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Evaluation of Biomarkers for the Diagnosis, Management and Follow-up of Women with Gestational Trophoblastic Disease

Thesis presented by

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List of abbreviations

APSN	Atypical placental site nodule
ART	Artificial reproductive techniques
CC	Choriocarcinoma
CHM	Complete hydatidiform mole
CI	Confidence interval
CLSI	Clinical and Laboratory standards Institute
CPD	Continuous professional development
Ct DNA	Circulating tumour DNA
CUMH	Cork University Maternity Hospital
CV	Coefficient of variation
D&C	Dilation and curettage
DDISH	Dual-colour dual-hapten in-situ hybridisation
EPSR	Exaggerated placental site reaction
EMA/CO	Etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine
EOTTD	European Organisation for the treatment of trophoblastic disease
EPU	Early pregnancy unit
EQA	External Quality Assessment
ERPC	Evacuation of retained products of conception
ESMO	European Society of Medical Oncology
ETT	Epithelioid trophoblastic tumour
FIGO	The International Federation of Gynecology and Obstetrics
FISH	Fluorescent in-situ hybridisation
FFPE	Formalin-Fixed Paraffin-Embedded
FRHM	Familial recurrent hydatidiform mole
GP	General practitioners
GTD	Gestational trophoblastic disease
GTN	Gestational trophoblastic neoplasia
HCPs	Healthcare professionals
hCG	human chorionic gonadotrophin

HM	Hydatidiform mole
HSE	Health Service Executive
IHC	Immunohistochemistry
IM	Invasive mole
IS	International Standard
ISH	In-situ hybridisation
IVF	In-vitro fertilisation
LIMS	Laboratory information management system
MDT	Multi-disciplinary team
MTX	Methotrexate
NCCP	National Cancer Control Programme
NEQAS	National External Quality Assessment
PHM	Partial hydatidiform mole
POC	Products of conception
POCT	Point of care testing
PSN	Placental site nodule
PSTT	Placental site trophoblastic tumour
PUL	Pregnancy of unknown location
QF-PCR	Quantitative Fluorescent Polymerase Chain Reaction
RCOG	Royal College of Obstetricians and Gynaecologists
RCPI	Royal College of Physicians of Ireland
RHM	Recurrent hydatidiform mole
RIA	Radioimmunoassay
SNP	Single nucleotide polymorphism
STR	Short tandem repeat
UAPI	Uterine artery pulsatility index
ULN	Upper limit of normal
WHO	World Health Organisation

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

This publication-based thesis is structured as a compilation of manuscripts, each representing different aspects of the overarching research theme which are integrated into the thesis as individual chapters. All manuscripts are published in peer-reviewed academic journals except for the last two manuscripts.

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Research Outputs

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Joyce CM, Fitzgerald B, McCarthy TV, Coulter J, and O'Donoghue K. Advances in the diagnosis and early management of gestational trophoblastic disease. BMJ Medicine, 1 (1) http://dx.doi.org/10.1136/bmjmed-2022-000321

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***Joyce CM,** *Hamid M, Carroll HK, Kenneally C, Mulcahy S, O'Neill MK, Coulter J and O'Reilly S. (*joint first author) Gestational Trophoblastic Tumours in Ireland: An Exemplar for the Management of Rare Tumours. Eur J Obstet Gynecol Reprod Biol. 2023 Jul; 286:76-84 PMID: 37224702

Articles submitted for peer review:

Joyce CM, O'Shea PM, Lynch R, Costelloe SJ, McCarthy TV, Coulter J, Hayes-Ryan D and O'Donoghue K. Proof-of-Concept study: remote capillary blood for hCG analysis in early pregnancy. Submitted to Eur J Obstet Gynecol Reprod Biol. (manuscript ID: EJOGRB-24-633)

Articles in preparation:

Joyce CM, O'Shea PM, Lynch R, Costelloe SJ, McCarthy TV, Coulter J, Hayes- Ryan D and O'Donoghue K. Use of Point of Care testing for hCG monitoring in early pregnancy.

Collaboration in articles not part of this thesis:

Crowley MT, Lonergan E, O'Callaghan P, Joyce CM, Morita M, Conlon N and O'Halloran DJ. IGF-2 mediated hypoglycaemia and the paradox of an apparently benign lesion: a case report and review of the literature. BMC Endocrine Disorders 2022; 22 (1): 262

McMahon, L, Maher, GJ, **Joyce, CM**, Niemann, I, Fisher, R and Sunde, L on behalf of the genetics working party of the EOTTD. When to consult a geneticist specialising in gestational trophoblastic disease. Gynecol Obstet Invest. 2023 May 26. PMID:37245506

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Research dissemination

Oral presentations:

"National Survey of Women on the Gestational Trophoblastic Disease Registry"
Cork University Maternity Hospital Grand Rounds in Cork. 14th January 2022
National Centre for Cancer Control Steering Committee. 17th January 2022

"Recent Updates in Gestational Trophoblastic Disease", Association of Clinical Biochemists Republic of Ireland Virtual Meeting. 4th March 2022

"Accuracy of morphology with ploidy determination using HER2 D-DISH compared to molecular genotyping for Partial Hydatidiform Mole diagnosis", ISSTD XXI World Congress on Gestational Trophoblastic Disease, Sydney, Australia. 19th to 22nd October 2022.

"An analysis of Incidence Rates of Hydatidiform Moles in Ireland suggests under reporting of Partial Hydatidiform Mole, ISSTD XXI World Congress on Gestational Trophoblastic Disease, Sydney, Australia. 19th to 22nd October 2022.

"Challenges in hCG Measurement", 10th European Organisation for the Treatment of Trophoblastic Diseases Meeting in University College Cork. 13th to 14th May 2022

""National Survey of Women on the Gestational Trophoblastic Disease Registry" for the Gestational Trophoblastic Disease, How I do it Webinar series, Royal College of Physicians, Institute of Obstetrics and Gynaecologists. 5th October 2022

"Use of capillary blood for hCG analysis in early pregnancy", 11th European Organisation for the Treatment of Trophoblastic Diseases Meeting in Warsaw Poland. 12th and 13th May 2023

"Gestational Trophoblastic Tumours provide an Exemplar for the Management of Rare Tumours in Ireland", All Ireland National Rare Disease Interdisciplinary Research Network at University College Dublin. 27th June 2023

Poster presentations:

"Experience of women on the Irish GTD Registry"
Irish Society for Gynaecological Oncology Annual Meeting in Clarion Hotel Cork. 12th November 2021
College of Medicine and Health, From Molecules to People (FM2P) Conference in University College Cork. 22nd September 2022

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"An analysis of Incidence Rates of Hydatidiform Moles in Ireland" suggests under reporting of Partial Hydatidiform Mole, BMFMS in Brimingham. 2022

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National Strategy for Accelerating Genetic and Genomic Medicine in Ireland, Genetic and Genomic Testing Guidance Working Group (2023-2024)

Endocrinology Subcommittee of the Irish endocrine Society (2021-2024)

Education, training and events

Year	Course	Provider
2020	PhD workshops (5) – Presenting your research with confidence, How to plan your PhD, Seven secrets of highly successful research students, Turbocharge your writing and Defeating Self-Sabotage.	Hugh Kearns
2019/20	PG7016 Systematic Reviews for Health Sciences	UCC Postgraduate Module
2019/20	PG6009 Graduate Information Literacy Skills.	UCC Postgraduate Module (5 credits)
2020/21	ST6013 Data Analysis and Statistics	UCC Postgraduate Module (10 credits)
2020/21	PG6001 STEPS - Scientific Training for Enhanced Postgraduate Studies	UCC Postgraduate Module (5 credits)
2020/21	PG6015 an Introduction to Research Integrity, Ethics and Open Science	UCC Postgraduate Module (5 credits)
2022	Travel Bursary to attend GTD World Congress In Sydney, Australia	UCC SEFS
2021	Introduction to Qualitative Evidence Synthesis.	Evidence Synthesis Ireland.

Abstract

Gestational Trophoblastic Disease (GTD) describes a spectrum of disorders arising from the abnormal proliferation of trophoblastic tissue. Human chorionic gonadotrophin (hCG) is an excellent biomarker for most forms of this disease as its concentration accurately reflects trophoblastic activity. Hydatidiform mole (HM) or molar pregnancy is the most common form of GTD which may be suspected on ultrasound but requires pathological confirmation for diagnosis. Prognosis is generally excellent however close surveillance with hCG monitoring is imperative to detect cases progressing to malignant disease, referred to as gestational trophoblastic neoplasia (GTN). Clinical management and treatment of women with GTN in specialist centres achieves cure rates approaching 100%, emphasising the importance of accurate hCG monitoring for early detection of disease persistence to ensure optimal outcome. This thesis aims to evaluate pathological, genetic, and biochemical biomarkers used in the diagnosis, monitoring, and follow-up of women with GTD.

In this thesis, a scoping review was performed to generate evidence and identify gaps in the GTD knowledge base to inform areas for future research. Key findings were the need to standardise hCG immunoassays for effective GTD surveillance, to improve diagnostic reporting pathways and to establish more accurate incidence rates. In order to identify patient priorities and inform initiatives to enhance the quality of care, a service evaluation was performed with women enrolled in the National GTD Registry invited to participate. Feedback revealed a knowledge gap regarding GTD amongst healthcare professionals outside GTD centres. This study also revealed shortcomings in psychological support and bereavement counselling offered to women after a molar pregnancy.

To enhance the diagnostic accuracy of HM reporting, an in-situ hybridisation ploidy assay was developed with a customised scoring system to aid partial hydatidiform mole (PHM) diagnosis. This innovative technique provides a reliable adjunct to morphological assessment for PHM classification. The accuracy of this ploidy technique was confirmed by evaluation with molecular short tandem repeat genotyping. A national pathology questionnaire was performed to gather information on GTD diagnostic rates. Data collected was cross-referenced with cases documented in the National GTD Registry. This revealed a concerning under-enrolment of women with GTD (42%) onto the National GTD

Registry by their clinicians. An audit of HM diagnostic rates over a 3-year period was performed following the Implementation of the new ploidy technique to aid diagnosis . This revealed a higher local PHM incidence rate (1 in 296 births) than reported nationally in the pathology audit (1 in 484 births).

An electronic questionnaire was distributed to all European laboratories offering hCG for GTD management to gather insights on 5 key areas: hCG methodology, quality parameters, reporting and operational procedures, and use of hCG for non-GTD testing. This revealed considerable inter- and intra-laboratory variability in practice, emphasising the need to promote standardisation and harmonisation of immunoassays for GTD-derived hCG. A review of five challenging cases managed at the Irish GTD centre was conducted to evaluate the role of serum hCG and molecular genotyping in guiding management decisions, treatment strategies, and risk stratification for women with molar pregnancy, GTN and GTD mimics. These complex cases confirmed the pivotal role of serum hCG and molecular genotyping in monitoring disease persistence, prognostic stratification, and clinical management

Novel strategies to measure hCG were pursued to address the lack of centralised hCG testing for GTD in Ireland. A proof-of-concept study for remote capillary blood collection was performed to evaluate the clinical efficacy and user acceptability of this method of collection for hCG monitoring in early pregnancy. The study confirmed the equivalence of capillary and venous blood hCG testing, demonstrated complete clinical concordance, thereby offering an alternative to venepuncture for hCG measurement. To explore the accessibility of hCG testing in the community, a point-of-care testing (POCT) device was evaluated for its suitability to triage women with pregnancy of unknown location (PUL) in the early pregnancy unit. The Abbott iSTAT®1 POCT instrument was chosen for this purpose and was clinically concordant with central laboratory hCG results facilitating the use of clinical decision thresholds for PUL.

The research presented in this thesis provides valuable clinical insights through a series of nine research studies and has broadened the knowledge base in GTD. The findings offer an opportunity to positively impact diagnostic practices. The knowledge gap regarding GTD amongst healthcare professionals revealed in the patient survey points to a need for targeted educational initiatives in this area. Widespread integration of the *HER2* ploidy

technique to support morphology and standardise HM reporting could improve diagnostic accuracy, addressing a suspected underdiagnosis of PHM nationally and possibly internationally. It also suggests the need to re-evaluate incidence rates following the introduction of advanced diagnostic techniques such as ploidy assessment and molecular genotyping.

This thesis identified an under-registration of women with GTD in Ireland supporting the need for initiatives such as mandatory registration or advocacy by professional organisations to increase registration rates. The inter and intra-laboratory variation in hCG immunoassays revealed in the European laboratory survey merits renewed harmonisation efforts through pilot external quality assurance and sample exchange programmes. The use of novel methods to enhance the accessibility of hCG monitoring for GTD in the community, through capillary blood collection or use of point-of-care analysis, warrants further study. The positive patient feedback indicated a preference for this approach, suggesting better compliance with serial test monitoring, a priority for GTD management.

