidiform mole and recurrent reproductive wastage.
ii. To ascertain whether choriocarcinomas can be identified as gestational or non-gestational using a polymerase chain reaction (PCR) based microsatellite DNA assay.
iii. To ascertain whether the cases of gestational choriocarcinoma are a result of molar or non-molar pregnancies using a PCR based microsatellite DNA assay.

1.4 STRUCTURE OF THE THESIS

This thesis is presented as a series of research articles which will be submitted for publication in various scientific journals.

The first article presented in Chapter 2, provides an overview of the demographic features of GTD in the public sector of the Free State Province, South Africa. In Chapter 3, the effect

of HIV infection on patients with GTD is evaluated to determine whether HIV should be used as an adverse prognostic indicator.

Chapters 4 and 5 investigate the genetic aetiology of GTD. Chapter 4 specifically evaluates the presence of variants in the *NLRP7* and *KHDC3L* genes in cases of hydatidiform mole with associated episodes of reproductive wastage while in Chapter 5, microsatellite analysis is performed on cases of choriocarcinoma to determine whether they are gestational or non-gestational in origin. Finally, in Chapter 6, the overall conclusions of the study and future perspectives for further research are provided.

CHAPTER 2

ARTICLE 1: DEMOGRAPHIC FEATURES OF PATIENTS WITH GESTATIONAL TROPHOBLASTIC DISEASE IN THE PUBLIC SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA: A 10-YEAR REVIEW

The article was prepared according to the journal submission guidelines for the *International Journal of Gynaecological Cancer* (cf. Appendix F).

DEMOGRAPHIC FEATURES OF PATIENTS WITH GESTATIONAL TROPHOBLASTIC DISEASE IN THE PUBLIC SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA: A 10-YEAR REVIEW

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Abstract

Introduction: Although there are a number of studies on gestational trophoblastic disease (GTD) from Nigeria, there are relatively few studies from the rest of the African continent. The aim of this study was therefore to determine the demographic features of patients seen at public sector hospitals in the Free State Province of South Africa.

Methods: A retrospective review was performed of all cases of GTD diagnosed by the Department of Anatomical Pathology, University of the Free State and National Health Laboratory Service between 1 January 2006 and 31 December 2015.

Results: There were a total of 226 cases of GTD with 200 hydatidiform moles (88.5%) and 26 choriocarcinomas (11.5%). No placental site trophoblastic tumours or epithelioid trophoblastic tumours were diagnosed in the study period. The incidence of hydatidiform mole and choriocarcinoma was 0.4/1000 deliveries and 0.05/1000 deliveries respectively. The mean age of patients with GTD was 27.7 years (SD 9.2 years). The majority of patients were Black females (91%) which is in keeping with the demographic profile of the Free State Province. The majority (53.8%) of cases were submitted with a clinical diagnosis of molar pregnancy while 24.1% presented with vaginal bleeding. Ninety nine percent of hydatidiform moles and 53.5% of choriocarcinomas were located in the uterine corpus, whereas four cases (15.4%) of choriocarcinoma presented with metastatic disease.

Discussion: This study showed a similar age range and clinical presentation to that reported in the local international literature. However, the incidence of both hydatidiform mole and choriocarcinoma is much lower than that found in most studies both in Africa and internationally. This may partly be due to a lack of clinical suspicion and under submission of products of conception for histological confirmation.

INTRODUCTION

Gestational trophoblastic disease (GTD) encompasses a group of disorders arising from placental villous trophoblast and includes hydatidiform mole, choriocarcinoma, placental site trophoblastic tumour and epithelioid trophoblastic tumour. Hydatidiform moles are further sub-classified as partial, complete or invasive (Kurman *et al.*, 2014).

The incidence of hydatidiform mole varies and is much higher in South East Asia than in North America, Europe and Australia (Bracken, 1987; Steigrad, 2003). A similar distribution is noted for choriocarcinoma (Altieri *et al.*, 2003; Steigrad, 2003). A higher incidence has also been seen in Hispanics, Eskimos and American Indians, although whether this is due to genetic factors, socioeconomic factors or differences in reporting is uncertain (Smith, 2003). Conflicting evidence has been published when comparing the rates of hydatidiform mole in Black and Caucasian women in the United States of America (Palmer, 1994; Steigrad, 2003). In the United Kingdom the incidence of complete mole is one per 1000 pregnancies while that of partial mole is 3 per 1000 pregnancies and choriocarcinomas occur in approximately one in 50 000 pregnancies. Placental site trophoblastic tumour and epithelioid trophoblastic tumour are much rarer and make up 0.2% of cases of GTD in the United Kingdom (Seckl *et al.*, 2010; Froeling and Seckl, 2014).

Risk factors for the development of hydatidiform mole include maternal age and a previous molar pregnancy. Although most hydatidiform moles occur in women in their 20's and 30's as this is the age at which most pregnancies occur, teenagers and women over 35 years of age have a higher risk. Girls under 16 years of age have a 6 times greater risk than women between the ages of 16 and 40 while women over 40 years of age have a five times greater risk. A third of pregnancies in women over 50 are hydatidiform moles (Steigrad, 2003; Sebire and Seckl, 2008; Hoffner and Surti, 2012). Boufettal *et al.* (2011) found that the risk in Moroccan woman was 6.8 times higher in woman under 20 years of age and 15 times higher in those over 40 years of age. However, some studies have shown that maternal age seems to play a greater role in complete hydatidiform moles than in partial hydatidiform moles (Sebire *et al.*, 2002). The risk of a woman with a previous molar pregnancy is up to 40 times that of the rest of the population (Steigrad, 2003).

Risk factors for choriocarcinoma include a history of hydatidiform mole and maternal age (Palmer, 1994). Women with a history of a previous molar pregnancy have a 1000 to 2000 times greater risk of developing choriocarcinoma (Palmer, 1994; Altieri *et al.*, 2003) and as with molar pregnancies older women also have an increased risk (Lurain, 2010).

Although there are numerous studies on GTD from Nigeria, studies from the rest of Africa are lacking (Agboola, 1979; Agboola and Abudu, 1984; Egwuatu and Ozumba, 1989;

Osamor *et al.*, 2002; Kyari *et al.*, 2004; Mayun, 2008; Audu *et al.*, 2009; Mbarara *et al.*, 2009; Mayun *et al.*, 2012; Mbarara *et al.*, 2012; Yakasai *et al.*, 2013; Kolawole *et al.*, 2016). In this paper, we analyzed the demographic features of patients seen at public sector hospitals in the Free State Province of South Africa between 2006 and 2015.

METHODS

A retrospective study was conducted. A search of the laboratory information system of the Department of Anatomical Pathology, University of the Free State (UFS) and National Health Laboratory Service, Bloemfontein, South Africa was performed for all cases of GTD diagnosed between 1 January 2006 and 31 December 2015. The department provides histology services to all the public sector hospitals and clinics in the Free State Province of South Africa. Cases that were occasionally received from other provinces were excluded from the study. The age, race, type of GTD, topography, β-human chorionic gonadotropin (β-hCG) level and clinical presentation noted by the submitting clinicians were obtained from the pathology reports.

Approval to perform the study was granted by the Health Sciences Research Ethics Committee of the UFS (HSREC81/2017). Statistical analysis was performed by the Department of Biostatistics, UFS. Results were expressed as frequencies and percentages. The chi-squared test was used to determine whether differences between groups were statistically significant ($p < 0.05$).

RESULTS

A total of 226 cases of GTD were received in the 10-year study period including 200 hydatidiform moles (88.5%) and 26 choricarcinomas (11.5%). No placental site trophoblastic tumours or epithelioid trophoblastic tumours were diagnosed. Of the hydatidiform moles, 32 (16%) were partial moles, 166 (83%) were complete moles and two (1%) were invasive moles. As a total of 491 164 deliveries were recorded for the region during this time, the incidence of molar pregnancy and choriocarcinoma was 0.4/1000 deliveries and 0.05/1000 deliveries respectively.

The mean age of patients with GTD was 27.7 years (SD 9.2 years). The median age of patients with hydatidiform mole was 24 years with an age range of 15 to 57 years. The median age of patients with choriocarcinoma was 30 years with an age range of 14 to 59 years ($p = 0.006$). There were 51 patients (25.5%) aged 20 years or younger with

hydatidiform mole, 113 patients (56.5%) between the ages of 21 and 34 and 36 patients (18%) 35 years of age and older. With regards to choriocarcinoma, two patients (7.7%) were 20 years of age or younger, 13 (50%) were between the ages of 21 and 34 years and 11 (42.3%) were 35 years of age or older. Of a total of 208, 190 self-identified themselves as Black. In addition, there were 10 Coloured patients, five Caucasian patients, two Asian patients and one Indian patient.

β-hCG levels were available for 104 patients with hydatidiform mole and 19 patients with choriocarcinoma (Table 1). Nineteen patients (18.3%) with hydatidiform mole and five patients (26.3%) with choriocarcinoma had β-hCG values of more than 500 000 IU/l.

Table 1. β-hCG levels in patients with hydatidiform mole and choriocarcinoma

β-hCG (IU/l)	Molar pregnancy	Choriocarcinoma
<100 000	32 (30.8%)	4 (21.1%)
100 000 – 500 000	53 (50.9%)	10 (52.6%)
>500 000	19 (18.3%)	5 (26.3%)
Total number of cases	**104**	**19**

The frequency of presenting symptoms is illustrated in Table 2. In 212 cases, the clinical presentation was noted on the request form. Of these 113 (53.3%) were submitted with a clinical diagnosis of hydatidiform mole while 52 cases (24.5%) presented with vaginal bleeding.

Table 2. Clinical presentation

Presentation according to pathology request form	Molar Pregnancy	Choriocarcinoma
Features of molar pregnancy	113 (59.7%)	0
Vaginal bleeding	39 (20.6%)	13 (56.5%)
Miscarriage	18 (9.5%)	3 (13%)
Ectopic pregnancy	2 (1.1%)	3 (13%)
Abdominal pain	3 (1.6%)	0
Amenorrhoea	2 (1.1%)	0
Very high β-hCG	4 (2.1%)	0
Raised β-hCG after a previous molar pregnancy	1 (0.5%)	1 (4.4%)
Preeclampsia	2 (1.1%)	0
Twin pregnancy	2 (1.1%)	0
Hyperthyroidism	2 (1.1%)	0
Myomatous uterus	1 (0.5%)	0
Dyspnoea and coughing	0	1 (4.4%)
Haemoptysis	0	1 (4.4%)
Nodules on small bowel	0	1 (4.4%)
Total number of cases	**189**	**23**

One hundred and ninety-eight (99%) of the 200 molar pregnancies were located in the uterine corpus while two (1%) occurred in the fallopian tube. With regards to the

choriocarcinomas, 14 (53.8%) of the 26 cases were located in the uterine corpus, four (15.4%) in the cervix and vagina and four (15.4%) in the fallopian tube. In addition, two (7.7%) presented with lung metastases and two (7.7%) with metastases to the bowel.

DISCUSSION

The incidence of hydatidiform mole in this study is much lower than that found in most studies from Africa (cf. Table 3). Only one study from Nigeria had a similar incidence of hydatidiform mole with 0.8 per 1000 deliveries (Egwuatu and Ozumba, 1989) while the remainder had incidences varying between 1.03 and 6 per 1000 deliveries. The incidence of hydatidiform mole is also lower than that seen in most of the international literature (Smith, 2003; Hoffner and Surti, 2012). The number of partial moles in this study was 16% that is similar to the 12% seen by Moodley and Marishane (2005) and 12.2% seen by Kolawole *et al.* (2016). However, other studies from Africa had a much higher number of partial moles in relation to complete moles with 87%, 49.5%, 47% and 71.8% in Tanzania and Nigeria (Osamor *et al.*, 2002; Mayun, 2008; Audu *et al.*, 2009; Kitange *et al.*, 2015). The lower incidence and number of partial moles in this study may partly be due to under submission of cases by clinicians as only products of conception with suspected abnormalities are submitted to our laboratory for evaluation and early complete moles as well as partial moles may be missed clinically.

Table 3. The incidence of hydatidiform mole in African countries

Province	No of cases of hydatidiform mole	Study period	Ratio per 1000 deliveries	References
South African Provinces				
KwaZulu-Natal	78	1994-2000	1.2	Moodley *et al.*, 2003
KwaZulu-Natal	50	1998-2002	1.16	Moodley and Moodley, 2005
Limpopo	84	2008-2011		Van Bogaert, 2013
Free State	200	2006- 2015	0.4	This study, 2020
Uganda				
	181	1967-1970	1.03	Leighton *et al.*, 1973
	94 (complete moles only)	1995-1998	3.42	Kaye, 2002
Tanzania				
	23	2013		Kitange *et al.*, 2015
Nigeria				
	29	1974-1977	2.6	Agboola, 1979
	26	1980-1981	5.4	Agboola and Abudu, 1984
	41	1976-1985	0.8	Egwuatu and Ozumba, 1989
	208	1966-1996		Osamor *et al.*, 2002

Province	No of cases of hydatidiform mole	Study period	Ratio per 1000 deliveries	References
	34	2000-2005	6.0	Mayun, 2008
	71	1996-2005	3.8	Audu *et al.*, 2009
	5	2004-2008	1.6	Mbarara *et al.*, 2012
	18	2008-2012	2.2	Kolawole *et al.*, 2016
Morocco				
	254 (complete moles only)	2000- 2009	4.3	Boufettal *et al.*, 2011

The incidence of choriocarcinoma of 0.05 per 1000 deliveries was also very low when compared to other studies from Africa although Moodley *et al.* (2003) reported an incidence of 0.5 cases per 1000 deliveries and Leighton *et al.* (1973) reported an incidence of 0.3 per 1000 deliveries (cf. Table 4). Studies from Mexico and Puerto Rico had incidences of 0.3 per 1000 deliveries while other international studies had incidences of between 0.6 and 20.2 per 1000 deliveries (Smith, 2003).

Table 4. The incidence of choriocarcinoma in African countries

Province	No of cases of choriocarcinoma	Study period	Ratio per 1000 deliveries	References
South African Provinces				
Western Cape	24	1968-1977		Davey and Fray, 1979
KwaZulu-Natal	34	1994-2000	0.5	Moodley *et al.*, 2003
KwaZulu-Natal	46	1998-2002	1.07	Moodley *et al.*, 2005
Limpopo	31	2008-2011		Van Bogaert, 2013
Free State	26	2006- 2015	0.05	This study, 2020
Uganda				
	52	1967-1970	0.3	Leighton *et al.*, 1973
Nigeria				
	16	1980-1981	3.3	Agboola and Abudu, 1984
	16	1991-2000		Kyari *et al.*, 2004
	10	2004-2008	3.1	Mbarara *et al.*, 2009
	43	1994-2003	1.0	Mayun *et al.*, 2012
	23	2008-2011		Yakasai *et al.*, 2013
	41	2008-2012	5.0	Kolawole *et al.*, 2016

No placental site trophoblastic tumours or epithelioid trophoblastic tumours were diagnosed in the study period confirming the rare nature of these tumours. The patients' ages in this study correlated with findings from other studies in Africa as well as with the international literature. Ninety one percent of patients were Black, which is in keeping with the findings of the 2011 Census in which 87.6% of the population in the Free State Province were Black. Only 2.2% of the study population were Caucasian, which is lower than the 8.7% identified in the 2011 Census (SSA, 2011). However, this may partly be due to the

fact that many Caucasian patients attend private health care facilities. This finding was also noted by Moodley and Marishane (2005) in their study in KwaZulu-Natal.

In contrast to the findings of a study by Moodley *et al.* (2003) in which 40% of cases had β-hCG levels of less than 100 000 IU/l only 29.3% of cases in our study had levels under 100 000 IU/l while 51.2% of cases had β-hCG levels of between 100 000 and 500 000 IU/l.

In the past many patients with hydatidiform mole presented with symptoms such as hyperthyroidism, hyperemesis, anaemia, pre-eclampsia and respiratory distress. However, nowadays most patients present with vaginal bleeding in early pregnancy and the diagnosis is made on antenatal sonar (Seckl *et al.*, 2010; Froeling and Seckl, 2014). In 53.3% of cases, the submitting diagnosis was that of hydatidiform mole diagnosed on clinical and sonographic features. The second most common mode of presentation was vaginal bleeding (24.5%). Only two patients presented with pre-eclampsia and two with hyperthyroidism. The diagnosis of choriocarcinoma is more difficult and patients can present with vaginal bleeding or with metastatic disease as seen in this study in which two cases presented with lung metastases and two with metastases to the bowel (Froeling and Seckl, 2014). As expected, the majority of cases involved the uterus with only two hydatidiform moles and four choriocarcinomas located in the fallopian tube.

In conclusion, the age and clinical presentation of patients with GTD using state health care facilities in the Free State Province of South Africa is similar to that reported in the literature. However, the incidence of both molar pregnancy and choriocarcinoma is very low. This may partly be due to under diagnosis with a lack of suspicion among clinicians as well as under submission of products of conception for histopathological evaluation and confirmation of GTD. Further research is required for confirmation.

REFERENCES

Agboola A. Trophoblastic neoplasia in an African urban population. *J Natl Med Assoc* 1979;71:935-937.

Agboola A, Abudu OO. Epidemiology of trophoblast disease in Africa – Lagos. *Adv Exp Med Biol* 1984;176:187-195.

Altieri A, Franceschi S, Ferlay J, Smith J, La Vecchia C. Epidemiology and aetiology of gestational trophoblastic diseases. *Lancet Oncol* 2003;4:670-678.

Audu BM, Takai IU, Chama CM, Bukar M, Kyari O. Hydatidiform mole as seen in a university teaching hospital: a 10 year review. *J Obstet Gynaecol* 2009;29:322-325.

Boufettal H, Coullin P, Mahdaoui, Noun M, Hermas S, Samouh N. Complete hydatidiform mole in Morocco: Epidemiological and clinical study. *J Gynecol Obstet Biol Reprod* (Paris) 2011;40:419-429.

Bracken MB. Incidence and aetiology of hydatidiform mole: an epidemiological review. *Br J Obstet Gynaecol* 1987;94:1123-1135.

Davey DA, Fray R. Choriocarcinoma and invasive mole. A review of 10 years' experience. *SA Med J* 1979;56:924-931.

Egwuatu VE, Ozumba BC. Observations on molar pregnancy in Enugu, Nigeria. *Int J Gynecol Obstet* 1989;29:219-225.

Froeling FEM, Seckl MJ. Gestational trophoblastic tumours: an update for 2014. *Curr Oncol Rep* 2014;16:408.

Hoffner L, Surti U. The genetics of gestational trophoblastic disease: a rare complication of pregnancy. *Cancer Genet* 2012;205:63-77.

Kaye DK. Gestational trophoblastic disease following complete hydatidiform mole in Mulago Hospital, Kampala, Uganda. *Afri Health Sci* 2002;2:47-51.

Kitange B, Matovelo D, Konje E, Massinde A, Rambau P. Hydatidiform moles among patients with incomplete abortion in Mwanza City, North western Tanzania. *Afri Health Sci* 2015;15:1081-1086.

Kolawole AO, Nwajagu JK, Oguntayo AO, Zayyan MS, Adewuyi S. Gestational trophoblastic disease in Abuth Zaria, Nigeria: A 5 year review. *Trop J Obstet Gynaecol* 2016;33:209-215.

Kurman RJ, Carcangiu ML, Herrington CS, Young RH, eds. WHO classification of tumours of female reproductive organs. Lyon:IARC;2014:155-167.

Kyari O, Nggada H, Mairiga A. Malignant tumours of female genital tract in North Eastern Nigeria. *East Afr Med J* 2004:81:142-145.

Leighton PC. Trophoblastic disease in Uganda. *Am J Obstet Gynecol* 1973;117:341-344.

Lurain JR. Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole. *Am J Obstet Gynecol* 2010;203:31-39.

Mayun AA. Hydatidiform mole in Gombe: a five year histopathological review. *Niger J Clin Prac* 2008;11:134-138.

Mayun AA, Rafindadi AH, Shehu MS. Choriocarcinoma in Northwestern Nigeria: a histopathological review. *Niger Postgrad Med J* 2012;19:215-218.

Mbamara SU, Obiechin NJA, Eleje GU, Akabuike CJ, Umeononihu OS. Gestational trophoblastic disease in a tertiary hospital in Nnewi, Southeast Nigeria. *Niger Med J* 2009;50:87-89.

Moodley M, Tunkyi K, Moodley J. Gestational trophoblastic syndrome: an audit of 112 patients. A South African experience. *Int J Gynecol Cancer* 2003;13:234-239.

Moodley M, Marishane T. Demographic variables of gestational trophoblastic disease in KwaZulu-Natal, South Africa. *J Obstet Gynecol* 2005;25:482-485.

Osamor JO, Oluwasola AO, Adewole IF. A clinicopathological study of complete and partial

hydatidiform moles in a Nigerian population. *J Obstet Gynaecol* 2002;22:423-425.

Palmer JR. Advances in the epidemiology of gestational trophoblastic disease. *J Reprod Med*. 1994;39:155-162.

Sebire NJ, Foskett M, Fisher RA, Rees H, Seckl M, Newlands E. Risk of partial and complete hydatidiform molar pregnancy in relation to maternal age. *Br J Obstet Gynaecol* 2002;109:99-102.

Sebire NJ, Seckl MJ. Gestational trophoblastic disease: current management of hydatidiform mole. *BMJ* 2008;337:1193.

Seckl MJ, Sebire N, Berkowitz RS. Gestational trophoblastic disease. *Lancet* 2010;376:717-729.

Smith HO. Gestational trophoblastic disease epidemiology and trends. *Clin Obstet Gynaecol* 2003;4:541-556.

Statistics South Africa (SSA) 2011. Census 2011. Available at https://www.statssa.gov.za/publications/P03014/P030142011.pdf (accessed 3 January 2020).

Steigrad SJ. Epidemiology of gestational trophoblastic diseases. *Best Prac Res Clin Obstet Gynaecol* 2003;17:837-847.

Von Bogaert LJ. Clinicopathological features of gestational trophoblastic neoplasia in the Limpopo Province, South Africa. *Int J Gynecol Cancer* 2013;23:583-585.

Yakasai IA, Ugwa EA, Otubu J. Gynecological malignancies in Aminu Kano Teaching Hospital Kano: A 3 year review. *Niger J Clin Prac* 2013;16:63-66.

CHAPTER 3

ARTICLE 2: GESTATIONAL TROPHOBLASTIC DISEASE AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION: A 10-YEAR RETROSPECTIVE ANALYSIS OF PATIENTS FROM THE FREE STATE PROVINCE, SOUTH AFRICA

The article was prepared according to the journal submission guidelines for the *International Journal of Gynaecological Cancer* (cf. Appendix F).

GESTATIONAL TROPHOBLASTIC DISEASE AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION: A 10-YEAR RETROSPECTIVE ANALYSIS OF PATIENTS FROM THE FREE STATE PROVINCE, SOUTH AFRICA

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Abstract

Introduction: Gestational trophoblastic disease (GTD) encompasses a group of disorders of placental villous trophoblast. A previous study suggested that human immunodeficiency virus (HIV) infection with a CD4 count of <200 cells/μl should be regarded as a poor prognostic factor in patients with GTD. The aim of this study was to describe our data on HIV status and GTD to try to confirm whether this is indeed the case.

Methods: A retrospective cohort study was performed. All patients treated for GTD at the Department of Oncology, National District Hospital, Bloemfontein, South Africa between January 2006 and December 2015 were included in the study.

Results: Thirty-three patients with a median age of 26 years were treated in the study period. Seventeen (51.5%) were diagnosed with choriocarcinoma and 16 (48.5%) with hydatidiform mole. Twenty patients (60.6%) were HIV negative and 13 (39.4%) were HIV positive. Three of the HIV positive patients had a CD4 count of less than 200 cells/μl. All the HIV positive patients received chemotherapy including those with a CD4 count of less than 200 cells/μl. The HIV negative patients and HIV positive patients with a CD4 count of more than 200 cells/μl had a similar outcome with 88.2% and 75.0% of patients being alive, nine months after diagnosis. In contrast, although the numbers are small the HIV positive patients with a CD4 count of less than 200 cells/μl had a significantly poorer outcome with only 33.3% alive at nine months ($p = 0.03$).

Discussion: Our findings therefore support the hypothesis that a low CD4 count should be regarded as a poor prognostic marker. In addition, HIV positive patients are also more likely to have metastatic disease.

INTRODUCTION

From 2017 to 2019 the human immunodeficiency virus (HIV) infection burden in sub-Saharan Africa rose from an estimated 64% to 67.5% of the global burden and the number of cases in Eastern and Southern Africa increased from 19.4 million to 20.6 million (Barnardt, 2019; UNAIDS, 2019). According to Statistics South Africa (SSA), the estimated overall prevalence rate of HIV infection in the South African population is 13.5%. Approximately one fifth of women between the ages of 15 and 49 years are HIV positive and there are approximately 7.97 million people living with HIV in South Africa (SSA, 2019). The Fifth South African National HIV Prevalence, Incidence, Behaviour and Communication Survey conducted by the Human Sciences Research Council, determined that 25.5% of adults between the ages of 15 and 49 years living in the Free State Province are HIV positive. The Free State Province has the second highest prevalence after KwaZulu-Natal (HSRC, 2017).