Chapter 1 – Introduction

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1.1 Background

Gestational trophoblastic disease (GTD) represents a group of rare pregnancy-related disorders associated with abnormal proliferation of the placental trophoblast. GTD spans the premalignant conditions of complete and partial hydatidiform mole (or molar pregnancy) to the malignant conditions collectively known as gestational trophoblastic neoplasia (GTN). GTN encompasses four distinct entities: invasive mole, choriocarcinoma, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). GTN is the most curable of all gynaecological malignancies with most women maintaining reproductive function and achieving 100% cure rates when clinical management is provided in a specialist GTD centre.(1)

The first description of molar pregnancy as "dropsy of the uterus" is attributed to Hippocrates around 400BC(2,3) In 1752, William Smelie coined the term "hydatidiform mole" to describe it's characteristic pathological appearance as a "bunch of grapes of different sizes".(4,5) Hydatidiform mole (HM) is derived from the Greek word "hydratis" meaning a drop of water and the Latin word "moles" meaning a mass reflecting the fluid-filled cystic mass found in the placenta of these conceptuses.(6)

GTN may occur after any pregnancy and its pathogenesis is unique in that a maternal tumour develops from gestational rather than maternal tissue.(7) The precise mechanism underlying hydatidiform mole (HM) formation is not yet fully elucidated. An error at fertilisation gives rise to an excess of paternal chromosomes in the zygote which results in trophoblastic proliferation and HM development.

The exact mechanism of progression from HM to GTN remains incompletely understood, prompting ongoing research efforts to identify and evaluate prognostic markers in this context. Collaborative efforts within the GTD scientific community have focused on defining genetic composition, standardising biochemical measurement of the biomarker human chorionic gonadotrophin (hCG) and harmonising pathological diagnostic criteria to improve the accuracy of GTD incidence rates and classification of this rare condition.

The Republic of Ireland has a population of 5 million people and is served by a network of 19 maternity hospitals and eight designated cancer centres. Cork University Hospital

(CUH) is one of the designated cancer centres. In 2017, the National Cancer Control Programme (NCCP) established the National Gestational Trophoblastic Disease Registry, Monitoring and Advisory Centre in Cork University Maternity Hospital (CUMH) which is co-located with CUH.

This introductory chapter offers a comprehensive overview of the factors influencing the development and management of this rare disease. It covers recent updates to GTD classification (section 1.1), as well as advancements in diagnostics from genetic (section 1.2), pathological (section 1.3), and biochemical (section 1.4) perspectives. Additionally, it addresses the challenges in clinical management (section 1.5) and the search for prognostic biomarkers (section 1.6) aimed at early detection of disease persistence. The scoping review in Chapter Two delves deeper into several of these subject areas, offering further insights and updates in specific areas.

1.2 Overview of GTD

1.2.1 GTD classification

The World Health Organisation's (WHO) 2020 Classification of Female Genital Tumours divides GTD into HM, GTN, tumour-like (non-neoplastic) lesions and abnormal (non-molar) villous lesions as shown in Figure 1.1(8) The HM category includes complete (CHM) and partial moles (PHM). The GTN category has been expanded to include mixed trophoblastic tumour which is a combination of two (or all three) of choriocarcinoma, PSTT and ETT.

Two new tumour-like lesions were added to the WHO classification of GTD in 2020; exaggerated placental site reaction (EPSR) and placental site nodule (PSN).(8) Larger PSNs (5-10mm) with more cellularity and a higher mitotic index are classified as atypical placental site nodule (APSN).(9) In a UK study of APSNs, 14% (3/21) were associated with GTN within 16 months of APSN diagnosis.(10) A recent French study suggests that APSN may not be a distinct clinical entity but represent a transitional stage between PSN and ETT. In this study two distinguishing transcriptional features of APSNs were identified, upregulation of *CCL19* and downregulation of *EPCAM* which may serve to identify cases requiring closer clinical surveillance.(11)

The exact mechanism underlying the progression from molar pregnancy to GTN is unclear. Choriocarcinoma is one of the most aggressive form of GTN with many having metastasised at the time of diagnosis.(12) In addition to gestational choriocarcinomas, which represent a form of GTN, there are also non-gestational choriocarcinomas and differences in their genetic origin dictates the prognosis and treatment regimens. Gestational choriocarcinoma may present after a term pregnancy (25%), a molar pregnancy (50%), or any miscarriage and is usually associated with very high serum hCG concentrations.(13) Gestational origin can be investigated by comparing DNA from the choriocarcinoma tissue (or circulating tumour DNA, ctDNA) to DNA from the woman's living offspring (or stored miscarriage placental tissue), using short tandem repeat (STR) genotyping. Gestational choriocarcinoma has frequently metastasised at the time of diagnosis (50% metastases to the lungs, 12% to the CNS and 8.5% to the liver), mandating chemotherapy but prognosis is generally favourable, with fertility preserved.(12,14) Non-gestational choriocarcinoma may be somatic or part of a mixed germ cell tumour and is associated with a poor prognosis given its high malignant potential.(15)

ETT and PSTT are extremely rare gestational neoplasms of intermediate trophoblastic origin accounting for 1-2% of GTNs. Prognosis hinges on identifying the antecedent pregnancy and a time interval of more than 48 months from the index pregnancy demands intensive chemotherapy.(16) These tumours predominantly arise in female pregnancies which suggests that the male derived X chromosome is not required for tumorigenesis.(17) PSTT originates from the implantation site intermediate trophoblast and the antecedent pregnancy may be latent from 2 weeks to 17 years with a prolonged interval associated with a poor prognosis.(18,19) ETT patients with an interval of ≥48 months since antecedent pregnancy also have a poor outcome.(20) Given the low production of serum hCG in these tumours, imaging is the preferred modality for tumour monitoring. Checkpoint immunotherapies may provide a new salvage pathway for women with recurrent or refractory PSTT/ETT following conventional treatment with high-dose chemotherapy and surgical resection.(19)

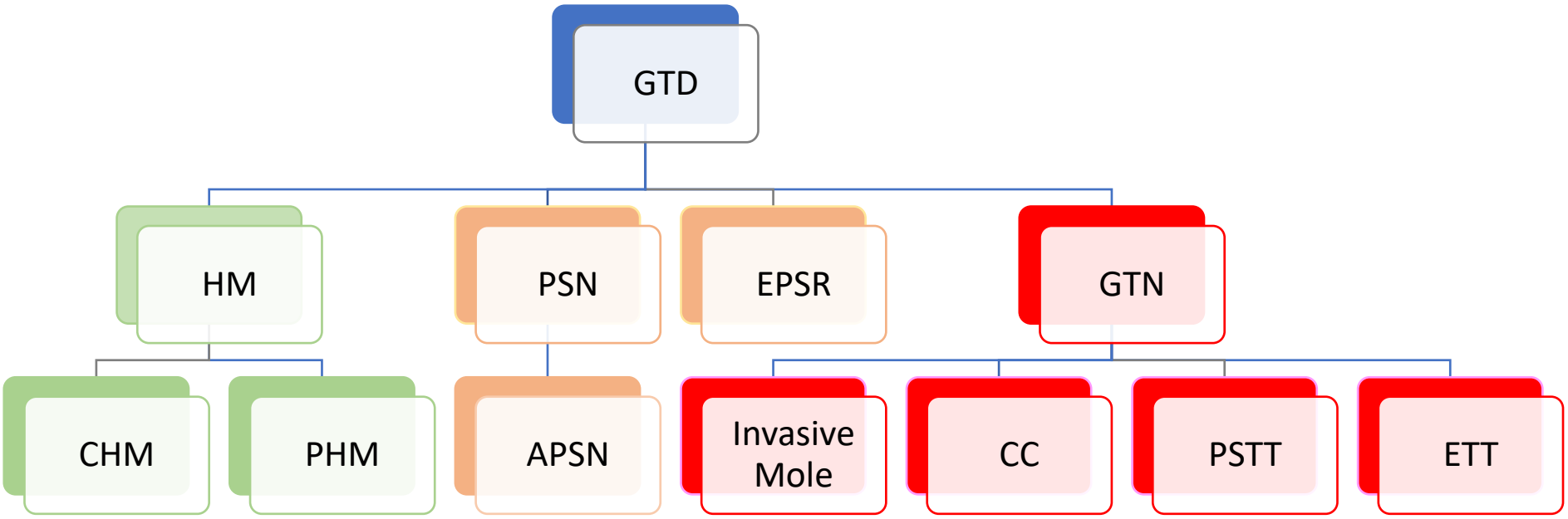

Figure 1. 1: Gestational Trophoblastic Disease WHO 2020 Classification. Adapted from Eiriksson et al. 2021.(21)

Abbreviations: *GTD, Gestational Trophoblastic Disease; HM, Hydatidiform Mole; CHM, Complete HM; PHM, Partial HM; PSN, Placental Site Nodule; APSN, Atypical Placental Site Nodule; EPSR, Exaggerated Implantation Site Reaction; GTN, Gestational Trophoblastic Neoplasia; IM, Invasive Mole; CC, Choriocarcinoma, PSTT, Placental Site Trophoblastic Tumour, ETT, Epithelioid Trophoblastic Tumour.*

Colour key: Green (non-neoplastic); Amber (tumour-like lesion); Red (neoplastic)

1.2.2 Incidence and Aetiology of GTD

GTD incidence rates vary worldwide, and international comparisons are hampered by a scarcity of centralised registries, use of inconsistent diagnostic criteria and lack of a universal dominator.(22) The total number of conceptions would provide the ideal denominator but this would be difficult to obtain.(23) The number of live births is often used as a surrogate for the total number of pregnancies.(24)

GTD incidence displays a distinct geographical distribution with highest rates in Southern Asia (10 per 1000 pregnancies) and lower rates in Europe and North America (1-2 per 1,000 pregnancies).(25,26) In the UK the calculated incidence of GTD is 1:714 live births but this may be underestimated due to underreporting, particularly in relation to partial moles.(27). Ireland has reported higher HM incidence rates of 1 in 521 pregnancies and 1 in 253 total births.(28,29) Moreover, the ratio of CHM:PHM of 1:3 in the UK is similar to those quoted in Ireland (1:2.8 and 1:3.4).(28–30) Further research studies are required to ascertain the true HM incidence rate in Ireland. The establishment of the Irish GTD Registry, Monitoring and Advisory Centre in 2017 has facilitated the collection of national GTD data to inform incidence rates and disease prevalence.

1.2.3 Risk Factors for the development of GTD

Risk factors for molar pregnancy include ethnicity (Asian descent) maternal age (<16 and >40years) and prior history of a HM.(26,31,32) A prior history of HM increases the risk of a second HM which is usually of the same type. The risk of a second CHM is 1% but this rises to 25% after two consecutive CHMs.(33) Notably, this risk is independent of the male partner which points to an underlying oocyte defect.(34) The risk of developing GTD has also been related to a dietary deficiency of animal fat and carotene (vitamin A precursor) which is also reported in countries with a high incidence of molar pregnancy. (35)(36) Some of these risk factors for GTD are included in the International Federation of Gynecology and Obstetrics (FIGO) prognostic scoring system for GTN.

The frequency of HM and prior miscarriage is higher than the frequency of two non-molar miscarriages in the population. (10-20% vs 2-5%). There is also a higher risk of aneuploidy in conceptuses of women with a HM and non-molar miscarriage than women with sporadic or recurrent miscarriages.(37) A recent study reported a higher frequency of aneuploidy in androgenetic dispermic CHMs.(38) This may be related to the extra pair of

centrioles in dispermic conceptions which may disturb chromosome segregation and lead to aneuploidy.(39) These data suggest a common germline genetic defect predisposing to HM, miscarriages and aneuploidy in some women.(37)

1.3 Genetic classification of hydatidiform mole

1.3.1 Origin of GTD

A hydatidiform mole (HM) is an abnormal conceptus with an extra copy of the paternal genome. HMs are classified as complete or partial based on their morphology and genetic composition as shown in Table 1.1.(40,41) Most CHMs have a diploid androgenetic genome and arise due to fertilisation of a single ovum devoid of nuclear DNA (enucleate) with either two sperm (20-25%) or a single sperm (75-80%) in which the haploid genome was duplicated. PHMs usually have a triploid monogynic diandric genome and usually arise from dispermic (>95%) fertilisation of a haploid ovum.(42) On rare occasions, tetraploid monogynic triandric PHMs are observed.(43)

The enucleate ovum in CHM could be explained by non-disjunction of chromosomes or chromatids during meiosis I or II which results in the expulsion of the maternal nuclear genome into one of the polar bodies.(44) However, an alternative popular hypothesis put forward by Golubovsky refutes the existence of an enucleate ovum and instead proposes a complex postzygotic event which excludes the maternal genome and results in diploidisation of triploids.(45) The postzygotic event could generate dispermic zygotes and give rise to mosaicism/chimerism.(45) This hypothesis is supported by the finding of rare CHMs with retained maternal chromosome 11.(46)

Heterozygous/dispermic complete moles are clinically more aggressive than homozygous CHMs with a higher serum hCG level and a higher risk of progression to GTN.(47) The increased risk may be related to a reduction in homozygosity of the paternal genome and the potential unmasking of recessive pathogenic variants. However, the risk of developing GTN after sporadic androgenetic CHM is not statistically different to the risk of developing GTN after a biallelic inherited form of CHM (discussed later) which suggests that abnormal imprinting rather than homozygosity for mutant alleles plays a more significant role in the malignant potential of CHM.(48)

1.3.2 Imprinting

Genomic imprinting plays a significant role in GTD, specifically in the development of HMs. It refers to an epigenetic phenomenon whereby certain genes are imprinted (silenced) resulting in a parent-of-origin specific gene expression. It supports the genetic conflict hypothesis in pregnancy which facilitates sex-limited expression of certain genes involved in fetal growth and development.(49) At gametogenesis, imprinted genes are modified and receive a reversible mark that differentiates the maternal and paternally derived alleles.(48) The finding of maternal alleles which have acquired paternal methylation patterns in some biparental CHMs suggests the error is not in erasing parental imprinting marks but in re-establishing the new maternal imprints during oogenesis or in their postzygotic maintenance.(48,50)

1.3.3 Recurrent HM

Recurrent HM (RHM) defines two or more HMs occurring in the same woman. Sporadic RHMs are generally androgenetic complete moles (AnCHM) but rare cases of recurrent partial moles have been reported.(51,52) Women with sporadic RHM may achieve a normal pregnancy and consideration of in-vitro fertilisation (IVF) using intracytoplasmic sperm injection (ICSI) followed by preimplantation genetic diagnosis (PIGD) could potentially avoid further HMs.(53,54) In women with recurrent AnCHMs, biallelic pathogenic variants have been identified in genes (*MEI1*, *TOP6BL*,/*C11orf80* and *REC114*) associated with meiotic double-strand breaks formation which may provide an explanation for androgenetic zygote formation.(55)

Familial recurrent hydatidiform mole (FRHM) is a rare autosomal recessive condition which predisposes women to HM and accounts for 0.6-2.6% of all HMs.(56) FRHMs are diploid biparental conceptuses with biallelic variants in maternal effect genes which are likely to set the maternal imprint in the genome.(57) The majority of variants (75-80%) occur in *NLRP7* (58) with some (5-10%) in *KHDC3L*(59) and a minority in *PAD16*.(60) Women with FHRM are unlikely to achieve a successful pregnancy and should be advised to consider IVF with donor oocytes to help them achieve a viable pregnancy.(33) A review of CHMs registered in a UK trophoblastic centre suggests that FHRM may be underreported as 1 in 640 women registered with a pathologically confirmed CHM had

FRHM.(33) The Irish GTD registry has no recorded cases of FHRM but a similar underestimation of FHRM may exist in Ireland.

1.3.4 Mosaic/Chimeras

The increased use of genetic analysis to aid HM diagnosis has facilitated the increased detection of mosaic/chimera placental tissue containing two diploid cell populations. Androgenetic/biparental mosaic conceptions are present in up to 2% of suspected HMs.(61) Different mechanisms have been proposed for these conceptions ranging from the fusion of two distinct zygotes to placentas with chimeric cell populations originating from a single tri-pronuclear zygote where parental genomes were not distributed equally to daughter cells in the first cell division.(62)

1.4 Pathological diagnosis of GTD

1.4.1 Morphology

Histopathological examination of all pregnancy tissue following early pregnancy loss is essential to detect all HMs, as ultrasound alone will miss a significant proportion, with approximately one-third of CHMs and two thirds of PHMs reported to be undetected by ultrasound.(63) Pathological diagnosis is required for confirmation of HM but is not required for GTN diagnosis. A GTN diagnosis may be based on imaging and hCG measurement as pathological tissue may be unavailable due to the risk of major haemorrhage during biopsy of the neoplastic tissue.

The morphological and genetic criteria of Szulman and Surti are used to distinguish between CHM and PHM as shown in Table 1.1.(40,41) Both HMs have two copies of the paternal genomes and their presence drives trophoblastic hyperplasia which is pathognomonic for this condition. There is significant inter-observer and intra-observer disagreement amongst pathologists when histological examination of pregnancy tissue alone is used for diagnosis.(64) HM must be distinguished from its mimics as villous hydropic transformation may be associated with other chromosomal abnormalities including monosomy X and trisomy 21.(65) In early CHM or PHM histological features are often poorly developed, thus ancillary techniques may be required to establish an accurate diagnosis.(66)

Table 1. 1: Clinicopathological features of Hydatidiform Moles

Characteristic	Complete Mole	Partial Mole
Karyotype	46 XX, XY	69 XXY, XXX, XYY
Genome	Diploid	Triploid
Ploidy status	Diandric	Diandric Monogynic
Ultrasonography	Enlarged uterus, diffuse villous hydrops	Enlarged placenta, focal cystic spaces
Clinical	Vaginal bleeding, bilateral theca lutein cysts	Incomplete or missed miscarriage
Pathology	Hydropic villi with cisterns, trophoblastic pseudo-inclusions stromal karyorrhexis no fetal tissue	Focal hydropic changes, trophoblastic proliferation scalloped outlines fetal tissue may be present
hCG IU/L	Very high >100,000 U/L	High <100,000 U/L

Adapted from Bartosch et al 2023.(66) U/L, units per litre

1.4.2 Ancillary techniques to aid Hydatidiform Mole diagnosis in Pathology

Ancillary techniques are often required to support a morphologically suspected HM diagnosis particularly during early gestational stages when classical histological features may be absent or poorly defined.(66) Immunohistochemistry (IHC) for the protein product $p57^{KIP2}$ (p57) of a paternally imprinted gene *CDKN1C* on chromosome 11 is frequently used to confirm a morphologically suspected CHM.(67) Expression of p57 is lost in the cytotrophoblast and villous stroma of a CHM confirming its diandric genome.(68) Rare examples of challenges to interpretation of p57 IHC include reports of retention of maternal chromosome 11 in a CHM and it's loss in a PHM.(46,69)

The widespread use of p57 immunohistochemistry (IHC) to aid HM morphological diagnosis has led to an increased detection of mosaic/chimera pregnancy tissue as

evidenced by irregular staining patterns.(70)Discordant and divergent p57 expression in placental villi may also reveal a HM with a coexistent twin pregnancy.(61,71)

Ploidy analysis using cytogenetics, flow cytometry, or in-situ hybridisation (ISH) may be used to confirm a triploid PHM and exclude PHM mimics (e.g. early CHM and miscarriage tissue with hydropic villi). Molecular genotyping can infer ploidy and confirm its genetic origin.(72) Given the diagnostic challenges associated with reporting HMs on morphology alone due to HM mimics, it is possible that pathology laboratories lacking access to ancillary techniques are under-reporting HMs. This situation poses a significant clinical risk particularly for CHMs which have a high risk of progressing to GTN.

1.5 Biochemical monitoring of GTD

1.5.1 Human Chorionic Gonadotrophin structure and biological function

hCG belongs to the glycoprotein hormone family which includes luteinising hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). These hormones share an alpha (α) subunit but are distinguished by their beta (β) subunit. The α and β subunits are non-covalently bound to form intact hCG which is produced by the placental villous syncytiotrophoblasts.(73) hCG is degraded and cleared by the kidneys as hCG β-core fragment.

Hyperglycosylated hCG (hCG-H) originally known as invasive trophoblast antigen (ITA) contains sialylated O- and N-linked oligosaccharides and is produced by extravillous cytotrophoblasts. Pituitary hCG, a sulphated variant of hCG, is also produced at low levels during the menstrual cycle by the gonadotropic cells of the anterior pituitary. (74)

There are a variety of hCG isoforms in serum and urine and any one isoform can predominate in GTD.(75)(76) The nomenclature for hCG-related molecules proposed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) hCG Working Group is illustrated in Figure 1.2.(77) These include intact hCG, free hCG β-subunit (hCGβ), free hCG α -subunit (hCG α), nicked hCG (hCGn), nicked free hCG β (hCGβn) and hCG β-core fragment (hCGβcf). The partially degraded products, hCGn and hCGβn, have polypeptide chain breaks between amino acids 44-45 and 47-48, respectively. In contrast, the urinary degraded form, hCGβcf, consists of a hCGβ fragment comprising amino acids 6-40 and 55-92, linked by disulfide bonds. (78,79)

The main function of hCG is the promotion of progesterone production by ovarian corpus luteal cells to maintain a pregnancy. hCGβ is produced by trophoblastic and non-trophoblastic (germ cell) malignancies and promotes tumour growth and blocks apoptosis.(80) hCGβ is an excellent biomarker for GTD as it reflects tumour burden and is highly sensitive to remnant trophoblastic activity indicating progression to GTN or disease recurrence.(81) However, the recognition of hCGβ by different commercial hCG immunoassays can vary markedly which can impact the accuracy of hCG measurement for trophoblastic disease management.(82)

hCG-H behaves in an autocrine manner similar to cytokines and promotes cytotrophoblastic cell growth and invasion during pregnancy implantation during the first trimester.(83) It also promotes trophoblastic invasion during choriocarcinoma and provides an excellent tumour marker for trophoblastic disease.(83) hCG-H appears to stimulate angiogenic activity through activation of the transforming growth factor-β receptor complex on cytotrophoblasts, independent of the LH/hCG hormone receptor pathway.(84)(85) Pituitary hCG mimics the actions of LH promoting follicular maturation, ovulation and progesterone production during the menstrual cycle.(74) Low level elevations of hCG may be a benign finding in perimenopausal and postmenopausal women and should be interpreted with the FSH level (>20 IU/L) to determine menopausal state.(86)

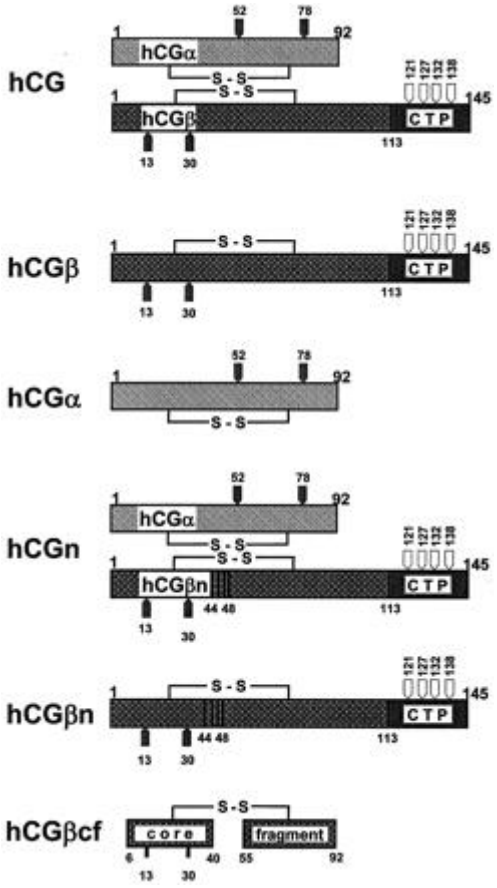

Figure 1. 2: Structure and IFCC nomenclature of hCG and important hCG metabolites. –S–S–, schematically depicted disulfide bonds; ▮, N-linked carbohydrate antennae; ▯, O-linked carbohydrate antennae; CTP, carboxyl-terminal peptide. Reproduced with permission from Oxford University Press.(77)

1.5.2 Challenges associated with hCG measurement

There is huge inter-assay variation in hCG measurement due to the heterogenous nature of hCG, use of different reagent antibody pairs and use of impure WHO international standards all of which poses a major challenge for accurate hCG measurement.(82)

Commercial hCG immunoassays are two-site sandwich assays with monoclonal antibodies to two conserved epitopes on the hCG β-subunit. These assays are validated solely for use in early pregnancy and often require an intact c-terminal peptide which may be cleaved in trophoblastic and non-trophoblastic disease.(87)(88) In contrast, competitive one-site radioimmunoassays (RIAs) use a polyclonal antibody (e.g. CX895) to one conserved

epitope. Although competitive RIAs are less specific, they are more likely to detect hCG variants with post-translational modification (eg. glycosylation, sulfation).

Serum hCGβ and its urinary degradation product hCGβcf are excellent tumour markers for trophoblastic disease and it is essential that all hCG assays used for GTD measure both total and free β-hCG.(88)(89) Ideally, the hCG assay used for GTD surveillance should detect all hCG isoforms in equimolar amounts.(90)

1.5.3 Centralised hCG testing

The well-established GTD centres (UK and Holland) have centralised pathology review and hCG testing with many countries including Ireland hoping to emulate their models. The European Society of Medical Oncology (ESMO) clinical practice guidelines for GTD recommend a centralised model of care to including centralised pathology review and hCG monitoring to achieve optimum GTN management.(91)

Ireland does not have centralised hCG testing or broad access to a cancer-specific hCG assay. In the absence of a centralised hCG monitoring service, harmonisation of laboratory practice for hCG monitoring in women with GTD has become a priority. For instance, the hCG upper limit of normal (ULN) is assay dependent but a threshold of <5 IU/L is generally used to establish normality during HM follow-up monitoring.(92) However, the hCG cut-offs used by some trophoblastic centres varies between <1 and <5 IU/L depending on their hCG assay and whether their laboratory uses the 95th or 99th percentile to establish their confidence interval. Consequently, women with GTD monitored in a laboratory using an ULN of <1 IU/L will have a longer follow-up period than women monitored in a laboratory using a ULN of <5 IU/L.