In 1992, a series of three cases of gestational trophoblastic neoplasia in HIV positive patients with an aggressive clinical course were published and it was proposed that HIV infection should be regarded as a poor prognostic indicator (Ojwang *et al.*, 1992). Since then there have been a handful of retrospective studies evaluating gestational trophoblastic disease (GTD) and the effect of HIV infection. Moodley *et al.* (2003a) published a series of 41 patients with choriocarcinoma of which 12 (29.3%) were HIV positive and they recommended that patients with a CD4 count of >200 cells/µl should receive standard treatment. In a second series, Moodley *et al.* (2009), evaluated 78 patients with GTD and concluded that HIV positivity with a low CD4 count should be included as a poor prognostic factor. Another series in 2011 reviewed 76 patients of which 14 (18.4%) were HIV positive. The five-year survival of the HIV positive group was 64.3% compared to 85.7% in the HIV negative group ($p = 0.141$). The authors noted that HIV positivity and poor treatment compliance were associated with a worse outcome (Tayib *et al.*, 2011). In a recent study by Makhathini *et al.* (2019), 29% of the patients were HIV positive but all had a CD4 count of more than 200 cells/µl. They did not find a statistically significant difference in survival between HIV positive and HIV negative patients, although the HIV positive patients presented at a later stage.

In light of these findings and the high HIV burden in our population we performed a retrospective review to describe our data regarding GTD and HIV status.

MATERIALS AND METHODS

A retrospective cohort study was conducted at National District Hospital and Universitas Academic Laboratories, Bloemfontein in the Free State Province of South Africa. All patients who were referred for the management of GTD to the Department of Oncology at National District Hospital between 1 January 2006 and 31 December 2015 were included in the study. The Department of Oncology provides services to all public sector cancer patients in the Free State Province. Cases were histologically confirmed by the Department of Anatomical Pathology, Universitas Academic Laboratories, National Health Laboratory Service (NHLS). Information was obtained from the patient files at the Department of Oncology as well as from the pathology information system of the NHLS. The information included age, race, type of GTD, site of involvement, presence and site of metastases, β-human chorionic gonadotropin (β-hCG) at diagnosis, gravidity, parity, haemoglobin level at diagnosis, thyroid functions at diagnosis, presence of a previous molar pregnancy, outcome of disease (remission or death), risk group, time from diagnosis to death when relevant, treatment provided, HIV status and CD4 count. Risk stratification was performed using the FIGO/WHO scoring system with a score of six or less regarded as low risk and a score of seven and above regarded as high risk (Ngan *et al.*, 2012; Seckl *et al.*, 2013)

Approval to perform the study was granted by the Health Sciences Research Ethics Committee of the University of the Free State (HSREC81/2017). Approval was also obtained from the NHLS and the Free State Department of Health.

The data was analysed by the Department of Biostatistics, UFS. Results were summarised by frequencies and percentages (categorical variables), and medians and ranges (numerical variables due to skew distributions). Ninety-five percent confidence intervals (CIs) were presented for main outcomes. Subgroup comparisons of categorical variables were done using chi-squared or Fisher's exact tests in the case of sparse cells. Product-limit survival estimates were calculated taking censoring into account, and compared using the logrank test.

RESULTS

Thirty-three patients with GTD were seen by the Department of Oncology during the study period, 17 (51.5%) with choriocarcinoma and 16 (48.5%) with hydatidiform mole. The mean age was 30.6 years with a median age of 26 years and an age range of 19-56 years. The age distribution of the patients per diagnosis is depicted in Table 1.

Table 1. Age distribution of patients

	19-29 years	30-39 years	40-49 years	>49 years	Total
Choriocarcinoma	10 (58.8%)	4 (23.5%)	3 (17.7%)	0 (0%)	17
Hydatidiform mole	8 (50%)	5 (31.2%)	2 (12.5%)	1 (6.3%)	16
Total	18 (54.5)	9 (27.3)	5 (15.2%)	1 (3%)	33

There were 30 (91%) Black patients and three (9%) Coloured patients. Of the molar pregnancies, 14 (87.5%) were complete moles while two (12.5%) were invasive moles. Twenty-eight cases (84.5%) were located in the uterine corpus while two (6%) occurred in the cervix and vagina and two (6%) in the fallopian tube. One case (3%) presented with lung metastases. Fifteen patients (45.5%) had metastatic disease and sites included lungs, brain, liver, bone, kidney and colon.

The median gravidity was two and the median parity was one, with the maximum number of pregnancies being seven. Two patients who were referred with a diagnosis of choriocarcinoma had a history of a previous hydatidiform mole. Seven patients (21.9%) had β-hCG levels of less than 100 000 IU/l, 17 (53.1%) had levels of between 100 000 and 500 000 IU/l and eight (25%) had levels of over 500 000 IU/l. Eight patients (25%) had normal haemoglobin levels while 17 (53.1%) were anaemic and seven (21.9%) had severe anaemia with a haemoglobin level of less than 7 g/dl. Thyroid functions were available in 16 patients of which six (37.5) were hyperthyroid and 10 (62.5%) had normal thyroid functions.

Twenty patients (60.6%) were HIV negative while 13 (39.4%, 95% CI 22.9% to 57.9%) were HIV positive. The HIV seroprevalence in this population was therefore 39.4%. The median CD4 count of the HIV positive patients was 350 cells/µl with a range of 78 to 1090 cells/µl. Three of the patients had a CD4 count of less than 200 cells/µl. Of the 15 patients with metastases, nine (60%) were HIV positive compared to four of the 18 patients without metastatic disease (22.2%) ($p = 0.03$). Table 2 summarizes the patients' characteristics according to HIV status.

Table 2. Patients' characteristics according to HIV status

	HIV positive n = 13	HIV negative n = 20	Total n = 33
Histology			
Complete mole	3 (23.1%)	11 (55%)	14 (42.4%)
Invasive mole	1 (7.7%)	1 (5%)	2 (6.1%)
Choriocarcinoma	9 (69.2%)	8 (40%)	17 (51.5%)
Metastases			
Lung	8 (61.55%)	6 (30%)	14 (42.4%)
Liver	2 (15.4%)	0	2 (6.1%)

	HIV positive n = 13	HIV negative n = 20	Total n = 33
Bone	2 (15.4%)	1 (5%)	3 (9.1%)
Brain	3 (23.1%)	1 (5%)	4 (12.1%)
Kidney	1 (7.7%)	0	1 (3%)
Colon	0	1 (5%)	1 (3%)
Risk group			
Low risk	6 (46.2%)	13 (65%)	19 (57.6%)
High risk	7 (53.8%)	7 (35%)	14 (42.4%)
Treatment			
Chemotherapy only	10 (76.9%)	14 (70%)	24 (72.7%)
Chemotherapy and Surgery	3 (23.1%)	5 (25%)	8 (24.2%)
None	0 (0%)	1 (5%)	1 (3.1%)
Status			
Alive	7 (53.8%)	15 (75%)	22 (66.7%)
Demised	4 (30.8%)	2 (10%)	6 (18.2%)
Lost to follow-up	2 (15.4%)	3 (15%)	5 (15.1%)

Nineteen patients (57.6%) were low risk while fourteen patients (42.4%) were high risk of which seven were HIV positive. All six patients who were confirmed to have demised were classified as high risk and were diagnosed with choriocarcinoma. All six patients had metastatic disease of which five had lung metastases, three had brain metastases and one had metastases to the liver, bone and kidneys. One patient died due to chemotherapy induced interstitial lung disease while the remainder died due to the metastases. The patient with chemotherapy induced interstitial lung disease was HIV negative.

Most patients (72.7%) were treated with chemotherapy only while eight (24.2%) received both chemotherapy and surgery. All the HIV positive patients received chemotherapy including those with a CD4 count of less than 200 cells/µl. One patient (3.1%) refused treatment. In addition, four patients received irradiation for metastatic disease. Of the 32 patients who had chemotherapy, five (15.2%) received only methotrexate while the remainder received multiagent chemotherapy. Three patients (9.4%) were treated with the EMA-CO regimen (etoposide, methotrexate, dactinomycin, cyclophosphamide, vincristine), six (18.8%) with PEB (vincristine, methotrexate, cisplatin) and 18 (56.3%) with methotrexate, vincristine and chlorambusil.

The patients were followed up at the Department of Oncology for between 1 and 120 months with a median of 30 months. Of the HIV negative patients, 94.1% (95% CI 82.7% to 100.0%) were alive at six months and 88.2% (95% CI 72.6% to 100%) were alive, nine months after diagnosis. Similarly, 87.5% (95% CI 64.1% to 100%) of the HIV positive patients with a CD4 count of over 200 cells/µl, were alive at six months while 75.0% (95% CI 44.4% to 100%), were alive at nine months. In contrast, only 66.7% (95% CI 12.3% to 100%) of HIV positive patients with a CD4 count of less than 200 cells/µl were alive at six months and 33.3% (95% CI 0% to 87.7%), were alive at nine months.

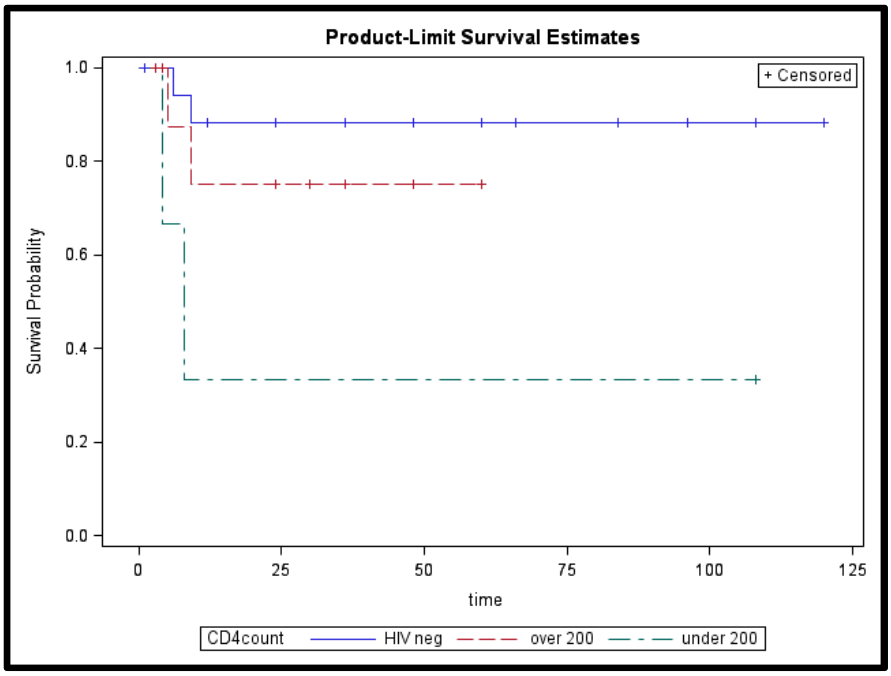

Figure 1. Product-Limit Survival Estimates for HIV positive and HIV negative patients. The HIV positive patients are divided into those with a CD4 count of less than 200 cells/µl and those with a CD4 count of ≥ 200 cells/µl.

The HIV positive patients with a CD4 count of less than 200 cells/µl therefore have a significantly poorer outcome than the other two groups ($p = 0.03$).

DISCUSSION

In light of the high HIV burden in South Africa a significant number of patients with GTD will be HIV positive. Thirty-three patients were included in this series of which 39.4% were HIV positive and 60.6% were HIV negative with an HIV seroprevalence of 39.4% (95% CI 22.9% to 57.9%). This is significantly higher than the 22.7% prevalence in females between the ages of 15 and 49 years as determined by SSA (SSA, 2019). In contrast to previous studies all the patients in this study had a known HIV status as this is tested routinely when the patients are first seen by the Department of Oncology. This is in accordance with the National Comprehensive Cancer Network (NCCN) clinical practice guidelines (Reid *et al.*, 2018).

The mean age of the patients was 30.6 years, which is in keeping with previous studies from South Africa in which the mean age, ranged from 28.5 years to 31 years (Moodley and Moodley, 2003b; Moodley *et al.*, 2009; Van Bogaert, 2013). Ninety-one percent of patients were Black, which is similar to the 2011 Census data in which 87.6% of the population in the Free State Province were Black. As in a study by Moodley and Marishane (2005), there were no Caucasian patients. This may partly be due to many Caucasian

patients attending private health care facilities.

Seventeen (51.5%) patients had choriocarcinoma while 16 (48.5%) were diagnosed with hydatidiform mole. This is in contrast to studies by Tayib *et al.* (2011) and Moodley *et al.* (2009) in which only 28% and 32% of patients had choriocarcinoma. This may partly be due to the local policy in which uncomplicated cases of hydatidiform mole are followed up by the Department of Obstetrics and Gynaecology and only cases requiring chemotherapy are referred to the Department of Oncology.

Five of the 33 patients (15%) were lost to follow up despite concerted efforts to determine whether the patients were alive or deceased which included phoning the patients and relatives using telephone numbers provided on initial presentation and contacting the Department of Home Affairs. Loss to follow up is a common problem in South Africa as seen in HIV and tuberculosis treatment programs as well as in other studies. (Hirasen *et al.*, 2018; Ambia *et al.*, 2019; Cubasch *et al.*, 2019). Badenhorst *et al.* (2018) evaluated causes of loss to follow up in patients with ankle fractures in the Northern Cape Province and found that increased travel distance and a positive HIV status made patients more likely to miss follow up visits. In addition, they determined that it was difficult to contact patients even when contact details were provided as patients often changed cell phone numbers without informing the hospital. A recent study on GTD noted that there was a high default rate in patients travelling more than 80.5 km for medical care and 40.7% of patients in this group were lost to follow up ($p = 0.014$) (Makhathini *et al.*, 2019).

Of the six patients who died, all were high risk with metastatic disease. Five patients died due to the metastases while one demised due to drug induced interstitial lung disease secondary to PEB. The treatment regime was changed to methotrexate, Oncovin and chlorambusil as soon as the interstitial lung disease was diagnosed but despite this, the patient demised nine months after initial diagnosis. Interstitial lung disease is a known complication of a number of chemotherapeutic agents and up to 10% of patients, receiving chemotherapy will develop a pulmonary adverse drug reaction (Limper and Rosenow, 1996). Any pattern of interstitial lung disease can occur including hypersensitivity pneumonitis, organizing pneumonia, diffuse alveolar damage, eosinophilic pneumonia, nonspecific interstitial pneumonia and granulomatous pneumonitis (Schwaiblmair *et al.*, 2012).

The HIV status was found to have a statistically significant influence on the presence of metastatic disease in our study as 60% of patients with metastases were HIV positive compared to 22.2% of patients without metastases ($p = 0.03$). The HIV negative patients and HIV positive patients with a CD4 count of more than 200 cells/µl had a similar outcome

with 88.2% and 75.0% of patients being alive nine months after diagnosis. In contrast, although the numbers are small the HIV positive patients with a CD4 count of less than 200 cells/µl had a significantly poorer outcome with only 33.3% alive at nine months ($p = 0.03$). This is in keeping with the findings of Moodley *et al.* in which a statistically significant increase in mortality was noted in HIV positive patients with a CD4 count of less than 200 cells/µl (Moodley *et al.*, 2009).

In conclusion, our findings support the recommendation by Moodley *et al.* (2009) that a low CD4 count should be regarded as a poor prognostic factor.

REFERENCES

Ambia J, Kabudula C, Risher K, Gomez-Olive FX, Rice BD, Etoori D, Reniers G. Outcomes of patients lost to follow-up after antiretroviral therapy initiation in rural north-eastern South Africa. *Trop Med Int Health* 2019;24:747-756.

Badenhorst DHS, van der Westhuizen CA, Britz E, Burger MC, Ferreira N. Lost to follow-up: Challenges to conducting orthopaedic research in South Africa. *SAMJ* 2018;108:917-921.

Barnardt P. Managing gestational trophoblastic neoplasm (GTN) and people living with HIV (PLWH). *South Afr J Gynaecol Oncol* 2019;11:21-24.

Cubasch H, Dickens C, Joffe M, Duarte R, Murugan N, Chih, MT, Moodley K, Sharma V, Ayeni O, Jaconsen JS, Neugut AI, McCormack V, Ruff P. Breast cancer survival in Soweto, Johannesburg, South Africa: A receptor-defined cohort of women diagnosed from 2009-11 *Cancer Epidemiol* 2018;52:120-127.

Hirasen K, Berhanu R, Evans D, Rosen S, Sanne I, Long L. High rates of death and loss to follow-up by 12 months of rifampicin resistant TB treatment in South Africa. *PLOS One* 2018;13: e0205463.

Limper A, Rosenow E. Drug-induced interstitial lung disease. *Curr Opin Pulm Med* 1996;2:396-404.

Human Sciences Research Council (HSRC). The fifth South African national HIV prevalence, incidence, behavior and communication survey, 2017. Available at http://www.hsrc.ac.za/uploads/pageContent/9234/SABSSMV_Impact_Assessment_Summary_ZA_ADS_cleared_PDFA4.pdf (accessed 6 November 2019).

Ojwang SBO, Otieno MRB, Khan KS. Human immunodeficiency virus in gestational trophoblastic neoplasias – Is it a poor prognostic risk factor? *East Afr Med J* 1992;69:647-648.

Makhathini S, Dreyer G, Buchmann EJ. Gestational trophoblastic disease managed at Grey's Tertiary Hospital: a five-year descriptive study. *South Afr J Gynaecol Oncol* 2019:1-5 https://doi.org/10.1080/20742835.2019.1667627.

Moodley M, Moodley J. Gestational trophoblastic syndrome and human immunodeficiency virus (HIV) infection: A retrospective analysis. *Int J Gynecol Cancer* 2003a;13:875-878.

Moodley M, Tunkyi K, Moodley J. Gestational trophoblastic syndrome: An audit of 112 patients. A South African experience. *Int J Gynecol Cancer* 2003b;13:234-239.

Moodley M, Marishane T. Demographic variables of gestational trophoblastic disease in KwaZulu-Natal, South Africa. *J Obstet Gynaecol* 2005;25:482-485.

Moodley M, Budram S, Connolly C. Profile of mortality among women with gestational trophoblastic disease infected with the human immunodeficiency virus (HIV). Argument for a new poor prognostic marker. *Int J Gynecol Cancer* 2009;19:289-293.

Ngan HYS, Seckl MJ, Berkowitz RS, Xiang Y, Golfier F, Sekharan PK, Lurain JR. Update on the diagnosis and management of gestational trophoblastic disease. *Int J Gynecol Obstet* 2015;131:S123-S126.

Reid E, Suneja G, Ambinder RF, ARd K, Baiocchi R, Barta SK, Carchman E, Cohen A, Gupta N, Johung KL, Klopp A, LaCasce AS, Lin C, Makarova-Rusher OV, Mehta A, Menon MP, Morgan D, Nathwani N, Noy A, Palella F, Ratner L, Rizza S, Rudek MA, Taylor J, Tomlinson B, Wang CJ, Dwyer MA, Freedman-Cass DA. Cancer in people living with HIV, Version 1.2018, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* 2018;16:986-1017.

Schwaiblmair M, Behr W, Haeckel T, Markl B, Foerg W, Berghaus T. Drug induced interstitial lung disease. *Open Respir Med J* 2012;6:63-74.

Seckl MJ, Sebire NJ, Fisher RA, Golfier F, Massuger L, Sessa C. Gestational trophoblastic disease: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013;24:vi39-vi50.

Statistics South Africa (SSA) 2019. Midyear population estimates 2019. Available at https://www.statssa.gov.za/publications/P0302/P03022019/pdf (accessed 6 November 2019).

Tayib S, van Wijk L, Denny L. Gestational trophoblastic neoplasia and human immunodeficiency virus infection: A 10 year review. *Int J Gynecol Cancer* 2011;21:1684-1691.

UNIAIDS Fact Sheet. World AIDS day 2019. Global HIV Statistics. Available at https://www.unaids.org/en/resources/fact-sheet (accessed 31 December 2019)

Van Bogaert LJ. Clinicopathologic features of gestational trophoblastic neoplasia in the Limpopo Province, South Africa. *Int J Gynecol Cancer* 2013;23:583-585.

CHAPTER 4

ARTICLE 3: *NLRP7* AND *KHDC3L* MUTATIONS IN SOUTH AFRICAN PATIENTS WITH HYDATIDIFORM MOLE AND RECURRENT REPRODUCTIVE WASTAGE

The article was prepared according to the journal submission guidelines for the *European Journal of Human Genetics* (cf. Appendix G).

NLRP7 AND *KHDC3L* MUTATIONS IN SOUTH AFRICAN PATIENTS WITH HYDATIDIFORM MOLE AND RECURRENT REPRODUCTIVE WASTAGE

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Abstract

NLRP7 and *KHDC3L* are maternal-effect genes expressed in all oocytes and preimplantation embryos. Variants in these genes are associated with recurrent hydatidiform moles and patients with *NLRP7* variants also present with other forms of reproductive wastage. To date more than 60 pathogenic *NLRP7* variants and 6 pathogenic *KHDC3L* variants have been described. However, there have been no documented cases from South Africa. In this study, seven Black African patients were screened for *NLRP7* and *KHDC3L* variants. The patients all had a history of a hydatidiform mole with one or more additional episodes of reproductive wastage. Three novel *NLRP7* variants were identified of which one was pathogenic and two were variants of unknown significance. The pathogenic variant, c.1224_1232delinsT, was a complex homozygous pathogenic variant consisting of a 9 bp deletion and a single base insertion in exon 4. The patient was 20 years old and had three previous hydatidiform moles and no normal pregnancies. This is the first reported pathogenic *NLRP7* variant in a South African patient.

INTRODUCTION

Hydatidiform mole is an abnormal human pregnancy characterized by impaired embryonic development, hydropic degeneration of chorionic villi and abnormal trophoblast proliferation, usually due to excess gene expression from the paternal genome (Manokhina *et al*., 2013; Reddy *et al.,* 2013). Complete hydatidiform moles (CHM) are predominantly sporadic, characterized by the absence of an embryo and are mostly diandric diploid in origin, while partial hydatidiform moles (PHM) are mostly diandric triploid and associated with limited embryonic and fetal development (Manokhina *et al*., 2013; Wang *et al*., 2013). Rare cases of CHM are diploid biparental (BiCHM). They are characterized by disrupted DNA methylation and an abnormal expression of some maternally imprinted genes (Puechberty *et al.,* 2009; Qian *et al*., 2011; Manokhina *et al*., 2013; Reddy *et al*., 2013).

Recurrent hydatidiform mole (RHM) is defined by the occurrence of repeated molar pregnancies in affected women. One to 6% of women with a previous mole will develop a second molar pregnancy, while 10-20% will have a second non-molar reproductive loss, usually a spontaneous abortion. RHM may be non-familial and occur in patients with no family history, or they may be familial (Deveault *et al.,* 2009; Rezaei *et al*., 2016). RHM is more common in certain geographic locations such as the Middle and Far East and is also increased in populations with a high consanguinity rate (Rezaei *et al.,* 2016).

Maternal-effect genes have been shown to have causative roles in RHM. Three maternal-effect genes, *NLRP7* (NM_001127255.1)*, KHDC3L* (NM_001017361) and *PADI6* (NM_207421.4) are responsible for recurrent and familial BiCHM via maternal imprinting. In 1999, the major gene, causing imprinting, *NLRP7 (NALP7)* (NACHT, leucine- rich region and pyrin domains-containing protein family 7) was mapped to chromosome 19q13.42 (Moglabey *et al.,* 1999*;* Murdoch *et al*., 2006). *NLRP7* encodes for a protein of 1037 amino acids (Hayward *et al*., 2009). A second and minor gene, causing imprinting, *KHDC3L* (KH domain containing 3-like) or *C6orf221* was identified in 2011 and mapped to chromosome 6q13 (Parry *et al*., 2011). *KHDC3L* has 3 exons and encodes for a protein consisting of 217 amino acids (Moein-Vaziri *et al*., 2018). Recently a third gene, *PADI6* (peptidyl arginine deiminase 6), which is located on chromosome 1p36.13 has been linked to hydatidiform moles and embryonic developmental arrest (Xu *et al*., 2016; Qian *et al*., 2018). All three genes have an autosomal recessive inheritance pattern (Fallahi *et al*., 2018).

NLRP7, KHDC3L and *PADI6* are expressed in all oocytes and preimplantation embryos and encode mRNA and proteins from the maternal genome that accumulate during oogenesis and control the transition from oocyte to embryo until the activation of the fetal

genome (Dean, 2002; Murdoch *et al.,* 2006; Akoury *et al.,* 2015). Their absence results in early embryonic arrest (Dean, 2002; Akoury *et* al., 2015). *NLRP7* and *KHDC3L* variants play a causal role in RHM. Pathogenic variants of *NLRP7* are present in 48 to 80% of patients with RHM while *KHDC3L* variants occur in 10 to 14% of cases which are negative for *NLRP7* variants (Reddy *et al*., 2016; Nguyen *et al.,* 2018; Parry *et al.,* 2011; Reddy *et al*., 2013).

Patients with *NLRP7* variants not only present with familial recurrent hydatidiform moles but also with late spontaneous abortions, stillbirths and normal pregnancies with intrauterine growth retardation (Murdoch *et al.,* 2006). Patients with recessive *KHDC3L* variants have only presented with spontaneous abortions. Variants in *KHDC3L* may be more severe than variants in *NLRP7* and may not present with other forms of reproductive loss or with live births (Rezaei *et al.,* 2016).

Functional *PADI6* variants have been reported in cases with primary infertility and early developmental arrest after *in vitro* fertilization. Patients have also presented with hydatidiform moles. Pregnancy outcome appears to be variable with some patients retaining their pregnancies for a number of weeks (Qian *et al.,* 2018). These differences may be due to the type of variant present. Missense variants have a milder effect on the protein than protein truncation variants and may allow the development of some embryonic tissue (Qian *et al.,* 2018). Similarly, patients with biallelic missense *NRLP7* variants with no functional effect on the protein can also show some embryonic tissue development (Qian *et al.,* 2018).

These maternal-effect variants may put healthy female variant carriers at risk of reproductive failure, and their offspring may develop aberrant methylation and imprinting disorders. These variants represent autosomal dominant maternal effect variants which lead to aberrant imprinting marks in the offspring (Sanchez-Delgado *et al.,* 2015; Soellner *et al*., 2017). The maternal effect is supported by fact that the same pregnancy outcome of recurrent BiHM is found even when different partners are involved (Reddy *et al.,* 2013).