To address the logistical challenges associated with transporting samples for centralised hCG testing, especially in the absence of a centralised hCG testing service, home testing alternatives such as capillary blood sampling, dried blood tests, and salivary sampling could be considered as has been done in diabetes care.(93,94)

1.5.4 Pitfalls in hCG measurement for GTD monitoring

The accurate measurement of hCG is subject to both analytical and biological interference.(95) Analytical interference can arise due to the presence of heterophilic antibodies or human anti-mouse antibodies (HAMA) in serum or urine giving rise to false positive/negative results.(96) Laboratory investigations include checking for linear dilution, precipitation of antibodies with polyethylene glycol (PEG), use of an alternative immunoassay, correlation with urine hCG and use of antibody blocking tubes.(97) Sandwich assays are also prone to a phenomenon known as the "high dose hook effect" which results in falsely low hCG results (false negative). This occurs due to saturation of the capture and/or detection antibody binding sites in the presence of very high hCG concentrations.(98) The ability to accurately report high hCG levels is of paramount importance as the pre-treatment hCG level is used to calculate the FIGO score which dictates the treatment regimen for GTN.(74)

Biological interference from pituitary hCG can result in persistently low level hCG elevations (false positive) in perimenopausal or post-menopausal women.(99) A trial of the oral contraceptive pill should suppress pituitary hCG and so confirm its interference.(100) In addition, a new pregnancy during the surveillance period after molar pregnancy or chemotherapy for GTN can complicate hCG interpretation Therefore, women are advised to use contraception and avoid pregnancy until hCG follow-up is complete.(27)

Very rarely, a persistent low level hCG elevation is due to familial hCG syndrome. This rare condition, first described by Cole in 2009, appears to show autosomal dominant inheritance with an incidence of approximately 1 in 60,000 families within the United States.(101) Affected individuals produce a mutated hCGβ with an altered C-terminal peptide (CTP) that can evade some hCG assays.(102) The hCG molecule produced is not biologically active and the syndrome does not have clinical sequelae as women have regular menstruation and apparently normal fertility.(102) Familial hCG syndrome is a diagnosis of exclusion, typically confirmed only after other causes of elevated hCG (intrauterine or ectopic pregnancy) have been fully investigated and supported by the demonstration of elevated hCG levels in other family members.(103)

1.6 Management of Molar Pregnancy

1.6.1 Clinical Management

Historically, molar pregnancy presented in the second trimester with vaginal bleeding, a "larger for dates" uterus with occasional passage of vesicles, pre-eclampsia and hyperthyroidism.(104) Advances in ultrasonography in pregnancy have resulted in earlier diagnosis at 9-11 weeks gestation with 88% of CHMs and 56% of PHMs correctly identified.(105,106) Vaginal bleeding is still one of the most common presenting features of molar pregnancy and suction curettage is the method of choice for evacuation of the retained products of conception (ERPC).(27) For women who have completed their families, hysterectomy may be considered as an alternative to suction curettage given the lower risk of subsequent GTN.(107) In Ireland, suction curettage is the main treatment used.

After molar evacuation, trophoblastic activity is monitored by serial measurement of hCG in serum and/or urine using the same hCG assay throughout the surveillance period concordant with clinical practice guidelines.(27)(108) Women with CHM who normalise their hCG level within 56 days (see Figure 1.3) have a lower GTN risk (<1%).(109)

Rarely, a twin pregnancy of a viable fetus and co-existent molar pregnancy may develop. In the UK, a twin pregnancy of a CHM and a coexisting fetus occurs in 1 in 20,000-100,000 pregnancies.(110) This pregnancy can result in a livebirth if managed appropriately in a tertiary unit by maternal fetal medicine specialists with ongoing surveillance and guidance from an expert GTD centre.(27,111) Most twin pregnancies co-exist with a CHM and have a higher risk of perinatal morbidity and progression to GTN.(111) Twin pregnancies with a PHM and co-existent viable fetus are extremely rare but they may also go to full-term and result in a livebirth.(112)

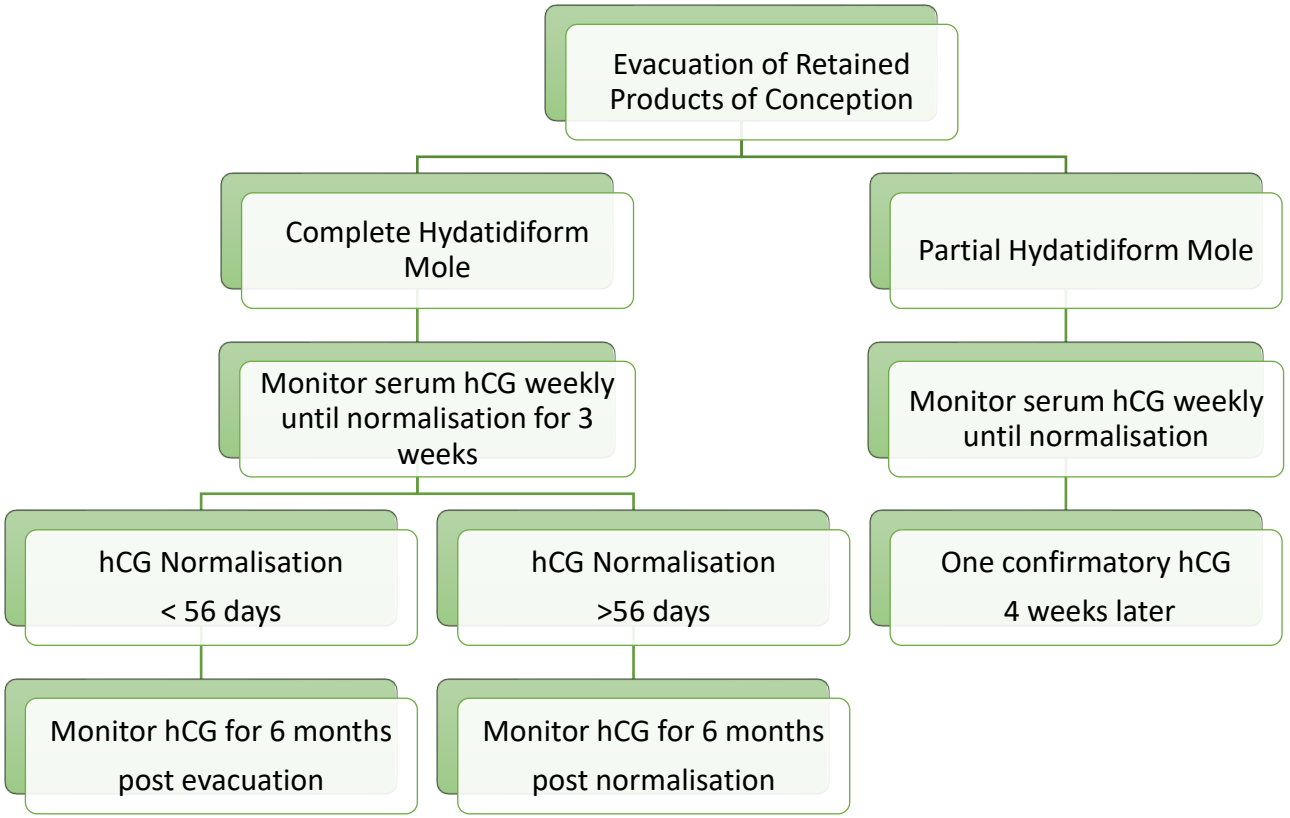

Figure 1. 3: hCG surveillance protocol for hydatidiform mole as recommended by the Irish and UK Management of Gestational Trophoblastic Disease Clinical Guidelines.(27)(108)

1.6.2 Experience of women with molar pregnancy

Research has demonstrated that molar pregnancy has a major effect on the psychological wellbeing of women and their partners during and after diagnosis.(113,114) In the Netherlands, a prospective study using the Hospital Anxiety and Depression Scale (HADS) reported a significantly higher HADS score in women with GTD than those with non-molar miscarriage, highlighting a greater need for psychological support services.(115)

Molar pregnancy presents a unique set of challenges. After experiencing the bereavement of pregnancy loss, women undergo a prolonged period of hCG surveillance, which can delay family planning. Furthermore, individuals affected by molar pregnancy face the possibility of developing a malignancy, in addition to grappling with concerns regarding disease recurrence and fertility.(116) The use of patient reported outcome measures (PROMs) in clinical practice could enable a better understanding of the impact of a GTD diagnosis on women and their families and better inform their psychosocial needs. In the UK, use of a bespoke GTD ePROM (ePAQ-GTD) programme in a nurse-led virtual clinic has facilitated discussions around taboo subjects such as sexual dysfunction and mental health issues. Feedback on use of ePROM showed benefits in relation to communication, empowerment and decision making.(117)

In Ireland, psychosocial support services were identified as a priority for cancer survivors with other malignancies.(118) In response, the National Cancer Control Programme (NCCP) launched a Psycho-Oncology Model of Care to help people with cancer and their families to improve access to psychological services and promote psychological wellbeing. (119) Psychol-Oncology is a discipline which addresses the psychosocial challenges (anxiety, depression, post-traumatic stress, fatigue and cognitive complaints) frequently experienced by people with a cancer diagnosis.(119) Similar resources may be needed to support women with GTN in Ireland. However, there is a knowledge gap in this area, highlighting the need for further studies to explore the long-terms effects of GTD on women and their partners in Ireland. Such research is essential to guarantee equitable access to appropriate services for all women with GTD.

1.6.3 Centralised registration

European clinical guidelines for GTD management recommend central registration with a specialist GTD centre.(100) UK and Irish guidelines also state that registration represents a minimum standard of care. (27,108) Although voluntary registration exists in most countries, some countries with mandatory registration still experience difficulties with compliance.(23) Since the establishment of the Swedish Cancer register in 1958, it has been mandatory to report all primary and some "precancerous" lesions (such as HM). Despite this, a number of studies have shown under-reporting of approximately 20% of all HM cases to the register.(120,121) Centralised registration offers a valuable database of patient information enabling research and clinical audit on various aspects such as incidence rates, risk factors, diagnostic tests, pathology trends, chemoresistance and patient outcomes.(122)

There is evidence that women managed by clinicians experienced in the management of GTD have better survival outcomes.(123) The mortality risk associated with low and high-risk GTN can be reduced when women are treated in a GTD reference centre or have their clinical management directed by experts at the reference centre.(124) A retrospective review of clinical outcomes in two Canadian GTD reference centres reported greater adherence to clinical guidance, more efficient initiation of chemotherapy and better surveillance completion.(125)

In the UK, GTD registration, pathology review, hCG monitoring and clinical management have been centralised to three expert centres since 1973.(30) The Irish National GTD Centre was established in 2017 and provides centralised management of care for all women with GTD who are registered by their referring clinicians. The centre offers supportive nursing care, disseminates patient information booklets, and maintains a national GTD register. It operates with a fully integrated multidisciplinary team (MDT) comprising of clinical nurse specialists, clinical biochemists, pathologists, radiologists and medical oncologists.(126) Currently, the centre does not offer centralised pathology review or centralised hCG testing as recommended in the ESMO Clinical Practice Guidelines for GTD.(91) However, the Irish GTD centre actively participates in the European reference network for rare gynaecological tumours (EURACAN) which enables

international MDT discussion of complex cases and clinical collaborations to improve treatment options for rare cancers.(127)

1.7 Management of Gestational Trophoblastic Neoplasia

1.7.1 Clinical management

GTN can develop in any pregnancy with approximately 50% occurring after molar pregnancy, 25% after miscarriage or ectopic pregnancy and 25% after term or preterm deliveries.(128) The risk of GTN is higher after CHM (15-20%) than PHM (0.5-1%).(129)(30) A recent 20-year review of low-risk GTN in Australia found a GTN incidence of 11.35% after CHM and 0.61% after PHM.(130) The risk of GTN developing after a live birth is even lower, occurring in approximately 1 in 50,000 births.(27)

Women with GTN generally require chemotherapy to cure their trophoblastic disease but a hysterectomy to reduce or eliminate tumour bulk may be an option in older women with GTN, if disease is confined to the uterus and preserving fertility is not a priority.(131). Women are risk assessed for chemotherapy regimens using the FIGO scoring system (see Table 1.2) which classifies women into a low or high risk category based on their predicted resistance to single agent chemotherapy.(132) Post-molar GTN is diagnosed based on the FIGO definition for GTN (see Table 1.3). The Dutch Society for Obstetrics and Gynaecology incorporate an additional criterion for GTN, based on their use of hCG regression curves as 15% of women will normalise without chemotherapy. They stipulate that at least one hCG measurement should be above the 95th percentile of a normal regression curve.(133)(134) Choriocarcinoma and post-molar GTN are chemo-sensitive and cure rates for this disease approach 100%.(132) Serum hCG monitoring is central to the diagnosis and management of GTN with treatment often initiated solely on the basis of trending hCG levels (see Table 1.4)

Table 1.2: FIGO/WHO 2000 staging and prognostic scoring sytem for Gestational Trophoblastic Neoplasia

FIGO stage	**Description**			
I	Gestational trophoblastic tumours strictly confined to the uterine corpus			
II	Gestational trophoblastic tumours extending to the adnexa or to the vagina, but limited to the genital structures			
III	Gestational trophoblastic tumours extending to the lungs with or without genital tract involvement			
IV	All other metastatic sites			
Risk scores	**0**	**1**	**2**	**4**
Age (years)	<40	>40	-	-
Antecedent Pregnancy	Mole	Abortion	Term	
Interval from index pregnancy, months	<4	4-6	7-12	>12
Pretreatment hCG mIU/ml	$<10^3$	$>10^3$-10^4	$>10^4$-10^5	$>10^5$
Largest tumour size including uterus[a], cm	-	3-4	≥5	-
Site of metastases including uterus	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

Low-risk score ≤6, High-risk score ≥7, ultra-high-risk score >12. Adapted from FIGO Committee on Gynecologic Oncology and reprinted with permission.(135) [a]Size of the tumour in the uterus. hCG, human chorionic gonadotrophin IU, international unit. To stage and allot a risk factor score, a patient's diagnosis is allocated to a stage as represented by a Roman numeral I, II, III and IV. This is then separated by a colon from the sum of all the actual risk factor scores expressed in Arabic numerals, e.g. Stage I:4, Stage IV:9. This stage and score will be allotted for each patient.