NLRP7 consists of an N-terminal pyrine domain, 9-10 leucine-rich repeats, a NACHT-associated domain (NAD) and a NACHT region. NLRP7 is involved in major histocompatibility complex class II inactivation. The other members of this family of genes are involved in inflammation and innate immunity (Hayward *et al*., 2009). *NLRP7* is rich in Alu repeats with approximately 48% of the *NLRP7* genomic structure made up of Alu sequences. Alu elements are predisposed to recombination and 13.5% of *NLRP7* mutations have been found to be Alu mediated (Reddy *et al*., 2016).

The presence of founder effects in *NLRP7* and *KHDC3L* in a population cause a 2 to 10-fold increase in the rates of HM (Qian *et al.,* 2011; Reddy *et al*., 2013; Fallahi *et al.,* 2018). More than 60 pathogenic *NLRP7* variants have been identified in patients with RHM and six

pathogenic variants have been described in *KHDC3L* in cases which are negative for *NLRP7* variants (Parry *et al.,* 2011; Reddy *et al*., 2013; Reddy *et al*., 2016; Nguyen *et al.,* 2018).

In Africa, *NLRP7* variants have been identified in Moroccan, Tunisian, Egyptian and Senegalese women while *KHDC3L* variants have been described in Tunisian women. (Kou *et al*., 2008; Deveault *et al.,* 2009; Puechberty *et al.,* 2009; Landolsi *et al*., 2011; Parry *et al.,* 2011; Slim *et al*., 2012; Reddy *et al*., 2016). A novel 4bp deletion, c.299_302delTCAA,p. Ile100Argfs*2 in homozygous state, resulting in a frameshift in exon 2 of the *KHDC3L* gene has been reported in a patient of African-American origin who had seven HMs with three different partners (Reddy *et al*., 2013). To date there have been no reports from Southern and Central Africa.

The aim of this study was to determine whether *NLRP7* and *KHDC3L* variants were present in a small cohort of women residing in the Free State Province of South Africa with a history of hydatidiform mole and at least one additional episode of reproductive wastage.

MATERIALS AND METHODS

Case selection

Approval to perform the study was granted by the Health Sciences Research Ethics Committee of the University of the Free State (HSREC81/2017 & HSREC72/2014). A Systematized Nomenclature of Medicine (SNOMED) search of the National Health Laboratory Service laboratory information system was performed for cases of hydatidiform mole. All patients with one or more additional episodes of reproductive wastage in the form of another hydatidiform mole or a miscarriage were contacted telephonically and asked to take part in the study. Patients who agreed were then seen at a local hospital or clinic where informed consent was obtained and a blood sample was taken.

DNA extraction

Peripheral blood (10-20 ml) was taken in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. The DNA extraction was performed using a salting out procedure (Miller *et al*., 1988). The blood was transferred into Nunc tubes and stored at –20°C until extraction was performed.

The frozen blood samples were thawed and the red cells were ruptured using 45 ml cold lysis buffer [0.3 M sucrose, 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 7.8, 5 mM $MgCl_2$, 1% (v/v) t-octylphenoxypolyethoxyethanol (Trixton X-100)]. The suspension was centrifuged (4 000 g) for 20 min at 8°C. The supernatant was removed

after which the obtained pellet was washed and suspended in 1X SET buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) containing 10 $\mu g.\mu l^{-1}$ proteinase K and 1% (w/v) sodium dodecyl sulphate (SDS). The solution was then placed in a 37°C incubator for 24 hours.

Following incubation, 1.4 ml of saturated NaCl (6 M) was added to the mixture. The solution was mixed vigorously by shaking, where after the tubes were centrifuged (4 000 g) for 15 min (15°C). The tubes were shaken vigorously for a second time and centrifugation was repeated. After centrifugation, the supernatant was transferred to a new tube which contained 2 volumes of 100% (v/v) ethanol.

The precipitated DNA was removed from the solution and transferred to an Eppendorf tube. The DNA was washed with 70% (v/v) ethanol for a minimum of two hours. The mixture was then centrifuged to produce a purified DNA pellet. The supernatant was removed and the DNA pellet was air dried in a 37°C incubator. The pellet was dissolved in 1xT.1E buffer solution.

The concentration and purity of the extracted DNA samples were determined using spectrophotometry (NanoDrop® ND-1000 Spectrophotometer v3.01, NanoDrop® Technologies Inc.) according to the manufacturer's instructions. The DNA samples were diluted to 50 $ng.\mu l^{-1}$ for use during the optimisation period of primer annealing temperatures using conventional polymerase chain reaction (PCR).

A 150 $ng.\mu l^{-1}$ DNA aliquot was prepared for high resolution melting analysis (HRMA) to equilibrate the DNA concentration before the final HRMA PCR dilutions (15 ng.µl-1) was prepared by adding DNA (150 $ng.\mu l^{-1}$ dilution) to 1xT.1E in a ratio of 1:9. The dilutions were stored at – 20°C.

NLRP7 and KHDC3L mutation screening

Real Time-based High-Resolution Melting Analysis (RT-HRMA)

Forward and reverse primer sets for all 11 exons of *NLRP7* and three exons of *KHDC3L* were ordered from Thermo Fisher Scientific and synthesized by Invitrogen™ (Appendix C). The primer sets covered all exons and exon-intron boundaries.

Each primer set was initially diluted in 1xT.1E pH 8 to a concentration of 20 µM for the conventional PCR during primer annealing temperature optimisation. Each primer dilution was then further diluted and aliquoted at a final concentration of 3 µM for the use in RT-HRMA.

PCR optimization for HRMA

A conventional gradient PCR protocol was used for the amplification of the initial PCR product for HRMA of each primer set. The PCR regime entailed one cycle at 95°C for 5 min, followed by 35 cycles at 94°C for 45 sec, the annealing temperature ranging from 56°C to 63°C for 1 min and 72°C for 45 sec, with a final elongation step at 72°C for 5 min.

Each 50 µl PCR reaction contained 200 ng template DNA, 20 µM exon specific primers, 250 µM deoxyribonucleotide triphosphate, 100 mM Tris-HCI (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCI and 1 U Taq DNA polymerase. PCR products were visualized using a 2% (w/v) agarose gel, using DNA molecular weight marker XIII (50bp ladder) to confirm the quality and specificity of the reaction. Electrophoresis was performed horizontally at 95 V in the presence of 0.05 $\mu g.ml^{-1}$ EtBr and 1xTBE.

High Resolution Melting Analysis was performed on the LightCycler® 480 II real-time instrument (Roche Molecular Systems, Inc., Basel). The DNA was diluted to 15 $ng.\mu l^{-1}$. The PCR reaction was set up per recommended instructions on the package insert of the LightScanner® Master Mix (BioFire Diagnostics, Inc., Salt lake City, Utah). Each 10 µl reaction contained 30 ng genomic DNA, 3 µM of each primer, 4 µl LightScanner® 2.5 X PCR Master mix, and molecular grade dH_2O. 25 mM $MgCl_2$ was used for further optimisation as needed.

The recommended amplification regime noted on the package insert for the LightCycler® 480 High Resolution Melting Master (version July 2009) was used.

Sanger sequencing

Samples demonstrating deviation from the baseline were bi-directionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) to determine possible variants.

The HRMA PCR products was purified using enzymatic PCR clean-up (Illustra™ ExoProStar™ 1-step from GE Healthcare Life Sciences, United Kingdom) according to the manufacturer's instructions. Each sequencing reaction contained 2 µl purified template, 1 µl BigDye® Ready Reaction terminator mix, 3 pmol of the respective primer and 2 µl BigDye® sequencing buffer.

The sequencing regime comprised of the following: one cycle at 96°C for 1 min, followed by 25 cycles at 96°C for 10 sec, 56°C for 5 sec and 60°C for 4 min. The final holding temperature was 4°C. The sequenced products were precipitated by adding 5 µl 125 mM EDTA and 60 µl 100% (v/v) ethanol and centrifuged at 14 000 rpm for 30 min, after which the supernatant was removed. The pellet was washed with 200 µl 70% (v/v)

ethanol and air dried.

Hi-Di™ formamide (Applied Biosystems, United States) (20 µl) was added to the dried products. An ABI 3500 Genetic Analyzer (Applied Biosystems, US) was used to analyze the products. Sequence analysis software (Chromas version 2.31, www.technelysium.com.au) was used to analyze the electropherograms. The sequences were aligned to the reference sequences (NM_001127255.1 for *NLRP7* and NM_001017361 for *KHDC3L*) with LALIGN (www.ch.embnet.org/software/LALIGN) and translated using the Expasy translate tool (http://au.expasy.org/tools/dna.html).

The variants identified were named according to the Human Genome Variation Society (http://www.HGVS.org/varnomen) guidelines and classified using the recommendations of the American Society of Medical Genetics and Genomics (ACMG) for the interpretation and reporting of single nucleotide variants (Richards *et al.*, 2019).

RESULTS

Seven Black African patients residing in the Free State Province of South Africa agreed to participate in the study. The median age of the patients was 25 years with an age range of 20 to 42 years. Four of the patients had a molar pregnancy and one additional miscarriage, one patient had a molar pregnancy and four miscarriages and two patients had more than one hydatidiform mole (Table 1). Histology was performed on all the hydatidiform moles and showed prominent hydropic degeneration, cistern formation and non-polar trophoblast proliferation. No fetal tissue was evident. p57 immunohistochemistry on all the cases was negative confirming them to be CHM as p57 is a paternally imprinted, maternally expressed gene. It was not possible to obtain a reliable family history in any of the cases.

Table 1. Age and number of molar pregnancies and miscarriages

Patient	Age	Number of hydatidiform moles	Number of miscarriages
1	38	1	1
2	23	1	1
3	21	1	1
4	26	1	1
5	25	1	4
6	20	3	0
7	42	2	0

A number of variants were identified in both *NLRP7* and *KHDC3L* (Table 2). A common missense variant in exon 3 of *KHDC3L* was present in six of the seven patients (85.7%). Three novel *NLRP7* variants were detected, two coding variants in exon 4 and exon 9 and one intronic variant in intron 8.

Frequencies depicted in Table 2 represent the African population as described in the 1000Genomes database (https://www.1000genomes.org/1000-genomes-browsers).

Table 2. *NLRP7* and *KHDC3L* variants

Pt	Gene	Location	Alleles	Variant	Protein	RefSNP	Class	Allele frequency
1	*NLRP7*	Exon 1	heterozygous	c.-359C>T		rs9941465	2	C 83.3% T 16.7%
	NLRP7	Exon 4	heterozygous	c.1725G>T	p.Leu575=	rs73055288	2	G 99% T 1%
	NLRP7	Exon 9	heterozygous	c.2682T>C	p.Tyr894=	rs269951	1	T 48.4% C 51.6%
	NLRP7	Exon 10	heterozygous	c.2811-23A>G		rs269933	1	A 47.8% G 52.2%
	NLRP7	Exon 11	heterozygous	c.2982-28delG		rs34438464	2	None
	KHDC3L	Exon 3	heterozygous	c.602C>G	p.Ala201Gly	rs561930	1	C 69.4% G 30.6%
2	*NLRP7*	Exon 2	homozygous	c.-39-16C>T		rs775886	1	C 36.3% T 63.7%
	NLRP7	Exon 9	heterozygous	c.2682T>C	p.Tyr894=	rs269951	1	T 48.4% C 51.6%
	NLRP7	Exon 10	heterozygous	c.2811-25G>C		rs775870	1	G 83.3% C 16.7%
	NLRP7	Exon 10	heterozygous	c.2811-23A>G		rs269933	1	A 47.8% G 52.2%
	NLRP7	Exon 11	heterozygous	c.2982-28delG		rs34438464	2	None
	KHDC3L	Exon 3	homozygous	c.602C>G	p.Ala201Gly	rs561930	1	C 69.4% G 30.6%
3	*NLRP7*	Exon 2	homozygous	c.-39-16C>T		rs775886	1	C 36.3% T 63.7%
	NLRP7	Exon 9	heterozygous	c.2682T>C	p.Tyr894=	rs269951	1	T 48.4% C 51.6%
	NLRP7	Exon 9	heterozygous	c.2706C>T	p.Ala902=	rs61746780	1	C 91.8% T 8.2%
	NLRP7	Exon 10	homozygous	c.2811-23A>G		rs269933	1	A 47.8% G 52.2%
	KHDC3L	Exon 3	homozygous	c.602C>G	p.Ala201Gly	rs561930	1	C 69.4% G 30.6%

4	*NLRP7*	Exon 2	heterozygous	c.-39-16C>T		rs775886	1	C 36.3% T 63.7%
	NLRP7	Intron 8	heterozygous	c.2642+29_ 2642+30delTG		Novel	3	None
	NLRP7	Exon 10	heterozygous	c.2811-25G>C		rs775870	1	G 83.3% C 16.7%
	KHDC3L	Exon 3	heterozygous	c.602C>G	p.Ala201Gly	rs561930	1	C 69.4% G 30.6%
5	*NLRP7*	Exon 2	heterozygous	c.-39-16C>T		rs775886	1	C 36.3% T 63.7%
	NLRP7	Exon 10	heterozygous	c.2811-25G>C		rs775870	1	G 83.3% C 16.7%
	KHDC3L	Exon 3	heterozygous	c.602C>G	p.Ala201Gly	rs561930	1	C 69.4% G 30.6%
6	*NLRP7*	Exon 4	homozygous	c.1224_1232delinsT	p.Arg409Alafs*116	Novel	5	None
	NLRP7	Exon 10	heterozygous	c.2811-25G>C		rs775870	1	G 83.3% C 16.7%
	KHDC3L	Exon 3	heterozygous	c.602C>G	p.Ala201Gly	rs561930	1	C 69.4% G 30.6%
7	*NLRP7*	Exon 9	heterozygous	c.2695C>T	p.Leu899Phe	Novel	3	None

Class 1: benign, class 2: likely benign, class 3: variant of uncertain significance, class 4: likely pathogenic, class 5: pathogenic. (Classification according to Plon et al., 2008)

DISCUSSION

A total of 12 *NLRP7* and *KHDC3L* variants were identified in the cohort of seven South African Black patients. Six *NLRP7* variants were common between the seven patients and four variants were present in homozygous state. All previously reported variants were classified as benign (class 1) or likely benign (class 2). The three novel *NLRP7* variants described in this study are classified as variants of unknown significance (class 3) and as pathogenic (class 5).

KHDC3L variant c.602C>G, p.(Ala201Gly) in exon 3

Six of the seven patients had the same *KHDC3L* missense variant in exon 3, c.602C>G. Two patients (patient 2 and 3) were homozygous for this variant. In the 1000 Genomes browser this variant was found in 30.6% of the African population (https://www.1000genomes.org/1000-genomes-browsers). In general, the ACMG guidelines indicates variants above 5% are expected to be benign and have a global occurrence (Richards *el al.*, 2015).

NLRP7 variant c.2642+29_2462+30delTG in intron 8

Patient 4, a 26-year-old patient with one HM and one additional miscarriage, presented with a novel heterozygous variant, c.2642+29_2462+30delTG. The c.2642+29_2462+30delTG variant is located in intron 8 of the *NLRP7* gene which is described by a sequence of seven AC repeats at genomic level. This variant has not been described previously and no population frequencies are available. *In silico* predictions by Human Splicing Finder (HSF) predict the deletion to disrupt an exon splicing silencer, but it is predicted to probably have no effect on splicing. Together with all the available evidence the variant tends towards a likely benign classification and until familial disease segregation studies and *in vitro* functional studies have been performed the variant remains classified as a variant of unknown significance (class 3).

NLRP7 variant c.2695C>T, p.(Leu899Phe) in exon 9

Patient 7, a 42-year-old patient with two hydatidiform moles and no other episodes of reproductive wastage, presented with a novel heterozygous missense variant, c.2695C>T. The c.2695C>T variant in exon 9 of the *NLRP7* gene is described as a substitution of a Leucine with a Phenylalanine at amino acid 899 of the NLRP7 peptide. The amino acid substitution is located between two leucine rich repeats namely LRR7 and LRR8, ranging

across amino acids 874 – 897 and 902 – 928 respectively, https://www.uniprot.org/uniprot/Q8WX94 (Rabian *et al.*, 2014). This variant has also not been described previously and no population frequencies are available. *In silico* predictions at DNA level with HSF and at protein level with Polyphen-2 predict this variant to probably have a pathogenic effect. Based on the available predictions this variant is likely pathogenic, but due to the discrepancies in the length of the available transcripts, *in vitro* functional studies are required to fully classify this variant as pathogenic. Until functional assays are performed this variant remains classified as a variant of unknown significance (class 3).

*NLRP7 variant c.1224_1232delinsT, p.(Arg409Alafs*116) in exon 4*

Patient 6, a 20-year-old patient with three previous hydatidiform moles and no other reproductive wastage, presented with a novel complex homozygous pathogenic variant, consisting of a 9 bp deletion and a single base insertion in exon 4. The effect on the amino peptide is described as a substitution at residue 409, but the insertion causes a frameshift that results in a truncated peptide 116 residue downstream. The frameshift is located in the NACHT domain of the NLRP7 protein that spans across amino acids 172 – 491. Mutations in the NACHT domain disrupt oligomerization (https://www.uniprot.org/uniprot/Q8WX94) (Rabian *et al.*, 2014). This novel variant is classified as a class 5 pathogenic variant.

The three novel *NLRP7* variants described in this study were not found in 121 336 global subjects and 10 400 African subjects in ExAC (https://gnomad.boradinstitute.org/) or in 5008 global subjects and 1322 African subjects in 1000 genomes browser (https://www.1000genomes.org/1000-genomes-browsers).

There is still no consensus as to whether heterozygous non-synonymous variants (NSVs) play a role in the development of recurrent hydatidiform moles. Deveault *et al.* (2009) suggested that some *NLRP7* NSVs can be associated with hydatidiform moles even when heterozygous. This was supported by Qian *et al.* (2011) who proposed that some, but not all, heterozygous *NLRP7* mutations are associated with reproductive wastage. Andreasen *et al.* (2013) stated that heterozygosity for particular NSVs could not be excluded as playing a role in familial hydatidiform moles and familial non-molar miscarriages while Soellner *et al.* (2017) suggested that heterozygous *NLRP7* variant carriers have an increased risk of reproductive wastage but do not develop hydatidiform moles. In addition, Slim *et al.* (2012) identified three new NSVs that were not found in the general population and stated that further studies were required to determine whether they were indeed pathogenic or

rather very rare variants. In contrast, Manokhina *et al.* (2013) found that *NLRP7* and *KHDC3L* variants were not a common risk factor for androgenetic hydatidiform moles, triploidy and recurrent miscarriages. In this study, patients one to six all had one or more NSV's of both *NLRP7* and *KHDC3L,* some of which were homozygous. All these variants were categorized as benign or likely benign and occurred in the normal population and the precise role they played is uncertain.

Our study was limited by the absence of a family history, small sample size and a mutation screening method limited to the detection and identification of variants on only a single indirect sample type, namely maternal blood. To further elucidate the causal role of the maternal effect genes, *NLRP7, KHDC3L* and *PADI6* in recurrent hydatidiform moles we will expand by increasing the sample size and ensure an extended family history. Additional screening methods for both identification and determination of the genetic imprinting status of variants will be used and blood, hydatidiform moles and/or other products of reproductive wastage from the patients and possible family members will be utilized when available.

In conclusion, we report a novel *NLRP7* homozygous pathogenic variant in a patient with three previous molar pregnancies. This is the first documented case of a pathogenic *NLRP7* variant in a South African patient.

REFERENCES

Akoury E, Zhang L, Ao A, Slim R. NLRP7 and KHDC3L, the two maternal-effect proteins responsible for recurrent hydatidiform moles, co-localize to the oocyte cytoskeleton. *Hum Reprod* 2015;30:159-169.

Andreasen L, Christiansen OB, Niemann I, Bolund L, Sunde L. *NLRP7* or *KHDC3L* genes and the etiology of molar pregnancies and recurrent miscarriages. *Mol Hum Reprod* 2013;19:773-781.

Dean J. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. *J Reprod Immunol* 2002;53:171-180.

Deveault C, Qian JH, Chebaro W, Ao A, Gilbert L, Mehio A, Khan R, Tan SL, Wischmeijer A, Couillin P, Xie X, Slim R*. NLRP7* mutations in women with diploid androgenetic and triploid moles: A proposed mechanism for mole formation. *Hum Mol Genet* 2009;18:888-897.

Fallahi J, Anvar Z, Razban V, Momtahan M, Namavar-Jahromi B, Fardaei M. Founder effect of *KHDC3L*, p.M1V mutation, on Iranian patients with recurrent hydatidiform moles. *Iran J Med Sci* [epub ahead of print].

Hayward BE, De Vos M, Talati N, Abdollahi MR, Taylor GR, Meyer E, Williams D, Maher ER, Setna F, Nazir K, Hussaini S, Jafri H, Rashid Y, Sheridan E, Bonthron DT. Genetic and epigenetic analysis of recurrent hydatidiform mole. *Hum Mutat* 2009;30:E629-E639.

Kou YC, Peng HH, Rosetta R, del Gaudio D, Wagner AF, Al-Hussaini TK, Van den Veyver IB. A recurrent intragenic genomic duplication, other novel mutations in *NLRP7* and imprinting defects in recurrent biparental hydatidiform moles. *Mol Hum Reprod* 2008;14:33-40.

Landolsi H, Rittore C, Philibert L, Missaoui N, Hmissa S, Touiyou I, Gribaa M, Yacoubi MT. Screening for *NLRP7* mutations in familial and sporadic recurrent hydatidiform moles: Report of 2 Tunisian families. *Int J Gynecol Pathol* 2011;30:348-353.

Manokhina I, Hanna CW, Stephenson MD, McFadden DE, Robinson WP. Maternal *NLRP7* and *C6orf221* variants are not a common risk factor for androgenetic moles, triploidy and recurrent miscarriage. *Mol Hum Reprod* 2013;19:539-544.

Miller S, Dykes D, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.

Moglabey YB, Kircheisen R, Seoud M, El Mogharbel N, Van den Veyver I, Slim R. Genetic mapping of a maternal locus responsible for familial hydatidiform moles. *Hum Mol Genet* 1999;8:667-671.

Murdoch S, Djuric U, Mazhur B, Seoud M, Khan R, Kuick R, Bagga R, Kircheisen R, Ao A, Ratti B, Hanash S, Rouleau GA, Slim R. Mutations in *NALP7* cause recurrent hydatidiform moles and reproductive wastage in humans. *Nat Genet* 2006;38:300-302.

Nguyen NMP, Khawajkie Y, Mechtouf N, Rezaei M, Breguet M, Kurvinen E, Jagadeesh S, Solmaz AE, Aguinaga M, Hemida R, Harma MI, Rittore C, Rahimi K, Arseneau J, Hovanes K, Clisham R, Lenzi T, Scurry B, Addor M, Bagga R, Nendaz GG, Finci V, Poke G, Grimes L, Gregersen N, York K, Bolze P, Patel C, Mozdarani H, Puechberty J, Scotchie J, Fardaei M,

Harma M, McKinley Gardner RJ, Sahoo T, Dudding-Byth T, Srinivasan R, Sauthier P, Slim R. The genetics of recurrent hydatidiform moles: new insights and lessons from a comprehensive analysis of 113 patients. *Mod Pathol* 2018; 31:1116-1130.

Parry DA, Logan CV, Hayward BE, Shires M, Landolsi H, Diggle C, Carr I, Rittore C, Touitou I, Philibert L, Fisher RA, Fallhian M, Huntriss JD, Picton HM, Malik S, Taylor GR, Johnson CA, Bonthron DT, Sheridan EG. Mutations causing familial biparental hydatidiform mole implicate *C6orf221* as a possible regulator of genomic imprinting in the human oocyte. *Am J Hum Genet* 2011;89:451-458.

Peuchberty J, Rittore C, Philibert L, Lefort G, Burlet G, Benos P, Reyftmann L, Sarda P, Touitou I. Homozygous *NLRP7* mutations in a Moroccan woman with recurrent reproductive failure. *Clin Genet* 2009;75:298-300.

Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV and Group IUGVW. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat* 2008;29:1282-91.

Qian J, Cheng Q, Murdoch S, Xu C, Jin F, Chebaro W, Zhang X, Gao H, Zhu Y, Slim R, Xie X. The genetics of recurrent hydatidiform moles in China: correlations between *NLRP7* mutations, molar genotypes and reproductive outcomes. *Mol Hum Reprod* 2011;17:612-619.

Qian J, Nguyen NMP, Rezaei M, Huang B, Tao Y, Zhang X, Cheng Q, Yang H, Asangla A, Majewski J, Slim R. Biallellic *PADI6* variants linking infertility, miscarriages, and hydatidiform moles. *Eur J Hum Genet* 2018;26:1007-1013.

Radian AD, de Almeida L, Dorfleutner A, Stehlik C. NLRP7 and related inflammation activating pattern recognition receptors and their function in host defense and disease. *Microbes Infect* 2013;15:630 – 639.

Reddy R, Akoury E, Nguyen NMP, Abdul-Rahman OA, Dery C, Gupta N, Daley WP, Ao A, Landolsi H, Fisher RA, Touitou I, Slim R. Report of four new patients with protein-truncating mutations in *C6orf221/KHDC3L* and colocalisation with *NLRP7*. *Eur J Hum Genet* 2013;21:957-964.

Reddy R, Nguyen NMP, Sarrabay G, Rezael M, Rivas MC, Kavasoglu A, Berkil H, Elshafey A, Abdalia E, Nunez KP, Dreyfus H, Philippe M, Hadipour Z, Durmaz A, Eaton EE, Schubert B, Ulker V, Hadipour F, Touitou I, Fardaei M Slim R. The genomic architecture of *NLRP7* is Alu rich and predisposes to disease-associated large deletions. *Eur J Hum Genet* 2016;24:1445-1452.

Rezaei M, Nguyen NMP, Foroughinia L, Dash P, Ahmadpour F, Verma IC, Slim R, Fardaei M. Two novel mutations in the *KHDC3L* gene in Asian patients with recurrent hydatidiform mole. *Hum Genome Var* 2016;3:16027. doi:10.1038/hgv.2016.27.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;17:405-424.

Sanchez-Delgado M, Martin-Trujillo A, Tayama C, Vidal E, Esteller M, Iglesias-Platas I, Deo N, Barney O, Maclean K, Hata K, Nakabayashi K, Fisher R, Monk D. Absence of maternal methylation in biparentla hydatidiform moles from women with *NLRP7* maternal-effect mutations reveals widespread placenta-specific imprinting. PLoS Genet 2015;11:e1005644. Doi:10.1371/journal.pgen.1005644.

Slim R, Coullin P, Diatta A, Chebaro W, Courtin D, Abdelhak S, Garcia A. *NLRP7* and the genetics of post-molar choriocarcinomas in Senegal. *Mol Hum Reprod* 2012;18:52-56.

Soellner L, Begemann M, Degenhardt, Geipel A, Eggermann T, Mangold E. Maternal heterozygous *NLRP7* variant results in recurrent reproductive failure and imprinting disturbances in the offspring. *Eur J Hum Genet* 2017;8:924-929.

Wang CM, Dixon PH, Decordova S, Hodges MD, Sebire NJ, Ozalp S, Fallahian M, Sensi A, Ashrafi F, Repiska V, Zhao J, Xiang Y, Savage PM, Seckl MJ, Fisher RA. Identification of 13 novel *NLRP7* mutations in 20 families with recurrent hydatidiform mole; missense mutations cluster in the leucine rich region. *J Med Genet* 2009;46:569-575.

Xu Y, Shi Y, Fu J, Yu M, Feng R, Sang Q, Liang B, Chen B, Qu R, Li B, Yan Z, Mao X, Kuang

Y, Jin L, He L, Sun X, Wang L. Mutations in *PADI6* cause female infertility characterised by early embryonic arrest. *Am J Hum Genet* 2016;99:744-752.

Zhang W, Chen Z, Zhang D, Zhao B, Liu L, Xie Z, Yao Y, Zheng P. KHDC3L mutation causes recurrent pregnancy loss by inducing genomic instability of human early embryonic cells. *PLOS Biology* 2019; 17(10):e3000468.

CHAPTER 5

ARTICLE 4: CHORIOCARCINOMA IN SOUTH AFRICAN WOMEN: ANALYSIS OF A SERIES WITH GENOTYPING

The article was prepared according to the journal submission guidelines for *American Journal of Surgical Pathology* (cf. Appendix H).

CHORIOCARCINOMA IN SOUTH AFRICAN WOMEN: ANALYSIS OF A SERIES WITH GENOTYPING

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Abstract

Choriocarcinomas can be either gestational or non-gestational. Gestational tumours arise from a previous pregnancy while non-gestational tumours arise from germ cells. The prognosis and treatment differ, and correct categorization is therefore important. Genotyping can be utilized to make this distinction, but this technique has not yet been used in Africa. In this study we genotyped 20 choriocarcinomas and 6 control cases of complete hydatidiform mole (CHM) using a short tandem repeat multiplex polymerase chain reaction (PCR) assay for 15 loci and a sex marker, amelogenin. All the patients were of African descent. In two cases amplification failed and the cases were excluded from the study. Of the remaining 18 cases, 17 were gestational and one was non-gestational. Of the gestational cases, 16 were purely androgenetic/homozygous XX compatible with a previous CHM while one arose from a previous normal pregnancy. In addition, a rare variant allelic repeat, 22.2 at locus FGA was identified in one case. This variant has a frequency of 0.0026 in the South African population. A number of problems were encountered with this technique including poor amplification and cross contamination between tumour and maternal tissue. However, despite these issues, interpretation was still possible.

INTRODUCTION

Choriocarcinoma is a malignant trophoblastic tumour characterised by the production of human chorionic gonadotropin (hCG) (Cheung *et al.*, 2009). Choriocarcinomas can be either gestational or non-gestational. The majority are gestational and arise from a previous pregnancy. The previous pregnancy may be a miscarriage, induced abortion, live birth, still birth, ectopic pregnancy or a hydatidiform mole (Fisher *et al.*, 1992; Zhao *et al.*, 2009). Non-gestational or primary choriocarcinoma represents a form of germ cell tumour which can arise from germ cells in the ovaries or from extragonadal sites in the midline such as the mediastinum and retroperitoneum. Occasionally, choriocarcinomas can also occur in association with a poorly differentiated carcinoma in parenchymal organs such as the gastrointestinal tract (Cheung *et al.*, 2009).

Gestational choriocarcinomas do not always arise from the immediately antecedent pregnancy (Fisher *et al.*, 1992; Fisher *et al.*, 1995). More than 50% of gestational choriocarcinomas are secondary to a previous complete hydatidiform mole (Hoffner and Surti, 2012). However, there may be one or more normal pregnancies or miscarriages between the complete hydatidiform mole and the development of the choriocarcinoma (Savage *et al.*, 2017). Although rare, choriocarcinomas can also occur secondary to a partial hydatidiform mole (Seckl *et al.,* 2000).

It is important to distinguish between gestational and non-gestational tumours as non-gestational tumours have a worse prognosis and different treatment, including different chemotherapy regimes, is required (Fisher *et al.*, 1992; Cheung *et al.,* 2009). It is often not possible to distinguish between gestational and non-gestational tumours based on histology. Although the clinical history may assist, a definite diagnosis can only be made with molecular genotyping. In addition, the International Federation of Gynaecology and Obstetrics and the World Health Organization (FIGO/WHO) scoring system for gestational choriocarcinoma allocates a lower score to those cases originating from a previous hydatidiform mole (Ngan *et al.*, 2012).

Microsatellite analysis has been successfully used to accurately categorize choriocarcinomas as gestational or non-gestational in a number of international studies (Cankovic *et al.,* 2006; Fisher *et al.,* 2007*;* Zhao *et al.,* 2009; Savage *et al.,* 2017). However, no study has been published applying this test method in choriocarcinoma in Africa.

Here we present a small series of 20 choriocarcinoma cases from South Africa analyzed with microsatellite genotyping using only patient tissue to facilitate clinical management.

MATERIALS AND METHODS

Case selection

Approval to perform the study was granted by the Health Sciences Research Ethics Committee of the University of the Free State (UFS) (HSREC81/2017).

Twenty histologically confirmed choriocarcinoma cases and six control cases diagnosed with CHM, with sufficient tumour and benign maternal tissue, available in paraffin blocks, were selected using the laboratory information system of the Department of Anatomical Pathology, UFS and National Health Laboratory Service (NHLS), Bloemfontein, South Africa. All patients were black Africans.

Slide dissection

Formalin-fixed paraffin-embedded (FFPE) tissue sections were mounted on glass slides and stained with haematoxylin and eosin using standard protocol. Areas of choriocarcinoma or hydatidiform mole were identified by histological examination and carefully demarcated and separate areas of maternal tissue from each case were selected for identification of maternal alleles. Microdissection was performed on 10-µm tissue sections for both the neoplastic and maternal tissues with a sterile scalpel blade and placed into separate microcentrifuge tubes. The paired tumour and maternal tissue samples were analysed in parallel.

DNA extraction

DNA was isolated using a commercial kit from Qiagen according to manufacturer instructions (Qiagen, Valencia, CA). FFPE sections were transferred to a 1.5 ml microcentrifuge tube and 1 ml xylene was immediately added to the sample. The samples were then vortexed vigorously for ten seconds followed by centrifugation at full speed (14 000 rpm) for two minutes at room temperature. The supernatant was removed by pipetting. Remaining xylene was removed by washing the pellet with 1 ml absolute ethanol (100%) by vortexing followed by centrifugation at full speed (14 000 rpm) for two minutes at room temperature. The supernatant was again removed by pipetting and the tubes were then opened and incubated at room temperature for ten minutes until the residual ethanol had evaporated. The pellets were then resuspended in 180 µl Buffer ATL and 20 µl of proteinase K was added. The samples were mixed by vortexing and incubated overnight at 56°C. The incubation period deviated from the manufacturer's instructions of three hours due to incomplete digestion at the specified time period. This was then followed by incubation at 90°C for one hour. The samples were then briefly centrifuged to remove drops from the inside of the lid. 200 µl of Buffer AL

was added to the samples and mixed thoroughly by vortexing followed by the addition of 200 µl of absolute ethanol after which the samples were again mixed by vortexing. The samples were then briefly centrifuged to remove drops from the inside of the lid. The entire lysate from each sample was transferred to a QIAamp MinElute column in a 2 ml collection tube without wetting the rim. The lids were closed, and the samples were centrifuged at 8000 rpm for one minute. The MinElute columns were then placed in new collection tubes and 500 µl of Buffer AW1 was added without wetting the rim. The lids were closed, and the samples were centrifuged at 8000 rpm for one minute after which they were placed in another clean collection tube and 500 µl of Buffer AW2 was added to each sample without wetting the rim. The samples were centrifuged for one minute at 8000 rpm after which they were again placed in a new collection tube. The samples were then centrifuged at 14 000 rpm for three minutes to dry the membrane completely. The MinElute columns were then placed in new microcentrifuge tubes and 100 µl of Buffer ATE was added to the centre of the membranes. The lids were closed, and the samples were incubated at room temperature for five minutes after which they were centrifuged at 14 000 rpm for one minute. Incubation of five minutes delivered significantly more DNA compared to the one minute advised by the manufacturer's instructions.

The quality of the extracted DNA was evaluated using spectrophotometry (NanoDrop® ND-100 Spectrophotometer v3.01, NanoDrop® Technologies Inc.). Genomic DNA was diluted to a concentration of 1 ng/µl.

Microsatellite genotyping

Paired tumour and benign maternal tissue samples were analyzed in parallel for genotype, using the AmpFlSTR™ Identifiler PCR Amplification System (Applied Biosystems, Foster City, California). The reaction consists of a short tandem repeat multiplex polymerase chain reaction (PCR) assay, using fluorescently labeled primers targeting 15 tetrameric repetitive polymorphic loci (D81179**,** D81179 , D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and a sex marker Amelogenin) in a single reaction producing short amplicons ranging from 100 to 350bp. Five different fluorescent dyes in the Identifiler™ kit allows for amplification of all loci in a single tube. Four fluorescent dyes (6-FAM, VIC, NED and PET) are labelled to PCR amplicons and a fifth dye (LIZ) is used to label the GeneScan-500 Size Standard (AmpFlSTR™ Identifiler™ PCR amplification kit User Guide, Lipata *et al.*, 2010).

The PCR reactions consisted of 1 ng genomic DNA amplified in a 25 µl reaction, containing 10µl of AmpFlSTR™ reaction mix, 5 µl of primer mix and 0.5 µl of AmpliTaq Gold DNA

polymerase. PCR conditions were 95°C for 11 minutes, followed by 28 cycles of 94°C for one minute, 59°C for one minute and 72°C for one minute with a final extension at 60°C for 60 minutes. One µl of amplified PCR product was mixed with 13 µl of Hi-Di and 0.5 µl of sizing ladder (GeneScan-500LIZ, Applied Biosystems, Inc.). Capillary electrophoresis was performed on an ABI3130 Genetic Analyzer (30cm capillary and POP-7 polymer) (Applied Biosystems, Inc.). Fragment analysis for both the maternal and tumour tissues was performed, using the GeneMarker software (SoftGenetics, LLC) for loci genotyping.

Comparative genotyping of the corresponding normal maternal tissue and the choriocarcinoma must be run in parallel in order to identify the genetic origin of the tumour tissue. The paternal genotype was unknown in all cases and the polymorphic loci in the choriocarcinoma, not present in the maternal tissue, were scored as paternal.

RESULTS

The results of the microsatellite analysis of the choriocarcinomas is provided in Table 1 while results of the control cases composed of complete hydatidiform moles are provided in Table 2.

Table 1. Results of the microsatellite analysis of the choriocarcinoma tissue

Choriocarcinoma cases																		
	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	THO1	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	Amel	D5S818	FGA	AP	
[1]Max allele size	≤153bp	≤232bp	≤279bp	≤334bp	≤136bp	≤187bp	≤240bp	≤288bp	≤351bp	≤125bp	≤190bp	≤245bp	≤316bp	X≤107bp; Y≤112bp	≤164bp	≤250bp		
Case																		
2N	14,15	30,35	11	7,9	15,16	7,8	10,11	12	16,21	13,14	15	6	14,16	X	10,12	19,23		
2T	13	33.2	10	12	14	8	11	11	20	14	16	6	14	X	12	22	CHM	monospermy
3N	12,14	30,32.2	11	11	16,17	7,8	12,14	9,11	16,19	11,13	15	11	18,20	X	8,10	23,24		
3T	13,14	31.2,32.2	10,11	10,11	15,17	6,8	12	9,11	19,23	13	14,15	10,11	16,20	X	10,13	24	NP	gestational
4N	12,15	28,29	10,11	7,13	14,16	7,8	11,12	9,12	23	11,14	15,18	11	10.2,17	X	11,12	22,25		
4T	12,15	28,29	10,11	7,13	14,16	7,8	11,12	9,12	23	11,14	15,18	11	10.2,17	X	11,12	22,25	P	maternal
5N	12,15	30	ND	ND	15	6,8	11	9	ND	10.2,14	14	12	16,17	X	12,13	22,23		
5T	13	32.2	ND	ND	15	7	ND	12	ND	13	16	11	ND	X	12	19	CHM	monospermy
6N	14,15	28,32.2	10,11	7,10	15,16	7,9	11,12	9	21,23	11,12.2	16	9,10	17,18	X	11,13	21,24		
6T	15	28	9	12	16	6	11	8	21	12	16	8	19	X	8	23	CHM	monospermy
7N	13,14	28	11	10	15,17	7,10	10,11	10	21,22	14	16,17	9	15,20	X	12,14	24,26		
7T	13	34.2	12	7	16	9	14	10	20	7	17	8	18	X	12	21	CHM	monospermy
8N	11,14	28,30.2	10,11	11,12	14,15	6,9	13	11,12	19,23	12,14	16,20	9,11	16,18	X	12,13	22,26		
8T	14	28	9	10	15	8	13	12	22	13	16	11	15	X	12	25	CHM	monospermy
9N	15	24.2,33.1	9,12	10,12	11	7,8	11	10	18,19	15	11	8,9	19	X	11,14	23,24		
9T	13	27	10	9	14	8	12	12	22	14	16	11	19	X	12	24	CHM	monospermy
10N	14,15	28	ND	ND	15,17	9	ND	11,12	ND	11,14	18	8,11	ND	X	12,13	22,23		
10T	14	28	ND	ND	15	8	ND	11	ND	12	16	9	ND	X	12	25	CHM	monospermy
11N	15	28,29	9,10	12,13	14,16	9	11,13	10,12	17,19	11,12	16	9,11	15,16	X	12	21,23		
11T	14	28	11	12	14	8	12	12	22	13	17	10	18	X	13	24	CHM	monospermy
12N	12,13	32.2,33	9,10	12	15	7	11,12	11,14	19,23	7,14	15,16	6,11	14,15	X	8,12	24,26		
12T	15	31	9	12	16	8	13	11	16	14	17	11	16	X	22	16	CHM	monospermy
13N	12,15	29,36.1	10,12	12	16,17	10	12	11	20,24	13,14	15,16	8,9	19	X	11,13	22,24		
13T	13	31	11	10	16	9	12	9	19	13	16	11	17	X	11	22	CHM	monospermy
14N	13	28	11,12	12,13	15,18	8,9	12,14	11	22	13,14	14,15	8,9	15,18	X	11,13	19,22		
14T	13	32.2	9	12	17	9	13	11	20	14.2	16	11	14.2	X	12	19	CHM	monospermy
15N	12,15	31.2,32.2	9	9	14,15	8	12,13	10,11	21,23	12,14	14,17	8,11	15	X	10,12	20		
15T	13	28	8	12	15	9	14	9	ND	15	11	9	16	X	8	19	CHM	monospermy
16N	11,13	32.2,33.2	8,10	10,12	16,17	7	11	9	22,26	10.2,14	16,17	10,11	15,19	X	12,13	16.2,25		
16T	14	28	9	12	14	7	13	13	22	14	16	6	17	X	11	24	CHM	monospermy
18N	13	29,32.2	8,10	8,13	16	7,8	12,14	12	21,22	12,14	14,18	8,9	15,16	X	12,13	23,26		
18T	13	28	9	12	18	8	11	11	21	14	19	8	18	X	11	22.2	CHM	monospermy
19N	14	31.2,32.2	8	11	15,17	6,8	11,12	11,12	22	12,13	18	9,11	13,16	X	11,12	19.2,20		
19T	14	31.2	11	12	15	7	13	9	17	15	16	8	19	X	8	23	CHM	monospermy
20N	13,14	31,31.2	9,12	9,10	15,17	8,9.3	11	9,10	20,21	14	15,18	6,11	14,15	X	12,13	23,24		
20T	15	30	10	12	15	7	12	13	22	12	15	11	20	X	8	25	CHM	monospermy

N = Normal or maternal tissue, T = tumour tissue, CHM = complete hydatidiform mole, ND = not detected, P= primary choriocarcinoma, AP = antecedent pregnancy, NP = normal pregnancy, [1]Maximum allele size as determined in this study (AmpFlSTR™ Identifiler™ PCR amplification kit User Guide)

Table 2. Microsatellite analysis of the control CHM group

Controls – Complete Androgenic Hydatidiform Moles																	
Case	**D81179**	**D21S11**	**D7S820**	**CSF1P**	**D3S1358**	**THO1**	**D13S317**	**D16S539**	**D2S1338**	**D19S433**	**vWA**	**TPOX**	**D18S51**	**Amel**	**D5S818**	**FGA**	**AP**
22N	14	28,32	10,12	11	16,17	8	12,14	10,11	19,25	10.2	15,17	6,11	12,13	X	12,13	22,25	
22T	13	31.2	9	9	15	7	12	11	21	13	15	8	18	X	8	26	monospermy
23N	15	29,31	10,11	11,12	14,15	6,7	12,13	11,12	21,25	13,14.2	16,18	6,9	17,19	X	8,13	19,27	
23T	13	30	10	12	15	8	12	10	24	12	18	8	19	X	11	22	monospermy
24N	14,15	29,31.2	9	9,12	15	8	12	11	19	12,15.2	16,17	9	15,17	X	8	21,23	
24T	13	32.2	10	12	12	9.3	13	8	16	14	19	11	18	X	12	26	monospermy
25N	10,14	29,30	11	9,12	14,16	9	11	12,13	18,24	13,15	14,17	22	25	X	10,12	21,25	
25T	14	29	12	10	16	6	8	11	18	15	15	11	17	X	11	24.2	monospermy
26N	14,15	28,30	10	10,12	15,18	6,8	19,12	11,12	17,18	14	14,18	20,11	13,16	X	12	22,24	
26T	12	32	9	13	15	7	9	11	18	14	14	8	13	X	12	22	monospermy
27N	13,14	28	9,10	10,12	15,16	8,9.3	11,12	11	21,23	11,13	16,19	8,11	15,20	X	11,12	23,27	
27T	14	28	9	7	16	7	13	12	16	14	16	11	18	X	11	26	monospermy

N = Normal or maternal tissue, T = tumour tissue

From the 20 cases of choriocarcinoma with sufficient tumour and maternal tissue, two cases (cases 1 and 17) failed amplification after various PCR attempts and were excluded from the study. The remaining 18 cases were suitable for analysis. Of these, one case (case 4) contained exclusively maternal alleles indicating a non-gestational origin compatible with a primary choriocarcinoma (Figure 1), one case (case 3) had one maternal and one non-maternal allele compatible with an antecedent normal pregnancy, while the remaining 16 cases showed only non-maternal alleles compatible with a previous complete androgenetic hydatidiform mole (Figure 2). No triploid cases indicative of a preceding partial hydatidiform mole were identified in this study. All six controls presented with exclusively non-maternal alleles confirming them as complete androgenetic hydatidiform moles (Figure 3).

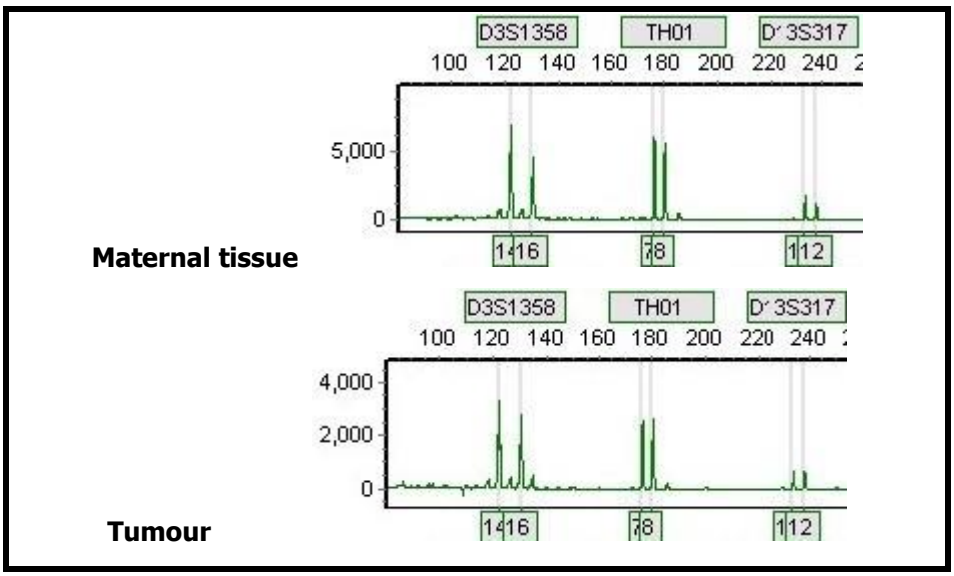

Figure 1. Microsatellite genotyping result for case 4. The alleles are the same in both the maternal and tumour tissue confirming a primary (non-gestational) choriocarcinoma

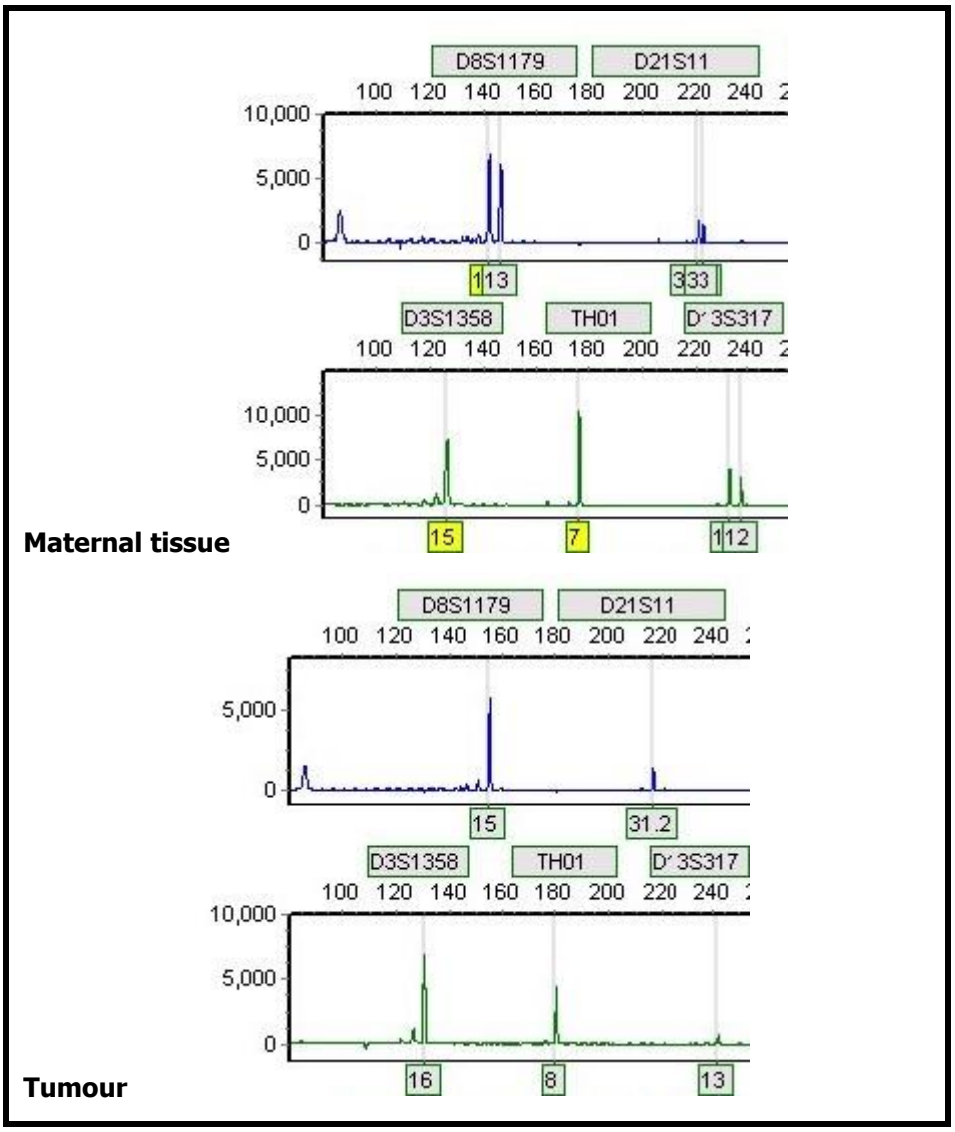

Figure 2. Microsatellite genotyping result for case 12. There are no matching alleles confirming that this represents a gestational choriocarcinoma arising from a previous complete hydatidiform mole.