In the UK and Ireland, intramuscular Methotrexate (MTX) is the preferred first-line treatment for low-risk GTN with intravenous Actinomycin-D (Act-D) reserved for those who develop MTX resistance (see Table 1.4) which is European GTD guideline concordant. (27,100) The absence of sufficiently powered prospective randomised control trials comparing MTX and Act-D has intensified the debate surrounding the optimal choice for first-line single-agent chemotherapy.(136) A recent systematic review reported that Act-D is probably more likely to achieve a primary cure than MTX in low-risk GTN.(137)

Table 1. 3: Definitions for GTN management

Category	Definition
Post-molar GTN	rise in hCG levels for 3 consecutive samples over a 2-week period plateau in hCG levels for 4 consecutive samples over a 3-week period histological diagnosis of choriocarcinoma
GTN recurrence†	rise in hCG level after normalisation/consolidation 2 sequential rises in hCG above the reference level 1-week apart
Primary MTX resistance in low-risk disease	After initial two courses, if hCG is rising change treatment If hCG plateau (<10% change), continue to third course if hCG still plateaued, change treatment
Acquired MTX resistance	After initial response, hCG plateau (<10% change) over two courses (4 weeks) or rise over at least 2 weeks

GTN, Gestational Trophoblastic Neoplasia; hCG, human chorionic gonadotrophin; MTX, methotrexate. † a new pregnancy is excluded. Adapted from FIGO criteria (132)(100)

Table 1. 4: Low-risk post-molar GTN hCG thresholds

Serum hCG level	Treatment offered
Pre-treatment hCG* >100,000 and <400,000 (138)	Single-agent chemotherapy will cure 30% of women and only prolongs treatment by 2 weeks if EMA/CO needed.
MTX resistance with hCG* ≤1000 IU/L (139)	Act-D as second single-agent therapy can spare women EMA/CO without compromising 100% survival outcome

*hCG values were derived using the Charing Cross hCG radioimmunoassay

Women with a high-risk FIGO score (≥7) are likely to be resistant to single agent chemotherapy and require multi-agent chemotherapy regimens consisting of Etoposide, Methotrexate, Actinomycin-D/Cyclophosphamide and Vincristine (EMA/CO) or EMA alternating with Etoposide and Cisplatin (EMA/EP).(140) The presence of brain metastasis continues to provide a poor prognosis for GTN with a median survival of 2.5 years reported in some trophoblastic referral centres.(141) From 2016 onwards, the number of consolidation cycles recommended following chemotherapy increased from two to three cycles in some countries.(142)

1.7.2 FIGO scoring system

In 2000, a modified FIGO anatomical staging system was combined with a revised World Health Organisation (WHO) prognostic scoring system to aid GTN risk classification and clinical management. The FIGO/WHO 2000 scoring system incorporates eight risk factors including maternal age, pre-treatment hCG level, obstetric history and diagnostic imaging results as shown in Table 1.2. (135) The FIGO 2000 criteria is not used to manage PSTT or ETT as these tumours behave differently to other types of GTN and are more chemotherapy resistant.

In 1983, the Dutch Working Party on Trophoblastic Disease established their own risk classification system for GTN.(143) This scoring system differs from FIGO in that antecedent term pregnancy and previous failure to chemotherapy are incorporated into absolute criteria. There is extensive overlap between both risk classification systems and further refinements are necessary to both to prevent overtreatment. The Dutch clinical

classification system was used until 2017, after which the FIGO/WHO scoring system was adopted and implemented nationally.(143)

In Ireland, GTN is managed according to the FIGO recommendations.(132) The FIGO scoring system is not perfect as 25-35% of women overall fail single agent therapy and need multi-agent chemotherapy to cure their disease.(137) In women with a FIGO score of 5-6, predictors of resistance include metastatic disease and choriocarcinoma (regardless of pre-treatment hCG level) and women with this medical history should be considered for primary multiagent chemotherapy.(144) However, approximately 60% of women with a FIGO score of 5-6 achieve remission with single-agent therapy.(144) Streamlining of the FIGO scoring system from eight to two factors (using pre-treatment hCG and history of failed chemotherapy or metastasis) has produced a simplified scoring model whose performance was acceptable when compared to FIGO in the PREDICT-GTN2 study.(145)

1.7.3 Immunotherapy

GTN cases with an ultra-high-risk FIGO score (≥12) and rare tumours (PSTT/ETT) which are chemotherapy resistant often require salvage therapy. Checkpoint immunotherapy (CPI) targeting inhibitory T cell receptors including anti-programmed death 1 (PD-1) and its ligand (PD-L1) has been effective in some of these cases. However, immunotherapy resistance has been observed in some patients with high PD-L1 expression but low tumour-infiltrating lymphocytes (TILs).(146)

In the TROPHIMMUN trial, avelumab (anti-PD-L1) cured half of the patients with single-agent chemo-resistant GTN sparing them multi-agent chemotherapy.(147) Cheng et al used Camrelizumab (anti-PD1) with Apatinib (tyrosine kinase inhibitor) in chemo-resistant GTN and 55% of women achieved full remission showing the benefits of combination chemo-immunotherapy.(148)

Pembrolizumab (anti-PD-1) is very effective in multi-drug resistant GTN and has low toxicity therefore it has been incorporated into the National Comprehensive Cancer Network (NCCN) guidelines.(149) In the future, immune checkpoint inhibitors may be used as third line therapy for low-risk GTN or as first line therapy for high-risk GTN.(150)

However, we don't know the impact of immunotherapy on women's fertility as this area lacks long-term data and further research is warranted.

1.7.4 Biomarkers

Biomarkers are measured as an indicator of normal biological processes, pathogenic processes or reponses to an exposure or intervention (see figure 1.4).(151,152) An ideal biomarker will guide diagnosis, staging, prognosis and be reliable, measurable, cost-effective and show a response to treatment (see Figure 1.5).(153,154) The pregnancy hormone hCG is an ideal biomarker for GTD as its concentration reflects trophoblastic actibity and correlates with disease burden.(155)

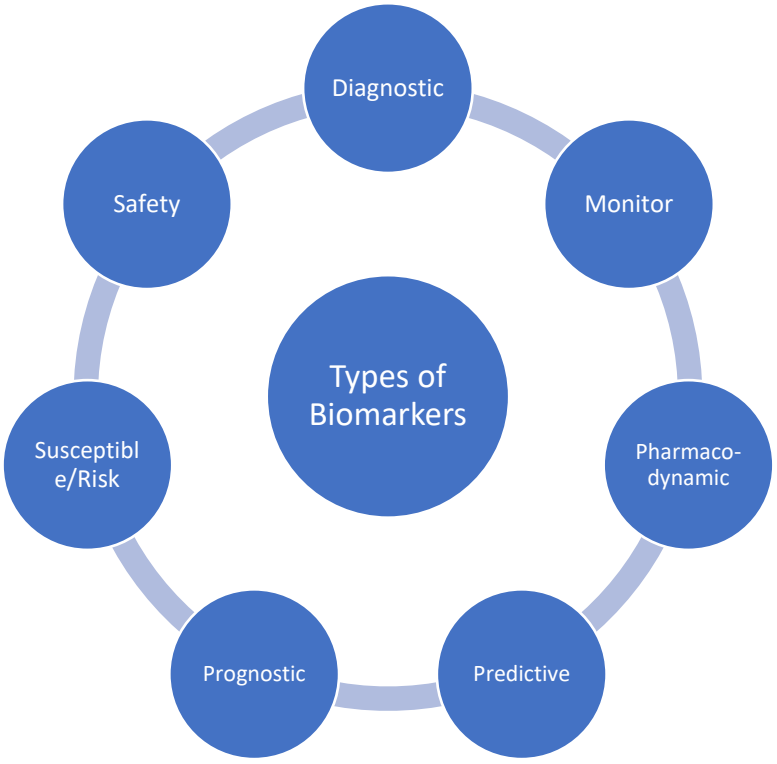

Figure 1. 4: Types of Biomarkers

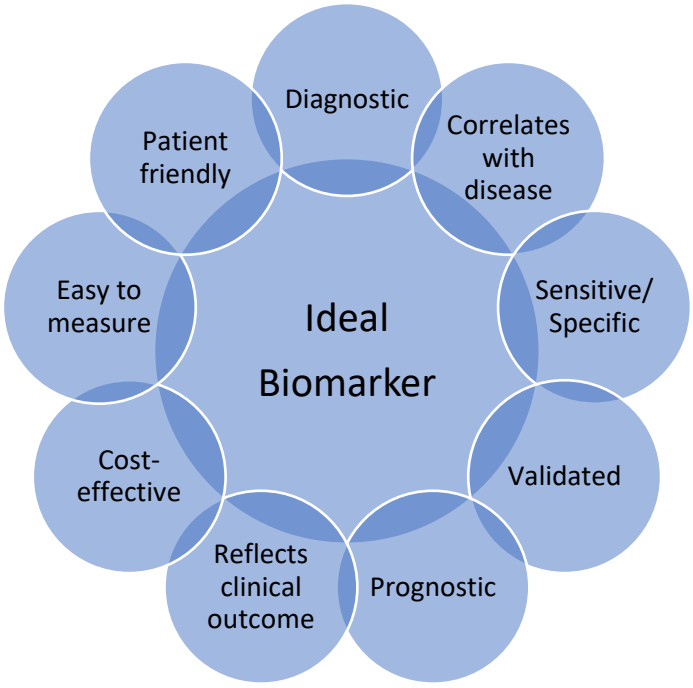

Figure 1. 5: Characteristics of an ideal biomarker

The search for new biomarkers to identify women who will develop post-molar HM has led to the development of novel approaches to recognise women at risk. In addition, shortcomings in the FIGO scoring system have prompted a search for better ways to predict which women will develop MTX resistance so they can be spared exposure to ineffective chemotherapy.