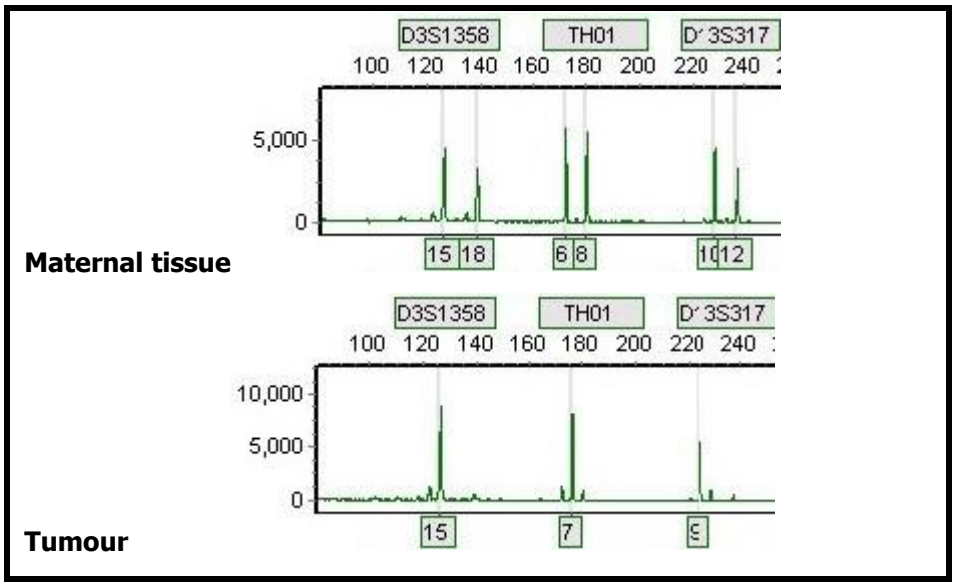

Figure 3. Microsatellite genotyping result for case 26. This is a control case of a complete hydatidiform mole. Only one peak is noted in the tumour tissue for all the STR markers, which is consistent with monospermy.

In three of the cases (cases 5, 10 and 15) not all the alleles could be amplified. Despite degraded DNA and consequential failed amplification across various loci (ranging from 240bp to 351bp) in cases 5 and 10, at least 9 loci in each case were still interpretable and could be genotyped. A single locus (D2S1338 - 351bp) of the tumour DNA of case 15 failed amplification. This locus contains the largest polymorphic fragment. This may be ascribed to poor amplification and was mostly due to DNA quality (degraded DNA) and was successfully addressed by re-extraction of DNA and PCR amplification using a lower DNA concentration to reduce possible amplification inhibition agents in the crude lysate (Figure 4). Despite the presence of degraded DNA interpretation was still possible.

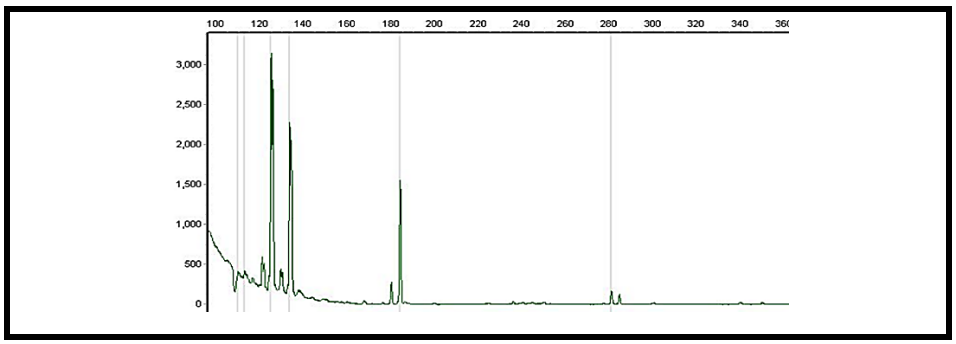

Figure 4. Example of microsatellite genotyping indicating poor amplification due to DNA degradation

Minor cross contamination between the two tissue types (present as small peaks) was noted in some cases (Figure 5). The maternal alleles could not be distinguished from tumour alleles as both amplified poorly and peaks were of equal height and were not genotyped.

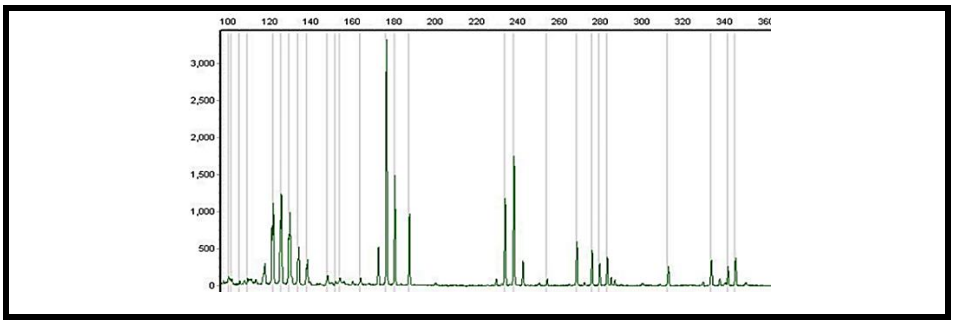

Figure 5. Minor cross contamination between the two tissue types (present as small peaks) was noted in some cases

For some cases the peak intensity was very low for fragments above 250 bp. Allele peaks below 200 relative fluorescent units (rfu's) were not genotyped in this study. Although poor amplification occurred, true peaks in tumour tissue were distinguishable from maternal alleles and random background peaks, as seen in the tumour tissue at 318 bp in Figure 6. Despite of the presence of maternal contamination interpretation was still possible.

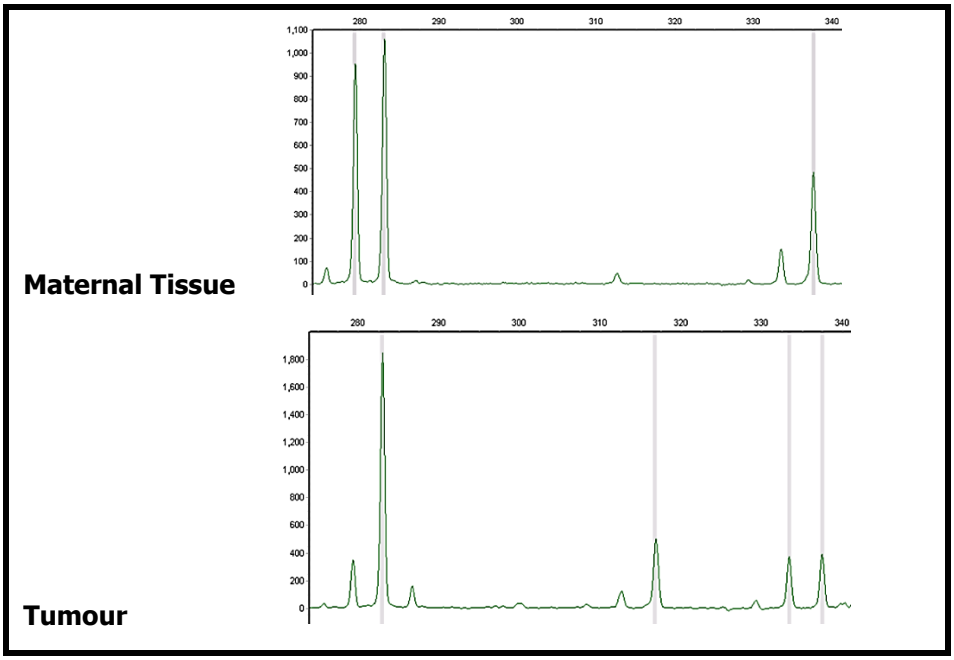

Figure 6. Microsatellite genotyping example of cross contamination of two tissue populations. The detectable relative fluorescent unit threshold was decreased to 200 rfu's.

Only twelve genotypes from the 16 choriocarcinoma cases (excluding cases 5 and 10) and three genotypes from the CMH control group were uninformative due to homozygosity between the maternal and tumour tissue. More than 95% (228/240 genotypes) and 96.7% (87/90 genotypes) of the genotypes across all loci in the choriocarcinoma and control groups respectively, were informative and allowed for accurate interpretation of the genotyping results and an unequivocal diagnosis.

Of interest, was the presence of a variant allelic repeat, 22.2 at locus FGA (case 18) which was not listed in the AmpFlSTR™ Identifiler™ PCR Amplification Kit User Guide as a known allele. Initially we excluded this allele from the genotype as various peaks of almost equal height were present at 200 rfu's. On further investigation we found the variant to be a true amplification product as it is a rare allele in the South African population which is only detected in 29/19,179 cases with a of frequency of 0.0026 (Unpublished data, Dr Andre de Kock, Head of the local Paternity and Kinship Testing Facility).

DISCUSSION

In our study 16 of the 18 cases (88.9%) were gestational choricarcinomas arising from a previous complete hydatidiform mole. This is in keeping with previous international studies (Zhao *et al.*, 2009; Savage *et al.*, 2017). All CHM were diploid androgenetic and homozygous at each locus indicating that the CHM arose from a single sperm that duplicated its genome. This is in concordance with other studies, indicating 81 to 90% of cases arise from monospermy (Bifulco *et al.,* 2008; Furtado *et al.,* 2013). Although the homozygous pattern does not definitely demonstrate diploidy, it does demonstrate complete androgenicity, which is probably the most important information required (Murphy *et al.*, 2009).

Case 3 was a gestational choriocarcinoma resulting from a previous normal pregnancy. Maternal and paternal alleles were expressed in equal proportion, suggesting it originated from a normal balanced biallelic gestation. Case 4 was a non-gestational choriocarcinoma containing only maternal alleles, devoid of any paternal alleles.

FFPE tissue can be significantly degraded, resulting in poor PCR amplification, especially with older specimens (>10 years) and particularly involving longer amplicons. Poor PCR amplification can result in inaccurate or biased allele ratios (skewed allelic ratio), which can confound interpretation. This may be sufficiently addressed by the institution of a requirement of peak heights. Murphy *et al*. (2009) institute rfu's of 300 at a 30 second injection. In our study we introduced a rfu of 200 to address the challenge for fragments smaller than 300 bp. Others reported interpretable results in older, damaged archival samples. Samples were between 2 and 10 years old at the time of DNA extraction (Cancovic *et al.*, 2006; Murphy *et al.*, 2009). Our laboratory receives samples from the entire Free State Province of South Africa and the quality of the formalin (not buffered to a more neutral pH) provided by the referral hospitals, in which the tissue is fixed and transported is not always of an appropriate standard.

Poor amplification is usually observed in larger sized amplicons (>300bp) (Furtado *et*

al., 2013). Cankovic *et al.* (2006) reported that amplification of shorter allelic fragments was successful in all samples, but amplification of fragments larger than 200bp was not possible in some cases. However, in spite of this challenge proper assignment of all alleles was accomplished and reliable comparison between tumour and maternal tissue was possible. Our data supports these studies and indicates amplification of all loci and allele size ranging from 107bp to 351bp, and although the majority of samples in our study were characterized by poor amplification of fragments larger than 200 bp interpretation was still possible.

Shared alleles (homozygosity) between maternal and tumour tissue and an unknown paternal genotype can make interpretation difficult and the locus completely uninformative. In contrast, between 95% and 96.7% of genotypes were informative and proper assignment of alleles was accomplished in our study.

The most frequently encountered problem with the genotyping assay was cross-contamination between maternal and tumour tissue owing to difficulty isolating these areas as pure populations by manual dissection. As contamination of maternal tissue cannot always be completely avoided during the microdissection for DNA extraction, minor maternal PCR amplified products can be seen in the analysis of the tumour tissue (Bifulco *et al.*, 2008; Furtado *et al.,* 2013). This was visible as small peaks correlating with the linked sample and does not usually pose a diagnostic challenge as these are quantitatively small and allele interpretation was possible (Furtado *et al.,* 2013). However, contamination with the maternal tissue is particularly problematic for loci at which the tissue is homozygous. This causes conflicting interpretation from different polymorphic loci and can result in allele ratios that appear to be consistent with triploidy, when in fact the case is androgenetic diploid (Murphy *et al.,* 2009). The diagnosis of a non-gestational tumour depends on showing that the genotype of the tumour reflects that of the patient and that the DNA analysed is from the tumour cells and not obscured by DNA from the host present in the same section. This can be a problem with microdissection from unstained sections where tumours are small and there is a high degree of infiltration by host cells, in particular host lymphocytes (Savage *et al.*, 2017). This can be eliminated by using laser capture microdissection. However, this was not available for use in this study.

The six control cases (cases 22 to 27) of CHM contained well preserved DNA, and all alleles could be detected. On average 1 to 9 alleles were common between the normal and tumour tissue. The androgenetic component was easily detected and distinguished from maternal alleles. Amelogenin was not informative for all of the CHM arose from monospermy and were XX. About 80% of CHM are monospermic arising by duplication of the parental

genome, these tumours are generally homozygous for all loci and easily recognizable as post-molar tumours (Savage *et al.,* 2017).

CONCLUSION

Microsatellites are short sequences of DNA that are arranged in repetitive units and dispersed throughout the genome. Polymorphic microsatellite markers are stably inherited, unique to an individual and are the same in all tissue from the same individual. Microsatellite genotyping is practical, cost-effective, requires a minimal amount of template DNA extracted from FFPE tissue and does not depend on the availability of the paternal genotype. The system produces short PCR amplicons of 100 to 350bp, suitable for FFPE samples and is highly specific and sensitive.

Although molecular genotyping is not required in all cases of choriocarcinoma as the majority are gestational, molecular genotyping can be useful in cases which do not respond to first line treatment and in cases in which the clinical history is suggestive of a non-gestational tumour so that the appropriate treatment can be implemented.

This is the first report of microsatellite genotyping on choriocarcinoma in South African women. Although the cohort of samples is small, we were able to identify a rare variant allelic repeat, 22.2 at locus FGA in the South African population.

REFERENCES

Bifulco C, Johnson C, Hao L, Kermalli H, Bell S, Hui P. Genotypic analysis of hydatidiform mole: an accurate and practical method of diagnosis. *Am J Surg Pathol* 2008;32:445-451.

Cancovic M, Gaba AR, Meier F, Kim W, Zarbo RJ. Detection of non-maternal components of gestational choriocarcinoma by PCR-based microsatellite DNA assay. *Gynecol Oncol* 2006;103:614-617.

Cheung ANY, Zhang HU, Xue WC, Siu MKY. Pathogenesis of choriocarcinoma: clinical, genetic and stem cell perspectives. *Future Oncol* 2009;5:217-231.

Fisher, RA, Newlands ES, Jeffreys AJ, Boxer GM, Begent RHJ, Rustin GJS, Bagshawe KD. Gestational and non-gestational trophoblastic tumours distinguished by DNA analysis. *Cancer* 1992;69:839-845.

Fisher RA, Soteriou BA, Meredith L, Paradinas FJ, Newlands ES. Previous hydatidiform mole identified as the causative pregnancy of choriocarcinoma following birth of normal twins. *Int J Gynecol Cancer* 1995;5:64-70.

Furtado LV, Paxton CN, Jama MA, Tripp SR, Lyon E, Jarboe EA, THaker HM, Geiersbach KB. Diagnostic utility of microsatellite genotyping for molar pregnancy testing. *Arch Pathol Lab Med* 2013;137:55-63.

Hoffner L, Surti U. The genetics of gestational trophoblastic disease: a rare complication of pregnancy. *Cancer Genet* 2012;205:63-77.

Lipata F, Parkash V, Talmor M, Bell S, Chen S, Maric V, Hui P. Precise DNA genotyping diagnosis of hydatidiform mole. *Obstet Gynecol* 2010;115:784-794.

Murphy KM, McConnell TG, Hafez MJ, Vang R, Ronnett BM. Molecular genotyping of hydatidiform moles. Analytic validation of a multiplex short tandem repeat assay. *JMD* 2009;11:598-605.

Ngan HYS, Seckl MJ, Berkowitz RS, Xiang Y, Golfier F, Sekharan PK, Lurain JR. Update on the diagnosis and management of gestational trophoblastic disease. *Int J Gynecol Obstet* 2015;131:S123-S126.

Savage J, Adams E, Veras E, Murphy KM, ROnnett BM. Choriocarcinoma in women. Analysis of a case series with genotyping. *Am J Surg Pathol* 2017;41:1593-1606.

Seckl MJ, Fisher RA, Salerno G, Rees H, Paradinas FJ, Foskett M, Newlands ES. Choriocarcinoma and partial hydatidiform moles. *Lancet* 2000;356:36-39.

Zhao J, Xiang Y, Wan XR, Feng FZ, Cui QC, Yang XY. Molecular genetic analysis of choriocarcinoma. *Placenta* 2009;30:816-820.

CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

GTD is a group of placental trophoblastic lesions which are always associated with pregnancy and are rarely seen in other species. These tumours generally respond well to treatment and even patients with metastatic disease can often be cured if the appropriate treatment is given timeously (Lurain, 2010; Hoffner and Surti, 2012). Rapid and accurate diagnosis is therefore essential.

GTD is uncommon and choriocarcinoma, ETT and PSTT are extremely rare (Smith, 2003). Large series are therefore limited. In addition, the use of different classification systems and terminology, the absence of central databases and the use of different denominators when determining incidence rates has made comparisons between older studies problematic (Bracken, 1987; Smith, 2003).

Apart from a number of studies from Nigeria on the demographic features of GTD, there is relatively little literature available from Africa and South Africa and no data are available from the Free State Province. Whether the local disease profile and genetic features are similar to that found in studies from Africa and the rest of the world is therefore unknown. The aim of this study was therefore to evaluate the local demographic and genetic features of patients with GTD to determine whether they conform or differ from the available local and international literature.

This study confirmed that patients with GTD seen at public sector health care facilities in the Free State Province have a similar clinical presentation to that seen in other studies from both Africa and the rest of the world. In 53.5% of cases, the referring clinician suspected a diagnosis of GTD based on clinical and sonographic features while an additional 24.1% of cases presented with vaginal bleeding. The age at which patients present is also similar to that found in other studies with a mean age of 27.7 years. This is to be expected, as these tumours are pregnancy related and therefore usually occur in the reproductive years. However, our study demonstrated a very low incidence of both hydatidiform mole and choriocarinoma and no ETT or PSTT were identified in the study period. The low incidence may partly be due to a low clinical suspicion as not all products of conception are submitted for histology in our setting due to budgetary constraints. Additional research is required and a large study is proposed in which all products of conception from public sector

health care facilities in the Free State Province will be submitted for pathological evaluation for a period of six months. This will allow us to determine whether the incidence is indeed very low or if clinicians need to be encouraged to submit more products of conception for pathological evaluation.

An HIV positive status with a CD4 count of less than 200 cells/µl has been postulated to be a poor prognostic factor in patients with GTD (Moodley *et al.*, 2009). Our study of 33 patients confirmed this hypothesis as only 33.3% of HIV positive patients with a CD4 count of less than 200 cells/µl were alive nine months after diagnosis in contrast to 88.2% of the HIV negative patients and 75% of the HIV positive patients with a CD4 count of more than 200 cell/µl ($p = 0.03$). In addition, HIV positivity was also found to have a significant influence on the presence of metastases ($p = 0.03$). The National Comprehensive Cancer Network (NCCN) clinical practice guidelines recommends that all patients with cancer should be screened for HIV. Treating the HIV infection can improve clinical survival so HIV therapy should be started or continued during treatment of GTD. It is also important to evaluate all the drugs a patient is receiving, as there may be drug-drug interactions between the HIV treatment and the chemotherapy and there may be overlapping toxicities (Reid *et al.*, 2018).

Fifteen percent of the patients treated for GTD at the Department of Oncology, National District Hospital, Bloemfontein were lost to follow up. This challenge has been noted in a number of other South African studies. Makhathini *et al.* (2019) found that 40.7% of patients travelling more than 80.5 km for treatment were lost to follow-up. The Free State Province encompasses an area of 129 825 km^2 and as patients with GTD from the entire Free State Province are treated at the local Department of Oncology many patients have to travel long distances for their appointments. Further investigation is required to confirm whether this is indeed a factor in our population.

In recent years three maternal-effect genes, *NLRP7, KHDC3L* and *PADI6* have been linked to recurrent hydatidiform mole and other forms of reproductive wastage (Moglabey *et al.*, 1999; Parry *et al.*, 2011; Xu *et al.*, 2016; Qian *et al.*, 2018). Patients with pathogenic variants of these genes almost always require assisted reproductive technologies and oocyte donation. *PADI6* was only identified as a causal gene in 2018 and has therefore not been evaluated in this study. This is the first study involving South African patients and three novel *NLRP7* variants were found, one pathogenic variant and two variants of

unknown significance. The pathogenic variant, c.1224_1232delinsT, was a homozygous variant with an insertion and deletion in exon 4. This resulted in a frameshift with a truncated peptide 116 residue downstream. Further in vitro studies are planned on the two variants of unknown significance to fully classify these variants and all seven patients will be evaluated for *PADI6* variants if additional consent is obtained. Mutation screening of the FFPE tissue of available hydatidiform moles from these patients will also be performed to identify the presence of variants and their imprinting status.

This study also provides the first report of molecular genotyping of choriocarcinomas in South African patients to classify them as gestational or non-gestational and to identify the nature of the underlying pregnancy in the gestational cases. This distinction is important as gestational choriocarcinomas secondary to a molar pregnancy have a better prognosis and different treatment regimes are used for gestational and non-gestational cases. Of 20 cases evaluated, 18 were suitable for analysis. Sixteen cases were compatible with a previous androgenetic complete hydatidiform mole, one case arose from a previous normal pregnancy and one case was a non-gestational choriocarcinoma. These findings correlate with those of previous international studies (Zhao *et* al., 2009; Savage *et al.*, 2017). Problems with cross contamination between maternal and tumour tissue were identified as manual dissection techniques were used but the data could still be interpreted. Poor amplification due to tissue degradation was also seen but this is a known result of formalin fixation.

A rare variant allelic repeat, 22.2 at locus FGA was identified in one case, which is not listed in the AmpFlSTR™ Identifiler™ PCR Amplification Kit User Guide as a known allele. This is important to note as local researchers may exclude the allele during analysis if they are unware of its presence at a very low frequency in the South African population.

Although this study is limited by the relatively small sample size, the findings will assist with future patient management and has highlighted a number of areas for further research. Both the *NLRP7* and *KHDC3L* screening and choriocarcinoma genotyping will be added to the routine diagnostic platform. Microsatellite genotyping will also be available in cases where the distinction between a partial hydatidiform mole and a normal pregnancy is not possible histologically as p57 immunohistochemistry is not helpful.

REFERENCES

Agboola A. Trophoblastic neoplasia in an African urban population. *J Natl Med Assoc* 1979;71:935-937.

Agboola A, Abudu OO. Epidemiology of trophoblast disease in Africa – Lagos*. Adv Exp Med Biol* 1984;176:187-195.

Akoury E, Zhang L, Ao A, Slim R. NLRP7 and KHDC3L, the two maternal-effect proteins responsible for recurrent hydatidiform moles, co-localize to the oocyte cytoskeleton. *Hum Reprod* 2015;30:159-169.

Allison KH, Love JE, Garcia RL. Epithelioid trophoblastic tumour: Review of a rare neoplasm of the chorionic-type intermediate trophoblast. *Arch Pathol Lab Med* 2006;130:1875-1877.

Altieri A, Franceschi S, Ferlay J, Smith J, La Vecchia C. Epidemiology and aetiology of gestational trophoblastic diseases. *Lancet Oncol* 2003;4:670-678.

Ambia J, Kabudula C, Risher K, Gomez-Olive FX, Rice BD, Etoori D, Reniers G. Outcomes of patients lost to follow-up after antiretroviral therapy initiation in rural north-eastern South Africa. *Trop Med Int Health* 2019;24:747-756.

Andreason L, Christiansen OB, Niemann I, Bolund L, Sunde L. *NLRP7* or *KHDC3L* genes and the etiology of molar pregnancies and recurrent miscarriage. *Mol Hum Reprod* 2013;19:773-781.

Arima T, Inamura T, Sakuragi N, Higashi M, Kamura T, Fujimoto S, Nakano H, Wake N. Malignant trophoblastic neoplasms with different modes of origin. *Cancer Genet Cytogenet* 1995;85:5-15.

Ashley I. Choriocarcinoma in a patient with human immunodeficiency virus: case presentation and review of the literature. *Mt Sinai J Med* 2002;69:334-337.

Audu BM, Takai IU, Chama CM, Bukar M, Kyari O. Hydatidiform mole as seen in a university teaching hospital: A 10 year review. *J Obstet Gynaecol* 2009;29:322-325.

Badenhorst DHS, van der Westhuizen CA, Britz E, Burger MC, Ferreira N. Lost to follow-up: Challenges to conducting orthopaedic research in South Africa. *SAMJ* 2018;108:917-921.

Barnardt P, Relling M. Gestational trophoblastic neoplasm and women living with HIV and/or AIDS. *S Afr J HIV Med* 2015;16(1),a344.

Barnardt P. Managing gestational trophoblastic neoplasm (GTN) and people living with HIV (PLWH). *South Afr J Gynaecol Oncol* 2019;11:21-24.

Bifulco C, Johnson C, Hao L, Kermalli H, Bell S, Hui P. Genotypic analysis of hydatidiform mole: an accurate and practical method of diagnosis. *Am J Surg Pathol* 2008;32:445-451.

Boufettal H, Coullin P, Mahdaoui, Noun M, Hermas S, Samouh N. Complete hydatidiform mole in Morocco: Epidemiological and clinical study. *J Gynecol Obstet Biol Reprod* (Paris) 2011;40:419-429.

Bracken MB. Incidence and aetiology of hydatidiform mole: an epidemiological review. *Br J Obstet Gynaecol* 1987;94:1123-1135.

Brown J, Naumann RW, Seckl MJ, Schink J. 15 years of progress in gestational trophoblastic disease: scoring, standardization and salvage. *Gynecol Oncol* 2017;144:200-207.

Buza N, Hui P. New diagnostic modalities in the histopathological diagnosis of hydatidifirm mole. *Diagn Histopathol* 2012;18:201-209.

Cankovic M, Gaba AR, Meier F, Kim W, Zarbo J. Detection of non-maternal components of gestational choriocarcinoma by PCR-based microsatellite DNA assay. *Gynecol Oncol* 2006;103:614-617.

Cheung AN. Pathology of gestational trophoblastic diseases. *Best Prac Res Clin Obstet Gynecol* 2003;17:849-868.

Cheung ANY, Zhang HU, Xue WC, Siu MKY. Pathogenesis of choriocarcinoma: Clinical, genetic and stem cell perspectives. *Future Oncol* 2009;5:217-231.

Cubasch H, Dickens C, Joffe M, Duarte R, Murugan N, Chih, MT, Moodley K, Sharma V, Ayeni O, Jaconsen JS, Neugut AI, McCormack V, Ruff P. Breast cancer survival in Soweto,

Johannesburg, South Africa: A receptor-defined cohort of women diagnosed from 2009-11 *Cancer Epidemiol* 2018;52:120-127.

Davey DA, Fray R. Choriocarcinoma and invasive mole. A review of 10 years' experience. *SA Med J* 1979;56:924-931.

Davis MR, Howitt BE, Quade BJ, Crum CP, Horowitz NS, Goldstein DP, Berkowitz RS. Epithelioid trophoblastic tumour: a single institution case series at the New England Trophoblastic Disease Centre. *Gynecol Oncol* 2015;137:456-461.

Dean J. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. *J Reprod Immunol* 2002;53:171-180.

Deveault C, Qian JH, Chebaro W, Ao A, Gilbert L, Mehio A, Khan R, Tan SL, Wischmeijer A, Couillin P, Xie X, Slim R*. NLRP7* mutations in women with diploid androgenetic and triploid moles: A proposed mechanism for mole formation. *Hum Mol Genet* 2009;18:888-897.

Egwuatu VE, Ozumba BC. Observations on molar pregnancy in Enugu, Nigeria. *Int J Gynecol Obstet* 1989;29:219-225.

Fallahi J, Anvar Z, Razban V, Momtahan M, Namavar-Jahromi B, Fardaei M. Founder effect of *KHDC3L*, p.M1V mutation, on Iranian patients with recurrent hydatidiform moles. *Iran J Med Sci* [epub ahead of print].

Fisher RA, Soteriou BA, Meredith L, Paradinas FJ, Newlands ES*.* Previous hydatidiform mole identified as the causative pregnancy of choriocarcinoma following birth of normal twins. *Int J Gynecol Cancer* 1995;5:64-70.

Fisher RA, Savage PM, MacDermott C, Hook J, Sebire NJ, Lindsay I, Seckl MJ. The impact of molecular genetic diagnosis on the management of women with hCG-producing malignancies. *Gynecol Oncol* 2007;107:413-419.

Fisher, RA, Newlands ES, Jeffreys AJ, Boxer GM, Begent RHJ, Rustin GJS, Bagshawe KD*.* Gestational and non-gestational trophoblastic tumours distinguished by DNA analysis. *Cancer* 1992;69:839-845.

Froeling FEM, Seckl MJ. Gestational trophoblastic tumours: an update for 2014. *Curr Oncol Rep* 2014;16:408.

Furtado LV, Paxton CN, Jama MA, Tripp SR, Wilson AR, Lyon E, Jarboe EA, Thaker HM, Geiersbach KB. Diagnostic utility of microsatellite genotyping for molar pregnancy testing. *Arch Pathol Lab Med* 2013;137:55-63.

Goldstein DP, Berkowitz RS. Current management of gestational trophoblastic neoplasia. *Hematol Oncol Clin N Am* 2012;26:111-131.

Hassadia A, Gillespie A, Tidy J, Everard RGNJ, Wells M, Coleman R, Hancock B. Placental site trophoblastic tumour: clinical features and management. *Gynecol Oncol* 2005;99:603-607.

Hayward BE, De Vos M, Talati N, Abdollahi MR, Taylor GR, Meyer E, Williams D, Maher ER, Setna F, Nazir K, Hussaini S, Jafri H, Rashid Y, Sheridan E, Bonthron DT. Genetic and epigenetic analysis of recurrent hydatidiform mole. *Hum Mutat* 2009;30:E629-E639.

Hirasen K, Berhanu R, Evans D, Rosen S, Sanne I, Long L. High rates of death and loss to follow-up by 12 months of rifampicin resistant TB treatment in South Africa. *PLOS One* 2018;13: e0205463.

Hoffner L, Surti U. The genetics of gestational trophoblastic disease: a rare complication of pregnancy. *Cancer Genet* 2012;205:63-77.

Horowitz NS, Goldstein DP, Berkowitz RS. Placental site trophoblastic tumours and epithelios trophoblastic tumours: biology, natural history and treatment modalities. *Gynecol Oncol* 2017;144:208-214.

Hui P, Buza N, Murphy KM, Ronnett BM. Hydatidiform moles: genetic basis and precision diagnosis. *Annu Rev Pathol Med* 2017;12:449-485.

Hui P. Gestational trophoblastic tumours. A timely review of diagnostic pathology. *Arch Pathol Lab Med* 2019;143:65-74.

Human Sciences Research Council (HSRC). The fifth South African national HIV prevalence, incidence, behavior and communication survey, 2017. Available at http://www.hsrc.ac.za/uploads/pageContent/9234/SABSSMV_Impact_Assessment_Summary_ZA_ADS_cleared_PDFA4.pdf (accessed 6 November 2019).

Kaye DK. Gestational trophoblastic disease following complete hydatidiform mole in Mulago Hospital, Kampala, Uganda. *Afri Health Sci* 2002;2:47-51.

Kaur B, Sebire NJ. Gestational trophoblastic tumours and non-neoplastic trophoblastic lesions: morphology and immunohistochemistry to refine the diagnosis. *Diagn Histopathol* 2018;25:53-65.

Kitange B, Matovelo D, Konje E, Massinde A, Rambau P. Hydatidiform moles among patients with incomplete abortion in Mwanza City, North western Tanzania. *Afri Health Sci* 2015;15:1081-1086.

Kolawole AO, Nwajagu JK, Oguntayo AO, Zayyan MS, Adewuyi S. Gestational trophoblastic disease in Abuth Zaria, Nigeria: a 5 year review. *Trop J Obstet Gynaecol* 2016;33:209-215.

Kou YC, Peng HH, Rosetta R, del Gaudio D, Wagner AF, Al-Hussaini TK, Van den Veyver IB. A recurrent intragenic genomic duplication, other novel mutations in *NLRP7* and imprinting defects in recurrent biparental hydatidiform moles. *Mol Hum Reprod* 2008;14:33-40.

Kurman RJ, Scully RE, Norris HJ. Trophoblastic pseudotumour of the uterus. An exaggerated form of 'syncytial endometritis' simulating a malignant tumour. *Cancer* 1976;38:1214-1226.

Kurman RJ, Carcangiu ML, Herrington CS, Young RH, eds. WHO classification of tumours of female reproductive organs. Lyon:IARC;2014:155-167.

Kyari O, Nggada H, Mairiga A. Malignant tumours of female genital tract in North Eastern Nigeria. *East Afr Med J* 2004:81:142-145.

Landolsi H, Rittore C, Philibert L, Missaoui N, Hmissa S, Touiyou I, Gribaa M, Yacoubi MT. Screening *NLRP7* mutations in familial and sporadic recurrent hydatidiform moles: Report of 2 Tunisian families. *Int J Gynecol Pathol* 2011;38:348-353.

Landolsi H, Rittore C, Philibert L, Hmissa S, Gribaa M, Touitou I, Yacoubi MT. *NLRP7* mutation analysis in sporadic hydatidiform moles in Tunisian patients. *NLRP7* and sporadic mole. *Arch Pathol Lab Med* 2012;136:646-651.

Leighton PC. Trophoblastic disease in Uganda. *Am J Obstet Gynecol* 1973;117:341-344.

Li HW, Tsao SW, Cheung ANY. Current understandings of the molecular genetics of gestational trophoblastic diseases. *Placenta* 2002;23:20-31.

Limper A, Rosenow E. Drug-induced interstitial lung disease. *Curr Opin Pulm Med* 1996;2:396-404.

Lipata F, Parkash V, Talmor M, Bell S, Chen S, Maric V, Hui P. Precise DNA genotyping diagnosis of hydatidiform mole. *Obstet Gynecol* 2010;115:784-794.

Lurain JR. Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole. *Am J Obstet Gynecol* 2010;203:31-39.

Lurain JR. Gestational trophoblastic disease II: classification and management of gestational trophoblastic neoplasia. *Am J Obstet Gynecol* 2011;204:11-18.

Makhathini S, Dreyer G, Buchmann EJ. Gestational trophoblastic disease managed at Grey's Tertiary Hospital: a five-year descriptive study. *South Afr J Gynaecol Oncol* 2019:1-5 https://doi.org/10.1080/20742835.2019.1667627.

Manokhina I, Hanna CW, Stephenson MD, McFadden DE, Robison WP. Maternal *NPRP7* and *C6orf221* variants are not a common risk factor for androgenetic moles, triploidy and recurrent miscarriage. *Mol Hum Reprod* 2013;19:539-544.

Mayun AA. Hydatidiform mole in Gombe: a five year histopathological review. *Niger J Clin Prac*. 2008;11:134-138.

Mayun AA, Rafindadi AH, Shehu MS. Choriocarcinoma in Northwestern Nigeria: A histopathological review. *Niger Postgrad Med J* 2012;19:215-218.

Mbamara SU, Obiechina NJA, Eleje GU, Akabuike CJ, Umeononihu OS. Gestational trophoblastic disease in a tertiary hospital in Nnewi, Southeast Nigeria. *Niger Med J* 2009;50:87-89.

McConnell TG, Murphy KM, Hafez M, Vang R, Ronnett BM. Diagnosis and subclassification of hydatidiform moles using p57 immunohistochemistry and molecular genotyping: Validation and prospective analysis in routine and consultation practice settings with development of an algorithmic approach. *Am J Surg Pathol* 2009;33:805-817.

Miller S, Dykes D, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.

Moein-Vaziri N, Fallahi J, Namavar-Jahromi B, Fardaei M, Momtahan M, Anvar Z. Clinical and genetic-epigenetic aspects of recurrent hydatidiform mole: A review of literature. *Taiwan J Obstet Gynecol* 2018;57:1-6.

Moglabey YB, Kircheisen R, Seoud M, El Mogharbel N, Van den Veyver I, Slim R. Genetic mapping of a maternal locus responsible for familial hydatidiform moles. *Hum Mol Genet* 1999;8:667-671.

Moodley M, Moodley J. Choriocarcinoma and human immunodeficiency virus (HIV) infection: a case report. *Int J Gynecol Cancer* 2001;11:329-330.

Moodley M, Tunkyi K, Moodley J. Gestational trophoblastic syndrome: An audit of 112 patients. A South African experience. *Int J Gynecol Cancer* 2003a;13:234-239.

Moodley M, Moodley J. Successful use of anti-retroviral therapy in combination with cytotoxic chemotherapy for persistent molar pregnancy: A case report. *Int J Gynecol Cancer* 2003b;13:246-248.

Moodley M, Moodley J. Gestational trophoblastic syndrome and human immunodeficiency virus (HIV) infection: A retrospective analysis. *Int J Gynecol Cancer* 2003;13:875-878.

Moodley M, Marishane T. Demographic variables of gestational trophoblastic disease in Kwa-Zulu-Natal, South Africa. *J Obstet Gynecol* 2005;25:482-485.

Moodley M. Placental site trophoblastic tumour with an antecedent molar pregnancy in association with human immunodeficiency virus infection. *Int J Gynecol Cancer* 2007;18:860-861.

Moodley M, Budram S, Connolly C. Profile of mortality among women with gestational trophoblastic disease infected with the human immunodeficiency virus (HIV). Argument for a new poor prognostic marker. *Int J Gynecol Cancer* 2009;19:289-293.

Murdoch S, Djuric U, Mazhur B, Seoud M, Khan R, Kuick R, Bagga R, Kircheisen R, Ao A, Ratti B, Hanash S, Rouleau GA, Slim R. Mutations in *NALP7* cause recurrent hydatidiform moles and reproductive wastage in humans. *Nat Genet* 2006;38:300-302.

Murphy KM, McConnell TG, Hafez MJ, Vang R, Ronnett BM. Molecular genotyping of hydatidiform moles. Analytic validation of a multiplex short tandem repeat assay. *JMD* 2009;11:598-605.

Ngan HYS, Seckl MJ, Berkowitz RS, Xiang Y, Golfier F, Sekharan PK, Lurain JR. Update on the diagnosis and management of gestational trophoblastic disease. *Int J Gynecol Obstet* 2015;131:S123-S126.

Nguyen NMP, Slim R. Genetics and epigenetics of recurrent hydatidiform moles: Basic science and genetic counselling. *Curr Obstet Gynecol Rep* 2014;3:55-64.

Nguyen NMP, Khawajkie Y, Mechtouf N, Rezaei M, Breguet M, Kurvinen E, Jagadeesh S, Solmaz AE, Aguinaga M, Hemida R, Harma MI, Rittore C, Rahimi K, Arseneau J, Hovanez K, Clisham R, Lenzi T, Scurry B, Addor MC, Bagga R, Nendaz GG, Finci V, Poke G, Grimes L, Gregersen N, York K, Bolze PA, Patel C, Mozdarani H, Puechberty J, Scotchie J, Fardaei M, Harma M, Gardner RJM, Sahoo T, Dudding-Byth T, Srinivasan R, Sauthier P, Slim R. The genetics of recurrent hydatidiform moles: new insights and lessons from a comprehensive analysis of 113 patients. *Mod Pathol* 2018;31:1116-1130.

Ober WB. Historical perspectives on trophoblast and its tumours. *Ann N Y Acad Sci* 1959;80:3-20.

Ojwang SBO, Otieno MRB, Khan KS. Human immunodeficiency virus in gestational trophoblastic neoplasias – Is it a poor prognostic risk factor? *East Afr Med J* 1992;69:647-648.

Osamor JO, Oluwasola AO, Adewole IF. A clinicopathological study of complete and partial hydatidiform moles in a Nigerian population. *J Obstet Gynaecol* 2002;22:423-425.

Palmer JR. Advances in the epidemiology of gestational trophoblastic disease. *J Reprod Med*. 1994;39:155-162.

Parry DA, Logan CV, Hayward BE, Shires M, Landolsi H, Diggle C, Carr I, Rittore C, Touitou I, Philibert L, Fisher RA, Fallahian M, Huntriss JD, Picton HM, Malik S, Taylor GR, Johnson CA, Bonthron DT, Sheridan EG. Mutations causing familial biparental hydatidiform mole implicate *C6orf221* as a possible regulator of genomic imprinting in the human oocyte. *Am J Hum Genet* 2011;89:451-458.

Peuchberty J, Rittore C, Philibert L, Lefort G, Burlet G, Benos P, Reyftmann L, Sarda P, Touitou I. Homozygous *NLRP7* mutations in a Moroccan woman with recurrent reproductive failure. *Clin Genet* 2009;75:298-300.

Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV and Group IUGVW. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat* 2008;29:1282-1291.

Qian J, Cheng Q, Murdoch S, Xu C, Jin F, Chebaro W, Zhang X, Gao H, Zhu Y, Slim R, Xie X. The genetics of recurrent hydatidiform moles in China: correlations between *NLRP7* mutations, molar genotypes and reproductive outcomes. *Mol Hum Reprod* 2011;17:612-619.

Qian J, Nguyen NMP, Rezaei M, Huang B, Tao Y, Zhang X, Cheng Q, Yang H, Asangla A, Majewski J, Slim R. Biallellic *PADI6* variants linking infertility, miscarriages, and hydatidiform moles. *Eur J Hum Genet* 2018;26:1007-1013.

Radian AD, de Almeida L, Dorfleutner A, Stehlik C. NLRP7 and related inflammation activating pattern recognition receptors and their function in host defense and disease. *Microbes Infect* 2013;15:630-639.

Reddy R, Akoury E, Nguyen NMP, Abdul-Rahman OA, Dery C, Gupta N, Daley WP, Ao A, Landolsi H, Fisher RA, Touitou I, Slim R. Report of four new patients with protein-truncating mutations in *C6orf221/KHDC3L* and co-localization with *NLRP7*. *Eur J Hum Genet* 2013;21:957-964.

Reddy R, Nguyen NMP, Sarrabay G, Rezael M, Rivas MC, Kavasoglu A, Berkil H, Elshafey A, Abdalia E, Nunez KP, Dreyfus H, Philippe M, Hadipour Z, Durmaz A, Eaton EE, Schubert B, Ulker V, Hadipour F, Touitou I, Fardaei M Slim R. The genomic architecture of *NLRP7* is Alu rich and predisposes to disease-associated large deletions. *Eur J Hum Genet* 2016;24:1445-1452.

Reid E, Suneja G, Ambinder RF, ARd K, Baiocchi R, Barta SK, Carchman E, Cohen A, Gupta N, Johung KL, Klopp A, LaCasce AS, Lin C, Makarova-Rusher OV, Mehta A, Menon MP, Morgan D, Nathwani N, Noy A, Palella F, Ratner L, Rizza S, Rudek MA, Taylor J, Tomlinson B, Wang CJ, Dwyer MA, Freedman-Cass DA. Cancer in people living with HIV, Version

1.2018, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* 2018;16:986-1017.

Rezaei M, Nguyen NMP, Foroughinia L, Dash P, Ahmadpour F, Verma IC, Slim R, Fardaei M. Two novel mutations in the *KHDC3L* gene in Asian patients with recurrent hydatidiform mole. *Hum Genome Var* 2016;3:16027. doi:10.1038/hgv.2016.27.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;17:405-424.

Sanchez-Delgado M, Martin-Trujillo A, Tayama C, Vidal E, Esteller M, Iglesias-Platas I, Deo N, Barney O, Maclean K, Hata K, Nakabayashi K, Fisher R, Monk D. Absence of maternal methylation in biparentla hydatidiform moles from women with *NLRP7* maternal-effect mutations reveals widespread placenta-specific imprinting. PLoS Genet 2015;11:e1005644. Doi:10.1371/journal.pgen.1005644.

Santoro G, Lagana AS, Micali A, Barresi V, Giacobbe V, Palmara V. Historical, morphological and clinical overview of placental site trophoblastic tumours: from bench to bedside. *Arch Gynecol Obstet* 2017;295:173-187.

Savage J, Adams E, Veras E, Murphy KM, Ronnett BM. Choriocarcinoma in women. Analysis of a case series with genotyping. *Am J Surg Pathol* 2017;41:1593-1606.

Schwaiblmair M, Behr W, Haeckel T, Markl B, Foerg W, Berghaus T. Drug induced interstitial lung disease. *Open Respir Med J* 2012;6:63-74.

Sebire NJ, Foskett M, Fisher RA, Rees H, Seckl M, Newlands E. Risk of partial and complete hydatidiform molar pregnancy in relation to maternal age. *Br J Obstet Gynaecol* 2002;109:99-102.

Sebire NJ, Seckl MJ. Gestational trophoblastic disease: current management of hydatidiform mole. *BMJ* 2008;337:1193.

Sebire NJ, Lindsay I. Current issues in the histopathology of gestational trophoblastic tumours. *Fetal Pediatr Pathol* 2010;29:30-44.

Seckl MJ, Fisher RA, Salerno G, Rees H, Paradinas FJ, Foskett M, Newlands ES. Choriocarcinoma and partial hydatidiform moles. *Lancet* 2000;356:36-39.

Seckl MJ, Sebire NJ, Berkowitz RS. Gestational trophoblastic disease. *Lancet* 2010;376:717-729.

Seckl MJ, Sebire NJ, Fisher RA, Golfier F, Massuger L, Sessa C. Gestational trophoblastic disease: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013;24:vi39-vi50.

Seckl MJ. Gestational trophoblastic disease: clinical presentation and treatment. *Diagn Histopathol* 2018;25:77-85.

Shih IM, Kurman RJ. Epithelioid trophoblastic tumour: A neoplasm distinct from choriocarcinoma and placental site trophoblastic tumour simulating carcinoma. *Am J Surg Pathol* 1998;22:1393-1403.

Shih IM, Seidman JD, Kurman RJ. Placental site nodule and characterisation of distinctive types of intermediate trophoblast. *Hum Pathol* 1999;30:687-694.

Shih IM, Kurman RJ. The pathology of intermediate trophoblastic tumours and tumour-like lesions. *Int J Gynecol Pathol* 2001;20:31-47.

Shih IM, Kurman RJ. Molecular basis of gestational trophoblastic disease. *Curr Mol Med* 2002;2:1-12.

Shih IM, Kurman RJ. p63 expression is useful in the distinction of epithelioid trophoblastic and placental site trophoblastic tumours by profiling trophoblastic subpopulations. *Am J Surg Pathol* 2004;28:1177-1183.

Slim R, Coullin P, Diatta AL, Chebaro W, Courtin D, Abdelhak S, Garcia A. *NLRP7* and the genetics of post-molar choriocarcinomas in Senegal. *Mol Hum Reprod* 2012;18:52-56.

Smith HO. Gestational trophoblastic disease epidemiology and trends. *Clin Obstet Gynaecol* 2003;4:541-556.

Snyman LC. Gestational trophoblastic disease: An overview. *SA J Gynaecol Oncol* 2009;1:32-37.

Soellner L, Begemann M, Degenhardt, Geipel A, Eggermann T, Mangold E. Maternal heterozygous *NLRP7* variant results in recurrent reproductive failure and imprinting disturbances in the offspring. *Eur J Hum Genet* 2017;8:924-929.

Statistics South Africa (SSA) 2019. Midyear population estimates 2019. Available at https://www.statssa.gov.za/publications/P0302/P03022019/pdf (accessed 6 November 2019).

Statistics South Africa (SSA) 2011. Census 2011. Available at https://www.statssa.gov.za/publications/P03014/P030142011.pdf (accessed 3 January 2020).

Steigrad SJ. Epidemiology of gestational trophoblastic diseases. *Best Prac Res Clin Obstet Gynaecol* 2003;17:837-847.

Tangtrakul S, Linasmita V, Wilailak S, Srisupandit S, Bullangpoti S, Ayudhya NIN. An HIV-infected woman with choriocarcinoma presenting with a nasal mass. *Gynecol Oncol* 1998;68:304-306.

Tayib S, van Wijk L, Denny L. Gestational trophoblastic neoplasia and human immunodeficiency virus infection: A 10 year review. *Int J Gynecol Cancer* 2011;21:1684-1691.

UNIAIDS Fact Sheet. World AIDS day 2019. Global HIV Statistics. Available at https://www.unaids.org/en/resources/fact-sheet (accessed 31 December 2019)

Vang R, Gupta M, Wu L, Yemelyanova AV, Kurman RJ, Murphy KM, Descipio C, Ronnett BM. Diagnostic reproducibility of hydatidiform moles: Ancillary techniques (p57 immunohistochemistry and molecular genotyping) improve morphologic diagnosis. *Am J Surg Pathol* 2012;36:443-453.

Von Bogaert LJ. Clinicopathological features of gestational trophoblastic neoplasia in the Limpopo Province, South Africa. *Int J Gynecol Cancer* 2013;23:583-585.

Wang CM, Dixon PH, Decordova S, Hodges MD, Sebire NJ, Ozalp S, Fallahian M, Sensi A, Ashrafi F, Repiska V, Zhao J, Xiang Y, Savage PM, Seckl MJ, Fisher RA. Identification of 13 novel *NLRP7* mutations in 20 families with recurrent hydatidiform mole; missense mutations cluster in the leucine rich region. *J Med Genet* 2009;46:569-575.

Yakasai IA, Ugwa EA, Otubu J. Gynecological malignancies in Aminu Kano Teaching Hospital Kano: A 3 year review. *Niger J Clin Prac* 2013;16:63-66.

Xu Y, Shi Y, Fu J, Yu M, Feng R, Sang Q, Liang B, Chen B, Qu R, Li B, Yan Z, Mao X, Kuang Y, Jin L, He L, Sun X, Wang L. Mutations in *PADI6* cause female infertility characterised by early embryonic arrest. *Am J Hum Genet* 2016;99:744-752.

Zhang W, Chen Z, Zhang D, Zhao B, Liu L, Xie Z, Yao Y, Zheng P. KHDC3L mutation causes recurrent pregnancy loss by inducing genomic instability of human early embryonic cells. *PLOS Biology* 2019; 17(10):e3000468.

Zhao J, Xiang Y, Wan XR, Feng FZ, Cui QC, Yang XY. Molecular genetic analyses of choriocarcinoma. *Placenta* 2009;30:816-820.

Zhao J, Lv WG, Feng FZ, Wan XR, Liu JH, Yi XF, Qu PP, Xue FX, Wu YM, Zhao X, Ren T, Yang JJ, Xie X, Xiang Y. Placental site trophoblastic tumour: A review of 108 cases and their implications for prognosis and treatment. *Gynecol Oncol* 2016;142:102-108.