1.7.4.1 Methotrexate resistance

Trophoblastic tumours like the placenta are highly vascularised. The uterine artery pulsatility index (UAPI) measured using Doppler ultrasound, offers a non-invasive estimate of tumour vascularity in GTN. Low UAPI values in low risk GTN is predictive of MTX resistance independent of FIGO score.(156) However, the reproducibility of accurate Dopler ultrasound in this setting is variable and not widely adopted. Further research into the mediators of vascular changes in GTN associated elevated levels of the angiogenic factor, bone morphogenetic protein-9 (BMP-9) with MTX resistance. Combined use of elevated serum BMP-9 and low UAPI values could be used to improve the prediction of MTX resistance in low-risk GTN.(157)

Endoglin, another vascular mediator, is a co-receptor of the transforming growth factor-β (TGF-β) family which modulates signalling between TGF-β co-factors ALK1 and ALK5. Worley et al successfully used anti-endoglin monoclonal antibody and bevacizumab to treat refractory choriocarcinoma and this provides another therapeutic target.(158)

Targeting the ataxia telangiectasia and Rad3-related (ATR) signalling pathway genetically or pharmacologically using CDK4 inhibition has been shown to re-sensitise MTX resistant choriocarcinoma cell lines *in vitro* and in orthotopic mouse models *in vivo*, providing therapeutic strategies which could be translated into the clinical setting.(159)

1.7.4.2 hCG regression curves

Recognising hCG as an excellent biomarker for GTD, given its correlation with tumour volume, numerous endeavours have been made to develop predictive hCG models to forecase the malignant potential of HMs and their resistance to MTX. (81,133,160–163)

hCG regression curves use the pattern of hCG fall after uterine evacuation of HM as a useful indicator of progression to GTN and resistance to single-agent chemotherapy. A serum hCG ratio at 5 weeks after uterine evacuation was found to identify 75% of patients with persistent GTD.(164) In the Netherlands, a comparison of hCG regression in women who achieved spontaneous remission to those who developed GTN facilitated the construction of linear regression curves. Using a radioactive hCG assay, the slope of the regression line gave an area under the curve (AUC) approaching 1 (0.906, 95% CI) and predicted 52% of patients women with GTN with 97.5% specificity.(165)

Regression curves using hCG measurements before the fourth and sixth course of single-agent chemotherapy have also provided excellent diagnostic accuracy (AUC 0.949, 0.975) to identify single-agent chemoresistance.(166) A further Dutch study used a single serum hCG measurement four weeks after ERPC in women with CHM to identify 66% who would progress to post-molar GTN with 97.5% specificity.(162) Mathematic modelling of hCG kinetics using weekly hCG results to measure residual hCG (hCGres) and predict MTX resistance also shows promise but requires further validation in larger datasets.(163,167)

1.7.4.3 DNA markers

Specific circulating microRNAs (has-miR-520b, has-miR-520f, has-miR-520c-3p) have been identified in a small cohort of women with CHM who either spontaneously resolved or

developed GTN. Therefore, microRNAs may provide a useful adjunct to hCG testing to monitor GTN response during treatment. A specific microRNA (mIR-21) which is overexpressed in HM tissue also promotes invasion in choriocarcinoma.(168). CHMs that progressed to GTN showed higher expression of miR-181 and lower expression of BCL2 and both may provide prognostic biomarkers for GTN risk.(169)

Circulating tumour DNA (ctDNA) can help confirm GTN in challenging cases.(170)(171) Single nucleotide polymorphism (SNPs) genotyping using ctDNA distinguished between gestational and non-gestational choriocarcinoma when biopsy was unavailable or inappropriate.(172) Circulating gestational trophoblasts (cGTs) may be superior to ctDNA in establishing the genetic constitution in HMs as it is not hampered by the presence of maternal DNA.(173,174)

1.8 Summary

1.8.1 Current knowledge gaps and service needs

Despite significant research efforts, the aetiology of GTD remains unclear and the precise role of genomic imprinting in the development of molar pregnancy has yet to be fully elucidated. Given the rarity of GTD, pregnant women and their healthcare providers may have limited awareness and understanding of molar pregnancy. Furthermore, the optimum surveillance period for hCG monitoring in women following molar pregnancy has yet to be determined. This could potentially result in unnecessary delay of future pregnancies, which could have significant ramifications for older women.

While the emotional and psychological needs of women experiencing pregnancy loss are widely acknowledged, the perspectives of women who have had a molar pregnancy have traditionally not been included in pregnancy loss surveys. In Ireland, women with GTN although integrated into cancer care pathways, are not included in cancer patient experience surveys. Furthermore, since the establishment of a National GTD Registry, Monitoring and Advisory Centre in Ireland in 2017, affected women on the register have not had an opportunity to provide feedback on the services offered or to identify resources lacking in their care experience. Additionally, there has been no audit conducted to assess compliance with voluntary registration at the centre.

The reliance on morphological criteria for diagnosing molar pregnancy is subjective, often necessitating the use of ancillary techniques to support the diagnosis. However, limited access to these additional techniques could impede the collection of accurate incidence rates, providing an argument for centralised pathology review. It has been reported that digynic triploidy shares some morphological features with PHM, raising concerns about potentially over-diagnosing PHM solely based on morphology and ploidy analysis without molecular genotyping.

The lack of a commercially available hCG assay for oncology has created uncertainty regarding the optimal hCG assay for monitoring women with GTD. Given the central role of serial hCG measurement in GTD management, the inherent analytical and biological variability in certain hCG assays has emphasised the need for a centralised hCG testing service dedicated to GTD monitoring in Ireland. Such a centralised service would not only ensure consistency of hCG results but would also facilitate research into the use of hCG mathematical models for predicting disease progression and chemotherapy resistance.

In the absence of a quantitative urine hCG assay for monitoring GTD in Ireland, centralising serum hCG monitoring may pose logistical challenges related to inter-laboratory sample transport. Exploring alternative blood collection methods, such as capillary blood collection by women in their own homes, could offer a solution. For example, capillary blood collection for HbA_1C measurement in diabetes care demonstrated good agreement with venous blood and reduced hospital visits during the COVID-19 pandemic. This option may also offer a more convenient means of hCG monitoring for women with GTD.

In my thesis, I will explore several areas for further study within the framework of the following overarching objectives:

1 Knowledge of GTD
    i. Are there any additional services needed in the National GTD Registry, Advisory and Monitoring centre to meet the needs of women with GTD?
    ii. What are the challenges encountered by women and their partners following a molar pregnancy?
2 Under-reporting of GTD

i. What is the availability of ancillary techniques to assist the morphological diagnosis of HMs in pathology laboratories in Ireland?
ii. Does digynic triploidy cause morphological confusion and generate false positives in ploidy assays?
iii. Does morphology supported by ploidy analysis distinguish diandric triploid from digynic triploid conceptions?
iv. What is the true incidence rate of HMs in Ireland?
v. What is the compliance rate for voluntary registration by their physicians of women with GTD in Ireland?

3 Biomarker evaluation
   i. Can we standardise hCG measurement and harmonise hCG reporting in women with GTD across Europe?
   ii. Can trends in hCG levels be used to manage challenging GTD cases?

4 Novel methods for hCG analysis
   a. Could capillary blood home sampling provide a means to centralise hCG testing for GTD in Ireland?
   b. Can point of care hCG testing be used to guide patient management in the early pregnancy clinic?

1.9 Thesis aims and objectives

This thesis will address the knowledge gaps identified and questions posed in the last section within the following chapters, as illustrated in Figure 1.6.

The introduction (Chapter One) will provide the background knowledge on GTD and will serve to illustrate the rationale for some of the study questions.

In Chapter Two, a scoping review is presented which involves synthesising the evidence and critically examining the literature to provide a robust foundation for the study's main objectives.

Chapter Three provides feedback from the first survey of women on the National GTD registry. This study explores the specific needs of women with molar pregnancy with a view to informing future planning of GTD services.

Chapter Four introduces a novel scoring system for *Her2* D-DISH ploidy determination

Chapter Five audits the accuracy of this the *HER2* D-DISH ploidy procedure by comparison to molecular genotyping

In Chapter Six, HM incidence rates are reappraised based on implementation of the *HER2* D-DISH ploidy assay. This chapter also presents the results of a national pathology survey to inform national incidence rates and registration rates with our national GTD registry.

Chapter Seven examines the measurement and reporting of hCG in women with GTD across Europe. This study seeks to identify the challenges in hCG measurement and offer perspectives on hCG harmonisation.

Chapter Eight recognises the input of a national GTD service on the clinical management of five complex GTD cases. This study highlights the central role of biochemical hCG measurement and molecular genotyping in ensuring successful outcomes for these women.

Chapter Nine presents a proof-of-concept study on the use of capillary blood sampling for hCG analysis in early pregnancy.

Chapter Ten assesses the performance of a hCG point-of-care analysers for use in a remote early pregnancy unit.

In the discussion section (Chapter Eleven), the study outcomes are summarised and avenues for future research are explored.

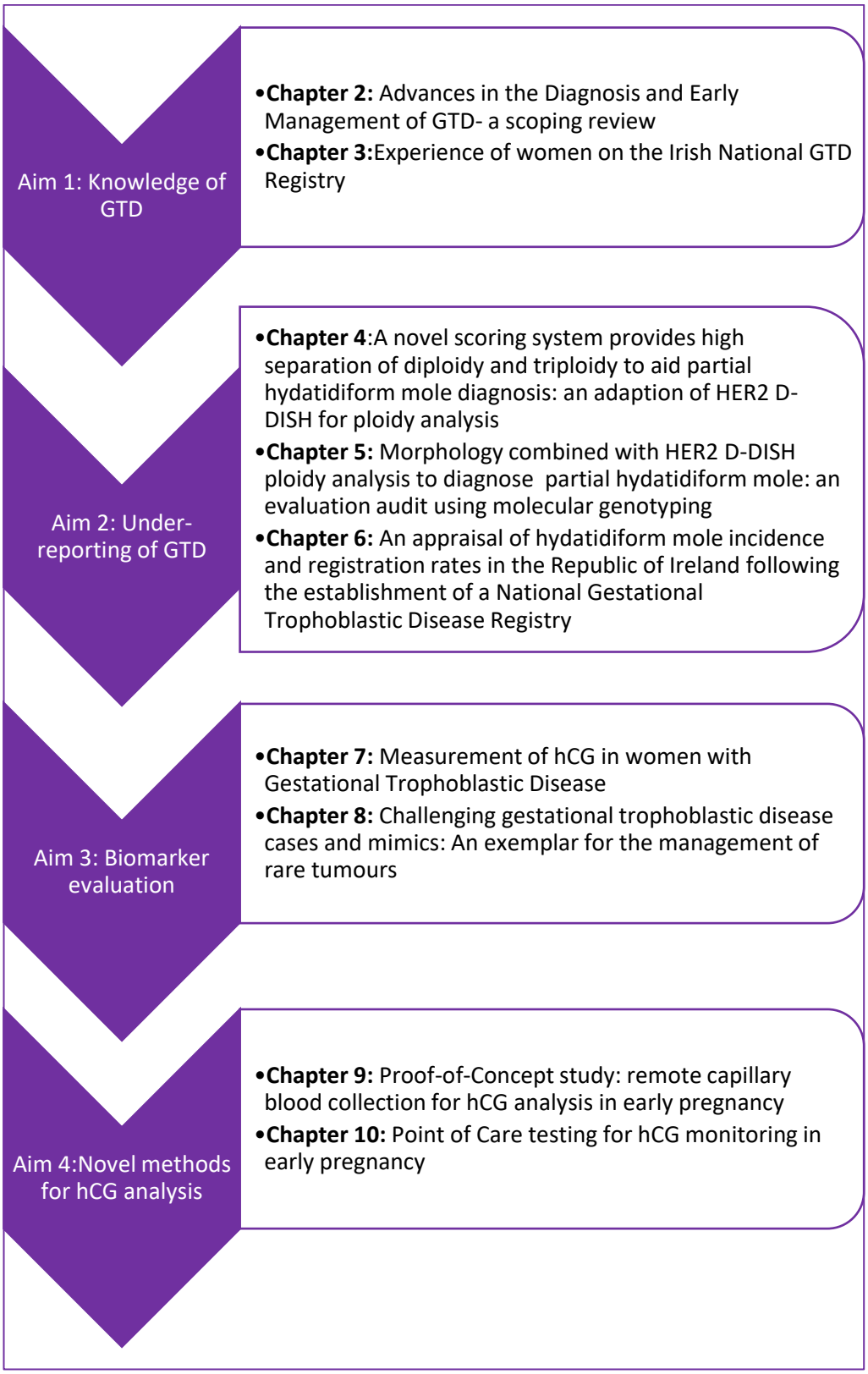

Figure 1. 6: Thesis Outline

Chapter 2 – Advances in the Diagnosis and Early Management of Gestational Trophoblastic Disease

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Chapter 2 - Advances in the Diagnosis and Early Management of Gestational Trophoblastic Disease

2.1 Abstract

Gestational trophoblastic disease (GTD) describes a group of rare pregnancy related disorders which span a spectrum of pre-malignant and malignant conditions. Hydatidiform mole (also termed molar pregnancy) is the most common form of GTD. It describes an abnormal conceptus containing two copies of the paternal genome which is classified as partial or complete depending on the presence or absence of the maternal genome, respectively. It typically presents in the first trimester with irregular vaginal bleeding and may be suspected on ultrasound, but confirmation requires histopathological evaluation of the products of conception. Most molar pregnancies resolve without treatment after uterine evacuation, but occasionally disease persistence develops with progression to gestational trophoblastic neoplasia. Close monitoring of women post-molar pregnancy with regular measurement of human chorionic gonadotrophin levels allows early detection of malignancy. Given the rarity of the disease, clinical management and treatment is best provided in specialist centres where very high cure rates are achievable. Here we review advances in the diagnosis and management of GTD and highlight updates to disease classification and clinical guidelines. We discuss the use of molecular genotyping for improved diagnostic accuracy and risk stratification and consider future biomarkers for the earlier detection of malignancy.

2.2 Introduction

Gestational trophoblastic disease (GTD) describes a heterogenous group of disorders that arise from abnormal proliferation of placental trophoblastic tissue. Diagnostic classification spans the pre-malignant conditions of complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM) to the malignant conditions of invasive mole (IM), choriocarcinoma (CC), placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT), collectively known as gestational trophoblastic neoplasia (GTN) (Figure 2.1). Both complete and partial moles have the potential for malignant transformation but the risk of GTN is higher for CHM (15-20%) than PHM (0.5-1%) (Table 2.1).(30,129,175) Maternal age and history of a previous molar pregnancy are two established risk factors for molar pregnancy. Hydatidiform moles (HMs) are classified as complete or partial based on their morphology and genetic profile.