LIST OF APPENDICES

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

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

03 October 2017

PROF J GOEDHALS
DEPT OF ANATOMICAL PATHOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Prof J Goedhals

HSREC 81/2017 (UFS-HSD2017/0787)
PRINCIPAL INVESTIGATOR: PROF J GOEDHALS
SUPERVISOR: M THERON
PROJECT TITLE: DEMOGRAPHIC AND GENETIC FEATURES OF GESTATIONAL TROPHOBLASTIC DISEASE IN THE PUBLIC SECTOR OF THE FREE STATE PROVINCE, SOUTH AFRICA

APPROVED

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved this protocol after all conditions were met. This decision will be ratified at the next meeting to be held on 31 October 2017.

2. The Committee must be informed of any serious adverse event and/or termination of the study.

3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.

4. A progress report should be submitted within one year of approval and annually for long term studies.

5. A final report should be submitted at the completion of the study.

6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.

7. 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 461 (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

MS MGE MARAIS
HEAD: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE ADMINISTRATION

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
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CONCENT TO PARTICIPATE IN A RESEARCH STUDY

NLRP7 and KHDC3L Mutations in South-African patients with hydatidiform mole

You have been asked to participate in the above-mentioned research study.

You have been informed about the study by .....

You may contact Dr W Vergottini (telephone number 0828866199) at any time if you have questions about the research.

You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have any questions about your rights as a research subject.

Your participation in this research project is voluntary, and you will not be penalized or loose benefits if you refuse to participate or terminate participation.

If you agree to participate, you will be given a signed copy of this document as well as the participation information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me and/or I have read the information document.

I, _____
understand what my involvement in the study means and I voluntarily agree to participate.

_____ _____
Signature of Participant Date

_____ _____
Signature of Witness Date

_____ _____
Signature of Study Doctor Date

_____ _____
Signature of Translator (*where applicable*) Date

Department of Anatomical Pathology UFS

Information Sheet

Study Title: NLRP7 and KHDC3L Mutations in South-African patients with hydatidiform mole

Dear Patient,

We, doctors from the Pathology and Human Genetics departments, are doing research on patients with a history of a molar pregnancy. Research is the process to learn the answer to a question. In this study, we want to learn if patients with a history of a molar pregnancy have a mutation in their NLRP7 or KHDC3L genes. Research in other countries has demonstrated a link between mutations of this gene and molar pregnancies and we want to learn if this is also true in patients from South Africa.

Why have you been asked to take part?

We are asking you to participate in this study because you have a history of a specific type of molar pregnancy and/or recurrent miscarriages.

What will the study involve?

Once you have read and understood this document, you will be asked to sign an informed consent document stating that you understand what your part in the study will be and what procedures will be done for the study. A doctor or nurse will take a blood sample from you. This sample will be analyzed at the genetics and anatomical pathology laboratories and tested for a mutation in the NLRP7 and KHDC3L genes. Sample slides of the histology of your previous pregnancy / pregnancies will also be reviewed by pathologists.

What are the risks?

The only risks that are involved in the study are those that could arise from obtaining a blood sample. These include pain, bleeding or infection. Obtaining blood samples form patients is a common, routine procedure. The risk of developing these complications is very small.

What are the benefits?

By participating in the study, you will be helping the doctors to understand the importance of mutations in the NLRP7 and KHDC3L genes in patients with molar pregnancies and recurrent miscarriages.

Do you have to take part?

No. Your participation in this study is voluntary and refusal to participate will involve no penalty or loss of benefit to which you are otherwise entitled. You may discontinue participation at any time during the study. Your treatment and follow-up will not be changed in any way.

Will your participation in the study be kept confidential?

Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis including groups such as the Ethics Committee for Medical research. No patient names will be used in the study data or results.

Results
You may request your test results and if they are positive genetic counselling will be made available to you.

Reimbursements:

Participation will not involve any extra costs to you as a patient. All procedures will be performed at no extra cost to you and will be paid for by the Pathology department. You will not receive any reimbursement for participation in the study.

Any further queries?

If you need any further information, you can contact me:

Dr. W Vergottini
Cell: 0828866199
Email: vergottini@live.com

If you agree to take part in the study, please sign the consent form overleaf.

TOESTEMMING OM IN 'N NAVORSINGSTUDIE DEEL TE NEEM

NLRP7 en KHDC3L Mutasies in Suid-Afrikaansepasiënte met hidatidiforme mola swangerskap(pe)

Jy is gevra om deel te neem in die bogenoemde navorsingstudie.

Jy is ingelig oor die studie deur .....

Jy kan Dr W Vergottini (telefoonnommer 0828866199) enige tyd kontak as jy vrae het oor die navorsing.

Jy kan die sekretariaat van die Etiekkomitee van die Fakulteit Gesondheidswetenskappe, UV by telefoonnommer (051) 4052812 kontak indien jy enige vrae oor jou regte as 'n studiedeelnemer het.

Jou deelname aan hierdie navorsingsprojek is vrywillig, en jy sal nie gestraf word of enige voordele verloor as jy weier om deel te neem of jou deelname beëindig nie.

As jy saamstem om deel te neem sal jy'n getekende afskrif van hierdie dokument, asook die deelname inligtingsblad wat 'n skriftelike opsomming van die navorsing is, ontvang.

Die navorsingstudie, insluitende die bogenoemde inligting is mondelings aan my verduidelik en / of ek het die inligtingsdokument gelees .

Ek, _____
verstaan wat my deelname in die studie behels en ek stem vrywillig in om deel te neem .

_____	_____
Ondertekening van 'n deelnemer	Datum
_____	_____
Handtekening van getuie	Datum
_____	_____
Handtekening van studiedokter	Datum
_____	_____
Handtekening van Tolk (waar van toepassing)	Datum

Departement Anatomiese Patologie UV

inligtingsblad

Studietitel: NLRP7 en KHDC3L Mutasies in Suid - Afrikaansepasiënte met hidatidiforme mola swangerskap(pe)

Geagte Pasiënt

Ons, dokters van die Patologie en Menslike Genetika departemente, doen navorsing op pasiënte met 'n geskiedenis van 'n mola swangerskap. Navorsing is die proses om die antwoord op 'n vraag te leer. In hierdie studie, wil ons leer of pasiënte met 'n geskiedenis van 'n molêre swangerskap 'n mutasie in hul NLRP7 of KHDC3L gene het .Navorsing in ander lande het getoon dat daar 'n skakel is tussen mutasies van hierdie gene en molêre swangerskappe, en ons wil leer of dit ook waar is in pasiënte van Suid-Afrika.

Hoekom vra ons jou om deel te neem?

Ons vra jou om deel te neem in hierdie studie want jy het 'n geskiedenis van 'n spesifieke tipe molêre swangerskap en / of herhaalde miskrame.

Wat sal die studiebehels?

Sodra jy die document gelees het en dit verstaan, sal jy gevra word om 'n ingeligte toestemming dokument te teken wat verklaar dat jy verstaan wat jou deelname in die studie sal behels en watter procedures gedoen sal word vir die studie. 'n Dokter of verpleegster sal 'n bloedmonster van jou neem. Jou bloedmonster sal by die Menslike Genetika en Anatomiese Patologie laboratoriums ontleed en getoets word vir 'n mutasie in die NLRP7 en KHDC3L gene. Histologiese skyfies van jou vorige swangerskap(pe) sal ook hersien word deur patoloë.

Wat is die risiko's betrokke?

Die enigste risiko's wat betrokke is in die studie, is die wat kan ontstaan as gevolg van die verkryging van 'n bloedmonster .Dit sluit in pyn, bloeding of infeksie. Verkryging van 'n bloedmonster is 'n algemene, roetine prosedure. Die kans om 'n komplikasie te ontwikkel is baie klein.

Wat is die voordele betrokke?

Deur deel te neem aan die studie sal jy dokters help om die belangrikheid van mutasies in die NLRP7 en KHDC3L gene, in pasiënte met molêreswangerskappe en herhaaldemiskrame, te verstaan.

Hoef jy deelteneem?

Nee. Jou deelname aan hierdie studie is vrywillig en die weiering om deel te neem sal geen boete of verlies van voordele,waarop jy andersins geregtig is, beinvloednie. Jy kan jou deelname op enigetyd tydens die studie staak. Jou behandeling en opvolg sal nie op enige manier verander word nie.

Sal jou deelname aan die studie vertroulikhanteer word?

Pogings sal aangewend word om persoonlike inligting vertroulik te hou. Absolute vertroulikheid kan nie gewaarborg word nie. Persoonlike inligting mag beskikbaar gemaak word as die wet dit vereis. Organisasies mag jou navorsing records vir gehalte-versekering en data-analise nagaan. Dit sluit in groepe soos die Etiekkomitee vir mediese navorsing. Geen pasiënt name sal gebruik word in die studie data of resultate nie.

Uitslae:
U kan u toets uitslae aanvra en indien die toetse postitief is sal genetiese berading vir U beskikbaar wees.

Vergoeding:

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Dr W Vergottini
Cell : 0828866199
E-pos: vergottini@live.com

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Diphethoho tsa NLRP7 le KHDC3L hara bakudi ba Afrika Borwa ba nang le hlahala e melang ka hara thari nakong ya kemaro.

O kopuwa ho ba le seabo diphuputsong tse hlalositsweng kahodimo.

O fumane hlakisetso mabapi le diphuputso ka.....

O ka iteyanya le Ngaka W Vergottini dinomorong tsena tsa mohala 0828866199 ka dinako tsohle haeba o na le dipotso tse mabapi le diphuputso.

O ka iteyanya le mongodi wa Komiti ya Lefapha la Mahlale a tsa Bophelo, nomorong ya mohala Unibesithing ya Foreisetata eleng (051) 4052812 haeba o na le dipotso mabapi le ditokelo tsa hao jwaleka ka monka-karolo diphuputsong.

Seabo sa hao diphuputsong ke sa boithaopo mme o ke ke wa fumantshwa kotlo kapa ho hanelwa menyetla haeba o hana ho nka karolo kapa ho kgaotsa ka seabo sa hao.

Haeba o dumela ho nka karolo o tla fuwa tokomane e saennweng mmoho le leqephe le nang le tshedimosetso, eleng kakaretso ya diphuputso.

Ke hlaloseditswe dintlha tse amanang le diphuputso, tse akaretsang tshedimosetso ka puo ya molomo kapa ke badile tokomane ya tshedimosetso.

E le nna, _____
Ke utlwisisa seabo sa ka diphuputsong tsena le hore ke sa boithaopo.

_____ _____
Tshaeno ya monka-karolo Letsatsi

_____ _____
Tshaeno ya paki Letsatsi

_____ _____
Tshaeno ya ngaka e tsamaisang diphuputso Letsatsi

_____ _____
Tshaeno ya mofetoledi (*ha ho hlokahala*) Letsatsi

Leqephe la Tshedimosetso

Sehloho sa Diphuputso: # Diphethoho tsa NLRP7 le KHDC3L hara bakudi ba hlahala e melang ka hara thari/popelo nakong ya kemaro

Mokudi ya Kgabane,

Re le dingaka tse hlahang Lefapheng la Dihlahlobo tsa Madi le Motheo wa Popo ya Motho re tshwarahane le diphuputso ka thuso ya bakudi ba bileng le bothata ba hlahala ya thari e melang nakong ya bokgatjhane/kemaro ka makgetlo a mmalwa nakong e fetileng. Sepheo sa diphuputso ke ho fumana dikarabo bakeng sa dipotso tse itseng. Diphuputsong tsena re batla ho sibolla diphethoho tse bang teng jineng ya NLRP7 le KHDC3L hara bakudi ba bileng le hlahala ya thari e melang nakong ya bokgatjhane. Diphuputsong tse ileng tsa etswa dinaheng tse ding di supa hore kamahano e teng dipakeng tsa phethoho ya jine mmoho le hlahala e melang popelong nakong ya kemaro. Ka hoo re batla ho sibolla haeba sena ke nnete hara bakudi ba Afrika Borwa.

Ke ka lebaka la eng ha o kopuwe ho ba le seabo?

Re kopa seabo sa hao diphuputsong tsena ka lebaka la hobane o bile le hlahala ya thari nakong ya bokgatjhane mmoho le ho lahlehelwa ke masea a eso belehwe ka makgetlo nakong e fetileng.

Na ebe ke eng se tla akaretswa ke diphuputso?

Ha o se o badile tokomane ena ka kutlwisiso o tla kopuwa ho saena lengolo la kutlwisisano ho supa hore o utlwisisa seabo sa hao diphuputsong mmoho le tsamaiso e tla latelwa bakeng sa diphuputso. Ngaka kapa mooki o tla hula madi bakeng sa diteko. Tekolo ya madi e tla etswa laboratoring ya dihlahlobo tsa madi bakeng sa dipatlisiso ka diphethoho tse etsahalang jineng ya NLRP7 le KHDC3L. Ditshwantsho tsa nako e fetileng nakong ya kemaro ya hao le tsona di tla hlahlojwa ke ditsebi tsa madi.

Ke kotsi efe e ka etsahalang?

Kotsi e le nngwe fela e ka etsahalang eka bakwa ke ho hulwa ha madi. Yona e akaretsa bohloko, phallo ya madi kapa tshwaetso. Ho nkuwa ha madi ho bakudi ke tsamaiso e bobebe e tlwaelehileng ya kamehla. Ha se ha ngata e ka bakang kotsi.

O ka una molemo jwang?

Ka ho ba le seabo diphuputsong o tla thusa dingaka ho utlwisisa bohlokwa ba diphethoho tse etsahalang jineng ya NLRP7 le KHDC3L tse bakang ho mela ha hlahala ya thari nakong ya bokgatjhane/kemaro eleng boemo bo qetellang bo bakile tahlehelo ya lesea le so belehwe.

Na o qobellwa ho ba le seabo?

Tjhee. Seabo sa hao diphuputsong ke sa boithaopo ebile ha ho kotlo kapa tahlehelo ya menyetla yeo tshwanetseng ka lebaka la ho hana ho ikamahanya le diphuputso. O ka kgaotsa seabo sa hao neng kapa neng ha o ikutlwa ho etsa jwalo ha diphuputso di ntse di tswela pele. Ha ho diphethoho tsa mofuta ofe kapa ofe tse tla latela phekolo ya hao ya kamora diphuputso.

Ekaba seabo sa hao diphuputsong se tla bolokwa e le sephiri?

Matsapa a tla etswa kahohle ho baballa tshedimosetso ya monka-karolo e le sephiri. Leha ho le jwalo ha ho tiiseletso bakeng sa ho boloka phethahalo ya sephiri sa bapallo ya tshedimosetso. Ditaba tse amanang le mokudi di ka hlahiswa ha ho hlokeha ka molao. Mekgatlo e akaretsang Komiti ya Boitshwaro bakeng sa Diphuputso tsa Bongaka e ka lekola pokello ya sephetho sa diphuputso. Mabitso a bakudi a ke ke a phatlalatswa pokellong le sephethong sa diphuputso.

Dipetho:

O na le tokelo ya ho kopa sephetho sa hlahlobo ya madi a hao. E baneng diphetho tsa hao di bontsha o na le di mutation tsa di jini tse boletsweng, o ka fumana thlakiso e fetelletseng maisana le dijini tsa hao.

Moputso:

Ha ho ditjeo tse ekeditsweng tse tla lefuwa ke mokodi ka lebaka la ho ba le seabo. Dihlahlobo kaofela di tla etswa ntle le ditjeo tse ekeditsweng ho mokodi empa di tla lefuwa ke Lefapha la Hlahlobo ya Madi. Ha ho tefo e lebeletsweng bakeng sa ho ba le seabo diphuputsong.

Bakeng sa dintlha ka botlalo?

Haeba o hloka tshedimosetso e phethahetseng iteyanye le nna:

Dr. W Vergottini
Cell: 082 886 6199
E-mail: vergottini@live.com

Haeba o dumela ho ba le seabo diphuputsong ka kopo saena foromo ya tumellano/kutlwisisano e leqepheng le kahodimo.

Forward and reverse primer sets for *NLRP7*

Fragments	Thermo Fisher Cat No./Lot No.	Sequence	Amplification size	Annealing temperature
Exon 1	A15629 – HS00357897	FWD – AAA TCA AAG ATC CTT CCA GCA TCC T	512 bp	61°C
	A15630 – HS00357897	REV – GTT TTT AAA GCT GGG AGC CAG GTA		
Exon 2	A15629 – HS00357896	FWD – AGG ACA CCC CAG GTT CTA CTT AC	490 bp	61°C
	A15630 – HS00357896	REV - GCT GGG CCA GAT TTT CAG TCT CT		
Exon 3	A15629 – HS00546856	FWD – CCA GAA ATG AAT AAA ACC AGG AAG AAG TG	247 bp	61°C
	A15630 – HS00546856	REV – CTG GCT GAC ACT TTA TGT ACA ATA ATG TCT		
Exon 4A	A15629 – HS00812246	FWD – GTT GCA GTC TGT CCA GTC CA	272 bp	58°C
	A15630 – HS00812246	REV – CAA CTG TCC TAT GGG CTG TCA		
Exon 4B	A15629 – HS00357890	FWD – CAG GAA GCC CTC CAC CCT TA	496 bp	58°C
	A15630 – HS00357890	REV – CTT GAA TCC CAG AAC ACC CAG GAA		
Exon 4C	A15629 – HS00357889	FWD – GAA CAG ACG GAG GTC GGA CT	495 bp	58°C
	A15630 – HS00357889	REV – GGG AGT TTG CTG AAG AGG AAG		
Exon 4D	A15629 – HS00357888	FWD – GAA GAT GTG CTT TGC ATT GCA GCA ATT	498 bp	58°C
	A15630 – HS00357888	REV – GGC TGT TCC TGC GTT TCC TCT G		
Exon 4E	A15629 – HS00357887	FWD – CCA GCC AGA GGG AAA TTC TGA C	491 bp	58°C
	A15630 – HS00357887	REV- GAA CCC CGA CCT GAT TCA AG		
Exon 5	A15629 – HS00591835	FWD – TCA GGG TCT TCC TTG CAA GAT G	272 bp	61°C
	A15630 – HS00591835	REV – GGT ACT AGT CCT AAG AGA TGA ACG TGT		
Exon 6	A15629 – HS00573473	FWD – CAA CAC GGT GCA GTG GAC	262 bp	61°C
	A15630 – HS00573473	REV – ATC ACT CCA AGT GGA ATC TCT TCT G		
Exon 7	A15629 – HS00551367	FWD – TCT ATA GCC CCA GAA CTA AAC CAG A	272 bp	61°C
	A15630 – HS00551367	REV – GGG TAT ACT CTG TCC TCC CAG AA		
Exon 8	A15629 – HS00815906	FWD – TCT CTC CTG CTT GAA TTC ATG TGC	274 bp	61°C
	A15630 – HS00815906	REV - AGT TGT GGA AAT GTT CTC ATC CTT CTA C		
Exon 9	A15629 – HS00799516	FWD – TAC CGT AGG TGT TTT AGG TTA CAG TTT G	236 bp	61°C
	A15630 – HS00799516	REV – ATA ACT GCT TCA CAG GGC GTT		
Exon 10	A15629 – HS00759579	FWD – AAC CCA TAC CTG AGT ATC TTC AAG GA	230 bp	61°C
	A15630 – HS00759579	REV – GCC ACT ACC TGC TCA GTG AAT		
Exon 11	A15629 – HS00708315	FWD – TCT GAC CTG CAT TCA TAA GAC ATC TTA G	274 bp	61°C
	A15630 – HS00708315	REV – TTC AGG CAT CCT GGG TAG TTG		

Forward and reverse primer sets for *KHDC3L*

Fragments	Thermo Fisher Cat No./Lot No.	Sequence	Amplification size	Annealing temperature
Exon 1	A15629 – HS00277217	FWD – ATA AGA AGG GCG CGG CTA GA	366 bp	60°C
	A15630 – HS00639676	REV – ATG GGT GGC AGA AAG AAG CC		
Exon 2	A15629 – HS00773004	FWD – CTC ACA GCC TCT CTG CTA CC	238 bp	60°C
	A15630 – HS00773004	REV - GCT CCA GGT AGC CC T ATT CC		
Exon 3A	A15629 – HS00277219	FWD – TCT GGG ATT TCT GGC TCC TAC TC	508 bp	58°C
	A15630 – HS00277219	REV – TAT GAA GGC ATC TCA GGC CCT GG		
Exon 3B	A15629 – HS00277220	FWD – AAA ATC CAC ATC CTC TCC CCA ACC	497 bp	58°C
	A15630 – HS00277220	REV – ATG CGC GCG GTT AAG GAG TA		

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   8.4. No amendment or waiver of any terms is binding unless set forth in writing and signed by the parties. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the licensing transaction described in the Order Confirmation, which terms are in any way inconsistent with any terms set forth in the Order Confirmation and/or in these terms and conditions or CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

   8.5. The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to support@copyright.com.

v 1.1

APPENDIX F: AUTHOR GUIDELINES FOR THE INTERNATIONAL *JOURNAL OF GYNAECOLOGICAL CANCER*

Authors

Submit manuscript >>

International Journal of Gynecological Cancer (IJGC), the official journal of the International Gynecologic Cancer Society and the European Society of Gynaecological Oncology, is the primary educational and informational publication for topics relevant to detection, prevention, diagnosis, and treatment of gynecologic malignancies. *IJGC* emphasizes a multidisciplinary approach, and includes original research, reviews, and video articles. The audience consists of gynecologists, medical oncologists, radiation oncologists, radiologists, pathologists, and research scientists with a special interest in gynecological oncology.

- Submission Policies
- Article Types
- Editorial Policies
- Article Processing Charges

Questions? Contact ijgc@jjeditorial.com

Submission Policies

For guidelines on policy and submission across our journals, please click on the links below:

- Manuscript Preparation
- Editorial Policies
- Patient Consent Forms
- License Forms
- Peer Review
- Submission and Production Processes

Authors may find it useful to consult our pre-submission checklist. Please review the article type requirements below and the Author Guide, prior to submitting your manuscript or revision.

Institutional Review Board Approval

Every research article, including every submitted article involving human participants, requires a statement of ethical or institutional review board approval within the manuscript text. Furthermore, a formal letter of ethical or institutional review board approval must be uploaded along with the manuscript files at initial submission. Find more information about ethical approval.

Author's Response

When submitting a revision, please include an Author's Response to the reviewers' comments. Please list each comment from each reviewer and provide a point-by-point response indicating how the comment has been addressed and the specific page(s) and line number(s) where the change was made in the manuscript text file. Additionally, please copy and paste the changed text in the Author's Response.

An example is below:

Reviewer 1, Comment 1: Please clarify what you mean by "borderline pathology."

Author's Reply: We have clarified this in the Methods section on page 6, lines 4-5.

Modified Text: "Patients with borderline mucinous and serous tumors were more likely to be partially satisfied, not satisfied or not at all satisfied compared to patients with cancer (p=0.027)."

Please note that authors may be required to provide data or cited works upon request for purposes of peer review.

Please note the following regarding the use of abbreviations for *IJGC*.

Abbreviations are always allowed for:

- Units of measure
- Clinical trial names
- Any name of a gene (e.g., BRCA) or serologic market (e.g., CA125)

Abbreviations are allowed but must be spelled out at first use for:

- Statistical terms (SPSS, Stata)
- The following organizations: NCI, WHO, FDA, CDC
- The following terms: HPV, Pap, MRI, USG, CT, PET/CT, HNPCC, HIPEC, RIFLE, FDG, EGOG, SLN, NCCN, FIGO, AUC, VIN, dVIN, HSIL, STIC, EOC, HGSC, and SCS

Abbreviations cannot be used – and must be spelled out at each use for the following: UVA, MAV, DFS,PFS, OS, OCCC, CRS, PCI, AKI, PCN, IRIS

The word count excludes the title page, abstract, tables, acknowledgements and contributions and the references. If you are not a native English speaker and would like assistance with your article there is a professional editing service available.

The *IJGC* is not currently accepting submissions on breast cancer. Additionally, given the high volume of submissions on the subject of HPV and

pre-invasive disease of the lower genital tract, we will restrict consideration for review and ultimate publication to those manuscripts reflecting novel data from either large prospective trials or high-level scientific multi-institutional efforts reflecting a high number of patients. We will also give higher priority to work that is impacting a segment or region of the world where HPV has been limited. Manuscripts reflecting findings that have already been published by others with similar results will not be considered for review and will be returned to authors.
When a paper has been submitted from the Editor, Deputy or Associate Editors' departments, they have no role in the reviewing or decision-making process. This also applies to any Associate Editors who are authors, in which instance the reviewing process is handled by the Editor in Chief.

Industry Authorship
The IJGC encourages the submission of manuscripts outlining results of research conducted in collaboration and/or supported by industry partnership. Manuscripts where authorship is shared among investigators both involved or uninvolved with industry sponsors will be evaluated in detail for compliance with updated Conflict of Interest statements. In addition, the number of authors who are directly or indirectly strictly linked with a company or pharmaceutical will be limited to no more than two authors. All authors must be in agreement with the final submission of such manuscript, and such authors shall agree to provide raw data if so requested by either Editorial team or Reviewers. Manuscripts exclusively written by members of a pharmaceutical company without any input from medical/surgical collaborators will not be considered for review.

File Formats
When submitting to IJGC, please ensure all files are submitted correctly with the corresponding file types selected. Doing so can help reduce the amount of time before your paper receives a decision.
Specific Examples:

- All tables should be submitted in the manuscript document after the references section. The main manuscript document (with tables) file should be in Word doc format.
- Each figure should be uploaded as a separate "Image" file. Figures may be in TIFF, EPS, PDF, or JPEG format.
- Highlights should be uploaded in one "Highlights" file. The Highlights document should be in Word doc format.

Dr. Pedro Ramirez, IJGC's Editor-in-Chief, provides authors with detailed instructions and considerations for preparing a manuscript for submission to IJGC.