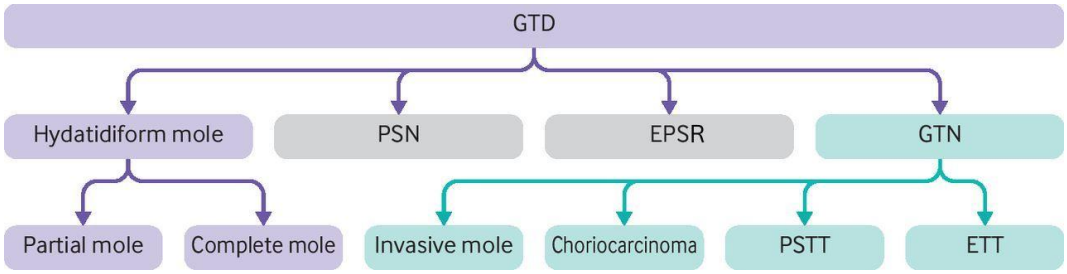

Figure 2.1: GTD Classification according to the World Health Organisation (WHO) 2020 Classification of Female Genital Tumours.(8) GTD = Gestational Trophoblastic Disease; PSN = Placental Site Nodule; EPSR = Exaggerated Placental Site Reaction; GTN = Gestational Trophoblastic Neoplasia; PSTT = Placental Site Trophoblastic Tumour; ETT = Epithelioid Trophoblastic Tumour.

Most cases of GTN occur after molar pregnancy but they can occur after any gestational event including miscarriage, ectopic or term pregnancies. GTN is the most curative of all gynaecological malignancies with cure rates approaching 100% even in the presence of metastatic disease (30) This review focusses on the diagnosis and early management of GTD and the reader is directed to other publications for expert opinion on the chemotherapeutic management of GTN.(91,100,176–178) The increased use of ancillary techniques has improved the accuracy of morphology-based GTD classification and risk stratification. Here we review advances in the diagnosis and management of GTD over the last decade.

2.3 Sources and selection criteria

We searched PubMed, Medline and CINAHL (EBSCO) for articles written in English and published from 2011 to 2021, using the search terms “gestational trophoblastic disease”, “hydatidiform mole”, “molar pregnancy” and “gestational trophoblastic neoplasia”. We considered articles in peer-reviewed journals and included articles based on study quality. We predefined the priority of study selection according to the level of evidence (clinical practice guidelines, systematic reviews and meta-analysis, cohort studies and expert opinion). Clinical management guidelines and expert reviews on this topic were consulted and highly cited studies and those of historical significance were included. Relevant publications outside the specified time-period were considered based on a review of bibliographies and expert opinion and the review bibliography was update to September 2022. In this article, we focus on the most common forms of GTD, hydatidiform mole and choriocarcinoma.

2.4 Incidence

The lack of centralised registries worldwide, combined with an under-reporting of cases to registries, variation in case definition and lack of a consensus-based denominator makes international comparisons difficult.(22,24,179) Incidence rates are typically derived from a mixture of live births, pregnancies, or deliveries but the total number of conceptions, although difficult to acquire would be more inclusive.(23)

The incidence of GTD varies worldwide with rates of 1-2 per 1,000 deliveries in Europe and North America compared to 10 per 1,000 pregnancies in India and Indonesia.(22,180) The incidence of GTD in the United Kingdom (UK) is 1 in 714 live births but incidence varies according to ethnicity with the highest incidence reported in women of Asian descent.(26,27,181). Genetic, social, cultural and dietary factors may be relevant as there is a higher frequency of GTD in regions of the world where malnutrition is common.(25,182) In the UK, the incidence of molar pregnancy is ~1 in 600 conceptions and the prevalence of PHM is higher than CHM with a ratio of 3:1.(31,129) However, the prevalence of CHM is age dependent with higher frequencies at the extremes of maternal age.(30,129)

Choriocarcinoma is the most aggressive form of GTN with a reported incidence of 3 per 100,000 deliveries in Europe and North America compared to 23 per 100,000 in Southeast Asia.(12,22) A systematic review of case reports revealed that 30% of choriocarcinomas had metastasised at the time of diagnosis.(183) More recently, a UK study reported metastasis in more than 50% of women with non-molar choriocarcinoma.(12) While most invasive moles originate from a CHM, only 25% of choriocarcinoma and 25% PSTT/ETT derive from a molar pregnancy.(60,184) The risk of developing choriocarcinoma post-molar pregnancy is higher for CHM (2-3%) than PHM (<1%).(185)(186) Hence, gestational choriocarcinoma should be considered in all premenopausal women with metastases of unknown primary.(1)

PSTT is a very rare form of GTN with an incidence of 1 in 100,000 deliveries in the UK and ETT is even rarer.(187–189) Owing to their rarity, PSTT/ETT are not discussed in detail in this review and readers are directed to other resources for details of their diagnosis and management.(13,18,19,190)

2.5 Clinical presentation

Women with molar pregnancy usually present with irregular vaginal bleeding in the first trimester. Ultrasound can identify the majority of CHMs (88%) but only detects some PHMs (56%) and this presents a diagnostic challenge.(106) Although ultrasound may be suggestive of a molar pregnancy, histopathological examination of the products of conception remains the gold standard for the diagnosis of molar pregnancy.(191) Human chorionic gonadotrophin (hCG) may be inappropriately high and reach levels >100,000 U/L in CHM (see Table 2.1). A review of cases presenting to the New England Trophoblastic Disease Centre over a 20-year period showed a reduction in the age at diagnosis of CHM from 12 to 9 weeks gestation.(105) Earlier diagnosis of GTD has led to changes in clinical presentation with women now rarely presenting with anaemia, hyperemesis, pre-eclampsia or hyperthyroidism.(16,192)

Table 2. 1: Typical Characteristics of Complete and Partial Moles

Characteristic	Complete Hydatidiform Mole	Partial Hydatidiform Mole
Ploidy	Diploid	Triploid
Genome	Diandric	Diandric monogynic
Karyotype	46 XX/XY	69 XXX/XXY/XYY
Ultrasound Imaging	Enlarged uterus, Bilateral theca lutein cysts Diffuse villous hydrops	Enlarged Placenta Focal cystic spaces Growth restricted fetus
hCG (U/L)	>100,000	<100,000
Pathology	Fetal tissue usually absent. Diffuse enlargement of villi. Cisterns. Diffuse trophoblast hyperplasia. Trophoblast pseudoinclusions. Bulbous, cauliflower-like villous outlines in earlier gestations. Villous stromal karyorrhexis in earlier gestations.	Abnormal fetus. Mixed population of enlarged and small villi. Occasional cisterns. Focal trophoblast hyperplasia. Trophoblast pseudoinclusions. Very irregular villi with fjord-like invaginations.
p57 Immunohistochemistry	Loss of p57 staining in cytotrophoblast and villous stromal cells.	No loss of p57 staining.
Risk of GTN progression	15-20%	0.5-1%

Obstetric management of molar pregnancy involves uterine evacuation and histopathological examination of the products of conception (POC). Follow-up serum or urine hCG monitoring is performed until hCG values return to normal. hCG is an ideal biomarker for GTD surveillance as its concentration accurately reflects disease burden. Most women with molar pregnancy are cured following uterine evacuation but some women develop disease persistence and progress to malignant disease requiring chemotherapy or further surgical intervention.

The diagnosis of GTN is largely based on a combination of obstetric history and elevated hCG levels.(193) Following molar pregnancy, a plateaued or rising hCG level is indicative of GTN. All forms of GTN (excluding PSTT and ETT) are highly vascular and biopsy is not recommended due to the risk of life-threatening haemorrhage. Identifying the origin of choriocarcinoma can be challenging and genetic profiling can help differentiate gestational from non-gestational choriocarcinoma, with the latter having a worse prognosis.

2.5.1 Twin Pregnancy

A twin pregnancy of a CHM with a co-existing viable fetus is rare occurring in 1 in 20,000-100,000 pregnancies.(110) In these cases, the most common combination is a CHM and normal fetus.(194) A UK study of 77 twin CHM pregnancies reported a livebirth rate of 40% and found that the risk of GTN did not increase beyond the first trimester obviating the need for early termination of pregnancy.(110) A subsequent study at that centre reported higher livebirth rates (51%) and a slightly higher malignancy risk in CHM twin (20.3%) compared to CHM singleton (16%) pregnancies. (195) Another larger multicentre study reported a higher malignancy risk (27%) in CHM twin pregnancies.(196,197) A systematic review of obstetric outcomes in CHM twin pregnancies revealed a higher risk of perinatal complications and low live birth weight with a third progressing to GTN.(111) Multidisciplinary management of these pregnancies in specialist centres is recommended to ensure early detection of pregnancy complications.(27)

2.5.2 “Risk factors” for the development of GTD

Potential risk factors for the development of molar pregnancy include ethnicity, maternal age, and prior HM.(31,32,198) Molar pregnancy is more prevalent in women at the extremes of reproductive age (<16 and >40 years). Adolescents have a slightly increased

risk (1:450) of molar pregnancy which increases to 1:157 (aged 40 years) and rises sharply to 1:8 (aged ≥50 years).(31,179,198) Most molar pregnancies are sporadic, but a prior history of HM increases the risk of a subsequent mole which is often the same type as the index mole.(33,199,200) Prior history of HM is associated with a ~1% risk of a further mole and this risk is associated with CHM rather than PHM. The risk increases to 15-20% after two HMs.(33,91) Of note, the risk is independent of male partner suggesting an underlying defect in oocyte function.(34) Supporting this concept, some recurrent HMs have an inherited methylation defect in a gene (*NLRP7*) associated with oocyte maturation and placental development.(201,202) There is also evidence of oocyte defects in other species. Female mice with an inherited meiotic abnormality in their oocytes (*MEI1*) produce androgenetic zygotes by extruding all maternal chromosomes and their spindles into the first polar body and a similar mechanism may exist in humans.(55)

Parity may be a potential risk factor for the development of post-molar GTN. Recent evidence suggests that women who have early diagnosis of PHM as their first gestational event are more likely to develop post molar GTN.(203) Studies have postulated a link between deficiency of carotene (vitamin A precursor) and a higher incidence of CHM in certain countries.(36,204) Animal studies have also shown that diet can reset the genetic imprint.(205) Deficiency of vitamin A and/or folates in early gestation (18-21days) is associated with absence of placental villous vascularity, as seen in CHM.(5) Hispanic women have a significantly lower risk of developing GTN after CHM suggesting a role for protective environmental or genetic factors.(206)

2.6 Genetic classification of hydatidiform mole

The genetic origin of HM is complex and is illustrated in Figure 2.2. CHMs have a purely androgenetic genome. They contain 46 chromosomes (diploid) with two copies of the paternal genome (diandric) and no contribution from the maternal genome. Heterozygous dispermic CHM are clinically more aggressive then homozygous monospermic CHM and have a significantly higher risk of neoplastic transformation.(47) These moles have higher mean hCG levels and a 3-fold increased risk of progression to GTN.(37,47,56) Rare cases of tetraploid CHM also exist and require the same close surveillance as diploid CHM.(207) PHMs have 69 chromosomes (triploid) and contain two paternal (diandric) and one maternal (monogynic) genome. Rare triandric tetraploid

PHMs have been reported which appear to arise from fertilisation of an ovum by three different sperm. (43)

2.6.1 Familial Recurrent Hydatidiform Mole

Familial recurrent hydatidiform mole (FRHM) is a rare autosomal recessive inherited disorder associated with a predisposition to molar pregnancy.(208) These recurrent CHMs have a diploid biparental origin with biallelic variants in maternal effect genes (*NLRP7* and *KHDC3L*) which disrupt genomic imprinting.(58,59). These genes are expressed in the oocyte and support embryonic development until gene expression in the embryo becomes active.(58) In Mexico, there is strong evidence of a founder effect in *NLRP7* (L750V) with most cases occurring in consanguineous families.(209) Familial recurrent CHM should be suspected in women presenting with two CHMs and genetic analysis should be requested to determine recurrence risk. (210) Women with FRHM usually require in-vitro fertilisation (IVF) with ovum donation to help them achieve a normal pregnancy.(201) Notably, a recent UK study found that 1 in 640 women registered with a complete mole had familial recurrent CHM.(33)

2.6.2 Origin of Hydatidiform Mole Formation

Genomic imprinting is a term used to describe the parental specific expression of certain genes. Some genes are "imprinted" (suppressed) when paternally inherited and expressed when maternally inherited and vice-versa. The "parental conflict hypothesis" was put forward to explain genomic imprinting in the placenta, whereby placental specific genes are paternally imprinted and maternally expressed.(211) Consistent with this hypothesis, the lack of a maternal genome and overrepresentation of the paternal genome in CHM results in impaired genomic imprinting and proliferation of the villous trophoblast.(212)