Article Types

- Original research
- Review
- Case study
- Letter
- Editorial
- Video article
- Clinical trial
- Corners of the world

Original research
Our intent is to publish high quality research as it relates to clinical trials, outcome analyses, translational research, cost utility analyses, etc. Meta-analyses and literature reviews should be submitted as Original Articles and require a PRISMA Checklist. Authors should use the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system for grading evidence when submitting a clinical guidelines article.

Original research should include a structured abstract of no more than 300 words with the following subsections: Introduction, Methods, Results and Discussion. The manuscript text should have the following headings: Introduction, Methods, Results and Discussion.
Original research should also include a Precis of 200 characters, which should briefly provide information on the value and impact of the study. Authors should also submit 3 Highlights of no more than 100 characters each, outlining the key findings and impact of the study. Authors may also include supplemental figures and tables.

Word Count: up to 2,700 words
Abstract: up to 300 words
Tables/Figures: up to 5 tables and/or figures
References: up to 35
Authors: up to 40 (o more than 8 from a single institution)

Review
Review articles will address a topic of major interest in the field of gynecologic oncology and should include an unstructured abstract of no more than 300 words and a Precis of no more than 200 characters. The Precis should briefly provide information on the value and impact of the study. Authors may also include supplemental figures and tables.

Abstract: up to 300 words
Word Count: up to 5,000 words
Tables/Figures: up to 7 tables and/or figures
References: up to 50
Authors: up to 5

Case study
By invitation only, Case Studies include a specific case of interest in the field of gynecologic oncology, a question and answer, and a discussion. They are limited to five authors total with two presenters, one discussant, one pathologist, and one radiologist.

Abstract: none
Word Count: up to 2,500
Tables/Figures: up to 1 table and 4 figures
References: up to 10 references
Authors: up to 5

Letter
Letters should be a short and concise communication commenting on a recently published Original Article in the Journal or commenting on a controversial current issue of concern to the readership. The letters **must be submitted within 1 month of publication of the Original Article in question**. A Letter to the Editor is not a site for publication of original results. A statement of potential sources of conflict of interest must accompany the letter and may be published along with the letter. The Editorial Board reserves the right to decline publishing insulting or inflammatory comments in letters to the editor.

Word Count: up to 200
References: up to 5
Authors: up to 3

Editorial
By invitation, Editorials have a limit of 500 words and a limit of 2 authors, and 5 references, including the article in question.

Word count: up to 500
References: up to 5
Authors: up to 2

Video article
This exciting new feature focuses on high-quality videos that includes any educational topic in Gynecologic Oncology. IJGC's aim is to provide gynecologic oncologists around the world with a unique educational opportunity using multimedia. Video articles may focus on radiological imaging, ambulatory procedures, pathology, surgical anatomy, exposure, innovation, reconstruction, step-by-step-procedures, complications and resolutions, as well as anatomic variations, tips and tricks in gynecologic oncology, robotics, or new devices. Video Articles may also illustrate ways of improving surgery in developing countries or implementing surgery in scenarios with low resources. Videos that have been presented at a meeting are eligible to be submitted to IJGC.

A video article should include a video that is between 5 and 8 minutes in duration and **no larger than 350 MB**. The video must be narrated in English and should not include music. The video may include slides, not exceeding 2 minutes in total. The first slide of the video must include the submission title and the authors' name(s) and institution(s). The last slide of the video must include the conclusions and acknowledgments. Whenever a video article shows a surgical procedure, it is recommended to add within the video (or as supplementary material) two tables showing the specific material needed and a summary of tips for carrying out the procedure. An individual high quality still image of the video should also be submitted that illustrates the technique demonstrated in the video. Additionally, the manuscript text should only be an unstructured summary of no more than 350 words, and must include references, no more than 4. Please list the length (in minutes), the size (in megabytes), and the type of video file (.mov, .mpg, .avi, or .mp4) in the title page. Video articles have a limit of 6 authors. Please include a caption for the video at the end of the manuscript text.
We encourage authors to include text and drawings in the video showing and pointing out the anatomical structures as well as schemas either of the procedure or the surgical field. Attractive educational content along with a high quality video and sound are greatly appreciated at

the time of the evaluation. Authors are encouraged to contact IJGC's Video Editor Luis Chiva (lchiva@unav.es) for any questions or assistance with creating a Video Article.

Summary: up to 350 words
References: up to 4
Authors: up to 6
Length: up to 8 minutes
File size: up to 350 MB

Clinical trial
These articles will look at ongoing clinical trials in the field of gynecologic oncology. The articles must have a main objective and the studies must have and/or be accruing patients. The articles should include an Introduction, explaining the rationale for the study; a Methods section (inclusion and exclusion criteria for the study must be clearly outlined), detailing the study design; and a Discussion section, describing how the study may change current standards of care and practice. Authors may also include supplemental figures and tables.
Word Count: up to 2,500 words
Tables/Figures: up to 5 tables and/or figures
References: up to 15
Authors: up to 30

Detailed instructions are below:

Abstract
Please use all of the subheadings listed below.

- Background (2 sentences)
- Primary Objective(s) (1-2 sentences). Please do not include secondary/exploratory objectives in abstract
- Study Hypothesis (1 sentence)
- Trial Design (3-4 sentences)
- Major Inclusion/Exclusion Criteria (2-3 sentences)
- Primary Endpoint(s) (1-2 sentence)
- Sample Size
- Estimated Dates for Completing Accrual and Presenting Results
- Trial Registration

Manuscript
Introduction (Heading)

- Should include brief background, rationale, and hypothesis (3-4 paragraphs)

Methods (Heading)

Trial Design (Subheading)

- Description of trial design/treatment plan
- Include funding source (if applicable)
- Setting including participating organizations and number of sites
- Include study schema as a figure

Participants (Subheading)

- Inclusion/exclusion criteria (major)
- Can be more detail than abstract but do not get into minutia

Primary Endpoints (Subheading)

- Include primary and secondary objective(s) and endpoints
- Important translational/exploratory endpoints (but not all)

Sample Size (Subheading)

- How sample size was determined
- When applicable, explanation of any interim analyses and stopping guidelines

Randomization and blinding (Subheading) (if applicable)

- Method used to generate the random allocation sequence
- Type of randomization
- If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how

Statistical Methods (Subheading)

- Brief details of analysis of primary and secondary endpoints (1-2 paragraphs)

Discussion (Heading)

- Brief summary of expected results and how they will change practice

Corners of the world
A brief article highlighted those in the field of gynecologic oncology who are doing impactful work in either their local community or abroad. *IJGC*'s goal is to show the global scope of our mission and to excite other who are doing great work. Though there is a limit of 5 authors for the article, an Acknowledgements list for those involved in the work may be included.
Abstract: none
Word Count: up to 500 words
Tables/Figures: up to 3 figures
References: none

References: up to 5

Editorial Policies
IJGC aims to operate a fast submission and review process, to ensure timely, up-to-date research is available worldwide. Submissions should be made through the Journal's online submission system, here. Articles should not be under review by any other journal when submitted to *IJGC.*

IJGC adheres to the highest standards concerning its editorial policies on publication ethics, scientific misconduct, consent and peer review criteria. To view all BMJ Journal policies please refer to the BMJ Author Hub policies page.

- Reviewing for *IJGC*
- Article Processing Charges
- Manuscript Transfer
- Data Checks
- Data Sharing
- ORCID IDs
- Supplements

Reviewing for *IJGC*
Peer review may seem like a thankless task, but without it research would be unreliable. *IJGC* and BMJ value reviewers and want to encourage good standards of review. We encourage reviewers to read the Reviewer Guide or view the video below to learn more as Dr. Pedro Ramirez, *IJGC*'s Editor-in-Chief, provides reviewers with detailed instructions and considerations for preparing review comments for *IJGC* manuscripts.

If you have any questions about reviewing, please contact our Editorial team at ijgc@jjeditorial.com.

Information on how to review for any BMJ Journal is also available here.
The IJGC reviewer guide

Article Processing Charges
During submission, authors can choose to have their article published open access. There are no submission, page or color figure charges. The costs for open access for members are $2,200 and for non-members the costs are $2,800.
For more information on open access, funder compliance and institutional programs please refer to the BMJ Author Hub open access page.

Manuscript Transfer
Your article will not automatically be transferred to *IJGC* if rejected from another BMJ Journal; however, you will be able to choose *IJGC* as an alternate journal when submitting an article to any BMJ Journal; any reviewer comments will be shared, resulting in a reduced time to decision.
Manuscripts will be evaluated separately by the *IJGC* editorial team, with different criteria for acceptance.

Data Checks
BMJ is a member of CrossCheck by CrossRef and iThenticate. iThenticate is a plagiarism screening service that verifies the originality of content submitted before publication. iThenticate checks submissions against millions of published research papers, and billions of web content. Authors, researchers and freelancers can also use iThenticate to screen their work before submission by visiting ithenticate.com.

Data Sharing
Authors of original research articles are encouraged to include a data sharing statement when submitting their article. The statement should explain which additional unpublished data from the study—if any—are available, to whom, and how these can be obtained.
At present there is no major repository for clinical data, but Dryad has declared its willingness to accept medical datasets. Authors can start the deposition process while submitting to any BMJ Journal. Dryad provides authors with a DOI for the dataset to aid citation and provide a permanent link to the data. Note that Dryad hosts data using a CC0 license so authors should check that this is suitable for the data that they are depositing. The DataCite organization has a growing list of other repositories for research data.

ORCID Policy
IJGC mandates ORCID IDs for the submitting author at the time of article submission; co-authors and reviewers are strongly encouraged to also connect their ScholarOne accounts to ORCID. We strongly believe that the increased use and integration of ORCID iDs will be beneficial for the whole research community. Please find more information about ORCID and BMJ's policy on our Author Hub.

Supplements
The BMJ Publishing Group journals are willing to consider publishing supplements to regular issues. Supplement proposals may be made at the request of:

- The journal editor, an editorial board

member or a learned society may wish to organise a meeting, sponsorship may be sought and the proceedings published as a supplement.
- The journal editor, editorial board member or learned society may wish to commission a supplement on a particular theme or topic. Again, sponsorship may be sought.
- The BMJPG itself may have proposals for supplements where sponsorship may be necessary.
- A sponsoring organisation, often a pharmaceutical company or a charitable foundation, that wishes to arrange a meeting, the proceedings of which will be published as a supplement.

In all cases, it is vital that the journal's integrity, independence and academic reputation is not compromised in any way.

For further information on criteria that must be fulfilled, download the supplements guidelines.

When contacting us regarding a potential supplement, please include as much of the information below as possible.

- Journal in which you would like the supplement published
- Title of supplement and/or meeting on which it is based
- Date of meeting on which it is based
- Proposed table of contents with provisional article titles and proposed authors
- An indication of whether authors have agreed to participate
- Sponsor information including any relevant deadlines
- An indication of the expected length of each paper Guest Editor proposals if appropriate

APPENDIX G: Submission guidelines for the *European Journal of Human Genetics*

ABOUT THE JOURNAL

Aims and Scope

The *European Journal of Human Genetics* is the official journal of the European Society of Human Genetics, publishing high-quality, original research papers, short reports and reviews in the rapidly expanding field of human genetics and genomics. It covers molecular, clinical and cytogenetics, interfacing between advanced biomedical research and the clinician, and bridging the great diversity of facilities, resources and viewpoints in the genetics community.

Key areas include:

- Monogenetic and multifactorial disorders
- Development and malformation
- Hereditary cancer
- Medical genomics
- Gene mapping and functional studies
- Genotype-phenotype correlations
- Genetic variation and genome diversity
- Statistical and computational genetics
- Bioinformatics
- Advances in diagnostics
- Therapy and prevention
- Animal models
- Genetic services
- Community genetics

This journal also publishes invited editorials and commentaries, announcements of societal and other European activities, and special issues of general interest for the human genetics community.

Journal Details

Editor-in-Chief:
Professor G.-J.B. van Ommen, Department of Human Genetics, Leiden University Medical Centre

Editorial office: ejhg@lumc.nl

Impact factor: 3.650 (*2018 Journal Citation Reports*, Clarivate Analytics, 2019)

Frequency: 12 issues a year

Abstracted In:

EBSCO Discovery Service
Google Scholar
OCLC
Summon by ProQuest
BIOSIS
Medline
Current Contents/Life Sciences
Science Citation Index
Science Citation Index Expanded (SciSearch)
SCOPUS
EBSCO Academic Search
EBSCO Advanced Placement Source
EBSCO Biomedical Reference Collection
EBSCO Consumer Health Complete
EBSCO STM Source
EBSCO Health Source: Nursing/Academic Edition
EBSCO TOC Premier

ARTICLE TYPE SPECIFICATIONS

Article Specification	Specifications
Article Please see 'Preparation of Articles' below for further details.	Unstructured abstract max 250 words; Main body of text (including Title & Abstract pages and References) not to exceed 20 A4 pages; Max 4 tables and 4 figures; Max of 50 references
Review Article	Unstructured abstract max 250 words ; Main body of text (including Title & Abstract pages and References) not to exceed 22 A4 pages; Max 4 tables and 4 figures; Max of 70 references
Brief Communication Please see 'Preparation of Articles' below for further details.	Unstructured abstract max 250 words; Main body of text (including Abstract) not to exceed 1,500 words; Max 1 tables or figures; Max of 20 references
Viewpoint Solicited or unsolicited opinions on any relevant Human Genetics topics, as judged by the editors.	No abstract required; Main body of text not to exceed 900 words; Max 1 tables or figures; Max of 10 references
Correspondence Letters to the Editor deal with material in published papers. When considered publishable, the letter will be sent first to the authors of the original paper so that their response may be jointly published	No abstract required; Main body of text not to exceed 900 words; Max of 10 references; Tables/Figures to be uploaded as Supplementary Material
Comment Solicited views on recently published material	
News	
Book Review	No abstract required; Max 500 words
Clinical Utility Gene Card Requirements and templates can be obtained via the editorial office: ejhg@lumc.nl	

PREPARATION OF ARTICLES

House Style: Authors should adhere to the following formatting guidelines

- Manuscript files must be in Word, Text or RTF format, not PDF or Latex.
- Text should be double spaced with a wide margin.
- All pages and lines are to be numbered.
- Do not make rules thinner than 1pt (0.35mm).
- Use a coarse hatching pattern rather than shading for tints in graphs.
- Colour should be distinct when being used as an identifying tool.
- Commas, not spaces should be used to separate thousands.
- At first mention of a manufacturer, the town (and state if USA) and country should be provided.
- Statistical methods: For normally distributed data, mean (SD) is the preferred summary statistic. Relative risks should be expressed as odds ratios with 95% confidence interval. To compare two methods for measuring a variable the method of Bland & Altman (1986, Lancet 1, 307–310) should be used; for this, calculation of P only is not appropriate.
- Units: Use metric units (SI units) as fully as possible. Preferably give measurements of energy in kilojoules or Megajoules with kilocalories in parentheses (1 kcal = 4.186kJ). Use % throughout.
- Abbreviations: On first using an abbreviation place it in parentheses after the full item. Very common abbreviations such as **FFA**, **RNA**, need not be defined. Note these abbreviations: gram **g**; litre **l**; milligram **mg**; kilogram **kg**; kilojoule **kJ**; megajoule **MJ**; weight **wt**; seconds **s**; minutes **min**; hours **h**. Do not add **s** for plural units.

Please note that original articles must contain the following components. Please see below for further details.

- Cover letter
- Title page (excluding acknowledgements)
- Abstract
- Introduction
- Materials (or Subjects) and Methods

- Results
- Discussion
- Acknowledgements
- Conflict of Interest
- References
- Figure legends
- Tables
- Figures

Adherence to data nomenclature, -sharing and clinical trial reporting is mandatory, please consult Editorial Policies.

Cover Letter

Authors should provide a cover letter that includes the affiliation and contact information for the corresponding author. Authors should briefly discuss the importance of the work and explain why it is considered appropriate for the diverse readership of the journal. The cover letter must state the material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration. If the manuscript has been previously considered for publication in another journal, please include the previous reviewer comments, to help expedite the decision by the Editorial team. Please also include a Conflict of Interest statement, see Editorial Policies for more details.

Format

Please note: Page allowance is calculated on margins of 3 cm all-round, double line spacing throughout (including references but not title page) and font/size of Times New Roman 12pt (or equivalent).

Title Page

The title page should contain:

- Title of the paper - brief, informative, of 145 characters or less and should not make a statement or conclusion
- Running title – should convey the essential message of the paper in no more than 50 characters. Should not contain any abbreviations
  Please note – the running head for a manuscript on all pages after the title page will be the shortened manuscript title followed by an ellipsis.
- Authors should disclose the sources of any support for the work, received in the form of grants and/or equipment and drugs.
- Full names of all the authors and their affiliations, as well as the e-mail address of the corresponding author. If authors regard it as essential to indicate that two or more co-authors are equal in status, they may be identified by an asterisk symbol with the caption 'These authors contributed equally to this work' immediately under the address list.
  Group Authorship/Collaborations - Please note that if in the list of authors you wish to include additional authors/collaborators/ groups/consortiums that aren't part of the core list of authors as 'on behalf of', 'for the' or 'representing the' you need to ensure you list the authors correctly within the paper to ensure these are deposited correctly in PubMed.
  - Groups where there is an 'on behalf of', or 'representing the', or 'for the' will appear in the HTML/PDF as follows: Author A, Author B, Author C and Author D on behalf of...
    The list of individual members should then appear in the Acknowledgements section and not under Notes or Appendix
  - A Group name who is an author in its own right should have the list of authors as usual and then all the individual authors of the group listed in their own section at the end of the article, NOT in Acknowledgement/Appendix or Notes

Abstract

Articles, Reviews and Brief Communications must be prepared with an unstructured abstract designed to summarise the essential features of the paper in a logical and concise sequence. 3-6 Keywords should also be included on this page.

Introduction

The Introduction should assume that the reader is knowledgeable in the field and should therefore be as brief as possible but can include a short historical review where desirable.

Materials (Subjects) and Methods

This section should contain sufficient detail, so that all experimental procedures can be reproduced, and include references. Methods, however, that have been published in detail elsewhere should not be described in detail. Authors should provide the name of the manufacturer and their location for any specifically named medical equipment and instruments, and all drugs should be identified by their pharmaceutical names, and by their trade name if relevant.

Results

The Results section should briefly present the experimental data in text, tables or figures. Tables and figures should not be described extensively in the text, either.

Discussion

The discussion should focus on the interpretation and the significance of the findings with concise objective comments that describe their relation to other work in the area. It should not repeat information in the results. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take.

Acknowledgements

These should be brief, and should include sources of technical assistance, critical advice or other assistance, which contributed to the final manuscript.

Conflict of Interest

Authors must declare whether or not there are any competing financial interests in relation to the work described. This information must be included at this stage and will be published as part of the paper. Conflict of interest should be noted in the cover letter and also on the title page. Please see the Conflict of Interest documentation in the Editorial Policy section for detailed information.

Funding

The funding section is mandatory. Authors must declare sources of study funding including sponsorship (e.g. university, charity, commercial organization) and sources of material (e.g. novel drugs) not available commercially. If no financial assistance was received in support of the study, please include a statement to this fact here.

References

Only papers directly related to the article should be cited. Exhaustive lists should be avoided. References should follow the Vancouver format: see additional examples below. In the text they should appear as numbers starting at one and at the end of the paper they should be listed (double-spaced) in numerical order corresponding to the order of citation in the text. Where a reference is to appear next to a number in the text, for example following an equation, chemical formula or biological acronym, citations should be written as (ref. X) and not as superscript. Example "detectable levels of endogenous Bcl-2 (ref. 3), as confirmed by western blot"

All authors should be listed for papers with up to six authors; for papers with more than six authors, the first six only should be listed, followed by *et al.* Abbreviations for titles of medical periodicals should conform to those used in the latest edition of Index Medicus. The first and last page numbers for each reference should be provided. Abstracts and letters must be identified as such. Papers in press may be included in the list of references.

Personal communications can be allocated a number and included in the list of references in the usual way or simply referred to in the text; the authors may choose which method to use. In either case authors must obtain permission from the individual concerned to quote his/her unpublished work.

Examples:
Journal article:
Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005; **6**: 353–360.

Journal article, e-pub ahead of print:
Bonin M, Pursche S, Bergeman T, Leopold T, Illmer T, Ehninger G *et al.* F-ara-A pharmacokinetics during reduced-intensity conditioning therapy with fludarabine and busulfan. Bone Marrow Transplant. 2007; e-pub ahead of print 8 January 2007; doi:10.1038/sj.bmt.1705565

Journal article, in press:
Gallardo RL, Juneja HS, Gardner FH. Normal human marrow stromal cells induce clonal growth of human malignant T-lymphoblasts. *Int J Cell Cloning* (in press).

Complete book:
Atkinson K, Champlin R, Ritz J, Fibbe W, Ljungman P, Brenner MK (eds). *Clinical Bone Marrow* and *Blood Stem Cell Transplantation*, 3rd edn. Cambridge University Press: Cambridge, UK, 2004.

Chapter in book:
Coccia PF. Hematopoietic cell transplantation for osteopetrosis. In: Blume KG, Forman SJ, Appelbaum FR (eds). *Thomas' Hematopoietic Cell Transplantation*, 3rd edn. Blackwell Publishing Ltd: Malden, MA, USA, 2004, pp 1443–1454.

Abstract:
Syrjala KL, Abrams JR, Storer B, Heiman JR. Prospective risk factors for five-year sexuality late effects in men and women after haematopoietic cell transplantation. *Bone Marrow Transplant* 2006; **37**(Suppl 1): S4 (abstract 107).

Correspondence:
Caocci G, Pisu S. Overcoming scientific barriers and human prudence [letter]. *Bone Marrow Transplant* 2006; **38**: 829–830.

Figure Legends

These should be brief, specific, appear on a separate manuscript page after the References section.

Tables

Tables should only be used to present essential data; they should not duplicate what is written in the text. **It is imperative that any tables used are editable**, ideally presented in Excel. Each must be uploaded as a separate workbook with a title or caption and be clearly labelled, sequentially. Please make sure each table is cited within the text and in the correct order, e.g. (Table 3).
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American Journal of Surgical Pathology

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SCOPE

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Journal article

1. Band NS, Dawson JM, Juliao SF, et al. In vivo macrophage recruitment by murine intervertebral disc cells. *J Spinal Disord.* 2001;14:339--342.

Book chapter

2. Crowe VR. Visual information analysis: frame of reference for visual perception. In: Kramer P, Hinojosa J, eds. *Frames of Reference for Pediatric Occupational Therapy*. Philadelphia, PA: Lippincott Williams & Wilkins; 1999:205–256.

Entire book
3. Dellman RM, Marentette LJ. *Atlas of Craniomaxillofacial Fixation*. Philadelphia, PA: Lippincott Williams & Wilkins; 1999.

Software
4. *Epi Info* [computer program]. Version 6. Atlanta, GA: Centers for Disease Control and Prevention; 1994.

Online journals
5. Friedman SA. Preeclampsia: a review of the role of prostaglandins. Obstet Gynecol [serial online]. January 1988;71:22-37. Available from: BRS Information Technologies, McLean, VA. Accessed December 15, 1990.

Database
6. G-PDQ [database online]. Bethesda, MD: National Cancer Institute; 1996. Updated March 29, 1996.

World Wide Web
7. Hostin LO. Drug use and HIV/AIDS [JAMA HIV/AIDS Web site]. June 1, 1996. Available at: http://www.ama-assn.org/special/hiv/ethics. Accessed June 26, 1997.

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CHAPTER 1

Literature review

Introduction

Gestational trophoblastic disease (GTD) is composed of a group of disorders arising from placental trophoblastic tissue (Brown *et al.*, 2017). The current World Health Organization (WHO) classification of gestational trophoblastic disease consists of hydatidiform mole, choriocarcinoma, placental site trophoblastic tumour (PSTT), epithelioid trophoblastic tumour (ETT), miscellaneous trophoblastic lesions and abnormal (non-molar) villous lesions. Hydatidiform moles are further sub-classified as complete, partial and invasive while the miscellaneous trophoblastic lesions consist of exaggerated placental site and placental site nodule and plaque (Kurman *et al.*, 2014).

Placental site nodule and plaque and exaggerated placental site are non-neoplastic while hydatidiform moles have a potential for malignant transformation and choriocarcinoma, PSTT and ETT are classified as neoplasms (Kurman *et al.*, 2014). The malignant lesions are collectively grouped under the term gestational trophoblastic neoplasia (GTN) (Seckl, 2018).

During embryogenesis trophoblast arises from the trophoectoderm (Shih *et al.*, 2002; Sebire *et al.*, 2010). Trophoblastic tissue can be divided into villous and extravillous trophoblast. Villous trophoblast covers the chorionic villi while all other trophoblast is classified as extravillous. There are three populations of trophoblast namely cytotrophoblast, syncytiotrophoblast and intermediate trophoblast (Shih *et al.*, 2001; Shih *et al.*, 2002; Cheung, 2003). Villous trophoblast is composed predominantly of cytotrophoblast and syncytiotrophoblast while extravillous trophoblast consists of intermediate trophoblast (Shih *et al.*, 2002).

Hydatidiform moles and choriocarcinomas arise from villous trophoblast while PSTT and ETT arise from intermediate trophoblast (Sebire *et al.*, 2010; Shih *et al.*, 2002).

Exaggerated placental site and placental site nodule and plaque are not included in studies on GTD in the literature. They will therefore not be included in the scope of this dissertation apart from a brief mention later in this chapter.

Hydatidiform mole

A hydatidiform mole is an abnormal pregnancy characterized by poor or no fetal development, trophoblast proliferation and hydropic villi. The term hydatid, which means drop-like, was first used by Aetius of Amida, a physician in Justinian's court in the sixth century. Additional case reports were recorded in the following centuries including that of Margaret, Countess of Henneberg who delivered what appeared to be a hydatidiform mole in 1276 (Ober, 1959).

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