Several theories have emerged to explain the origin of HMs. One theory is that complete moles originate from an empty ovum which is fertilised by one or two sperm (Figure 2.2).(213) However, the finding of rare CHMs with retained maternal chromosome 11 suggests that the ovum is not completely devoid of all maternal DNA.(212) An alternative theory proposed by Golubovsky postulates that all HMs originate from the fertilisation of a normal ovum by two 2 sperm to create a triploid conceptus. A complex post-zygotic

event then excludes the maternal genome and results in diploidisation to create a CHM.(45,213)

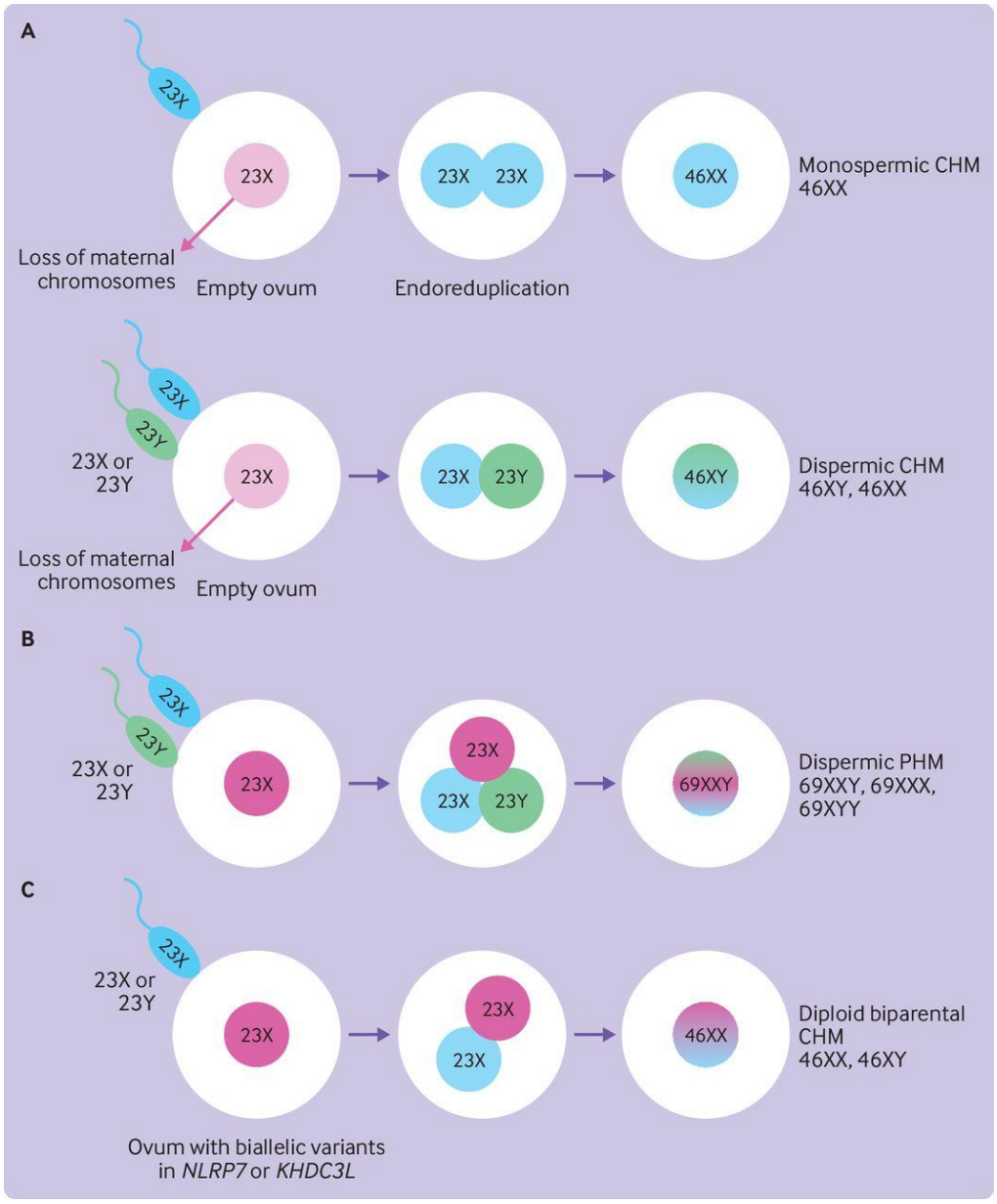

Figure 2. 2: Genetic origin of Hydatidiform Moles

A) CHMs have a diploid genome. Most CHMs (80-90%) arise from fertilisation of an "empty" ovum by a haploid sperm which undergoes endoreduplication to achieve diploidy (homozygous monospermy). Some CHMs (10-20%) result from fertilisation of the "empty" ovum by two sperm resulting in diploidy (heterozygous dispermy) B) PHMs have a triploid genome. Most PHMs (>95%) arise from fertilisation of a single oocyte by two different sperm (heterozygous dispermic). C) FRHM due to biallelic variants in maternal effect genes (NLRP7 or KHDC3L) results in biparental recurrent CHM.

Note: Haploid sperm may be 23X or 23Y but are only shown as 23X in these Figures.

2.7 Pathological classification

According to the World Health Organisation (WHO) 2020 Classification of Female Genital Tumours, GTD can be subdivided into molar pregnancies/hydatidiform moles, GTN, tumour-like (non-neoplastic) lesions and abnormal (non-molar) villous lesions.(8) The pathogenesis of GTD is unique as the maternal tumour arises from gestational tissue rather than maternal tissue.(3) Histopathological classification of POCs into CHM, PHM and non-molar gestations can be challenging based on morphology alone and ancillary techniques (e.g. immunohistochemistry, ploidy analysis, molecular genotyping) are used to aid diagnosis.

Placental site nodule (PSN) and exaggerated placental site reaction (EPSR) are classified as benign tumour-like trophoblastic lesions. These lesions usually present as incidental findings in tissue following hysterectomy or endometrial biopsy. PSN are placental remnants left in the uterine wall which fail to fully regress and disappear following a normal pregnancy.(193) Larger PSN-like lesions with atypical histopathological features are described as atypical placental site nodule (APSN). A recent UK study recorded neoplastic transformation in 14% of APSNs within 16 months of diagnosis.(10) As a result, APSN cases are registered with GTD centres, have central pathology review, hCG monitoring and women are offered a hysterectomy if deemed appropriate.(193)

Choriocarcinoma can be gestational or non-gestational and both have different routes of metastasis and treatment regimens. Non-gestational choriocarcinoma can be somatic or germ cell-derived and has a high propensity for metastasis.(214) Accurate classification of non-gestational choriocarcinomas is achieved using molecular genotyping to confirm the absence of paternal DNA.(215)

The diagnosis of CHM, PHM, PSN and APSN is made on the basis of histopathological confirmation.(27) In contrast, diagnosis of GTN does not usually rely on histopathological confirmation as biopsies are not always available. The treatment of invasive mole and choriocarcinoma is often initiated on the basis of a rising hCG level even in the absence of other clinical evidence of disease recurrence.(78)

2.8 Pathological diagnosis

In the past, molar pregnancies presented later in gestation with full expression of their various morphological features. Earlier clinical diagnosis of GTD due to improved ultrasound sensitivity often makes pathological differentiation of early CHM and PHM from non-molar gestations more challenging. The features of mole may be more subtle in early molar and non-molar pregnancies, which are often both genetically abnormal, and show morphological appearances that overlap to varying degrees. As a result, ancillary techniques are often required to assist morphological diagnosis.

Morphologically, CHM is characterised by the presence of a diffuse population of enlarged, hydropic villi with cistern formation. Fetal tissue is generally absent. Villi are irregular with formation of trophoblast pseudoinclusions. There is prominent non-polar or circumferential proliferation of villous trophoblast and the included extravillous trophoblast shows cytological atypia (Figure 2.3). In CHM presenting earlier in gestation the hydropic changes, villous enlargement and trophoblast proliferation may be less developed and, in these cases, useful diagnostic features include the presence of villi with bulbous, “cauliflower-like” or “knuckle-like” outlines. These villi often have abnormal dense, blueish, myxoid stroma with prominent karyorrhectic nuclear debris.(69,212)

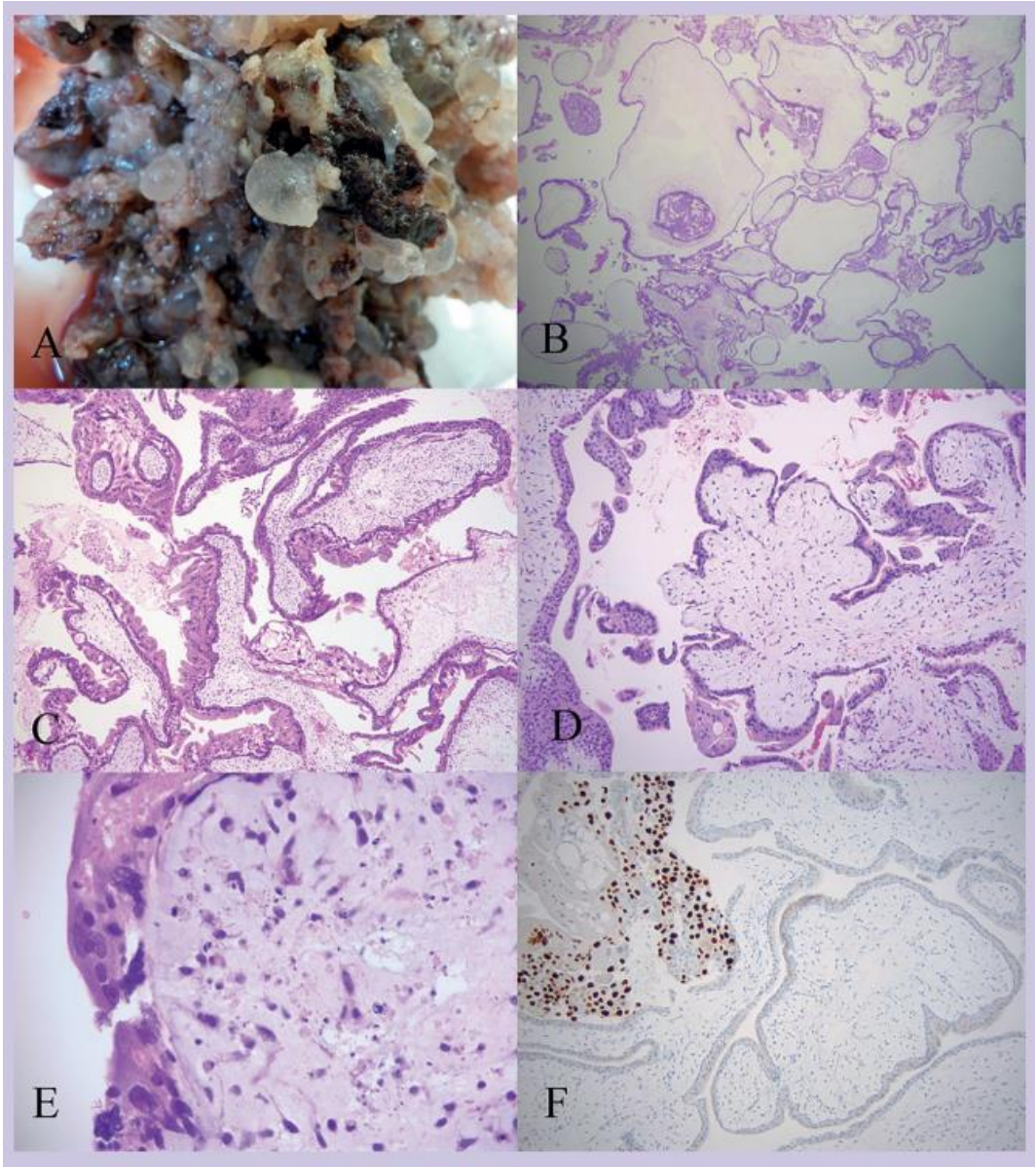

Figure 2. 3: Complete Hydatidiform Mole.
A) In this gross image of a POC, extensive formation of vesicles is evident giving a bunch of grapes effect. B) The villous population is diffusely abnormal with numerous enlarged hydropic villi. C) The villi in this view are highly irregular and most show non-polar or circumferential trophoblast hyperplasia. D) In this early CHM the central villous shows the typical cauliflower-like outline with bulbous, knuckle-like projections. E) On high power of this early CHM the abnormal, dense, myxoid stroma is evident with prominent stromal karyorrhexis. F) p57 immunohistochemistry shows loss of staining in villous cytotrophoblast and stromal cells with preservation of staining in extravillous trophoblast (upper left).

In contrast to CHM, PHM is characterised by a mixed population of enlarged hydropic villi with some cisterns and small more normal appearing or fibriotic villi. An abnormal fetus may be present. Villous outlines are irregular and scalloped with "fjord-like" indentations and formation of trophoblast pseudoinclusions (Figure 2.4). Villous trophoblast abnormalities, in contrast to CHM, are less prominent with focal non-polar or circumferential trophoblast hyperplasia.(69,212)

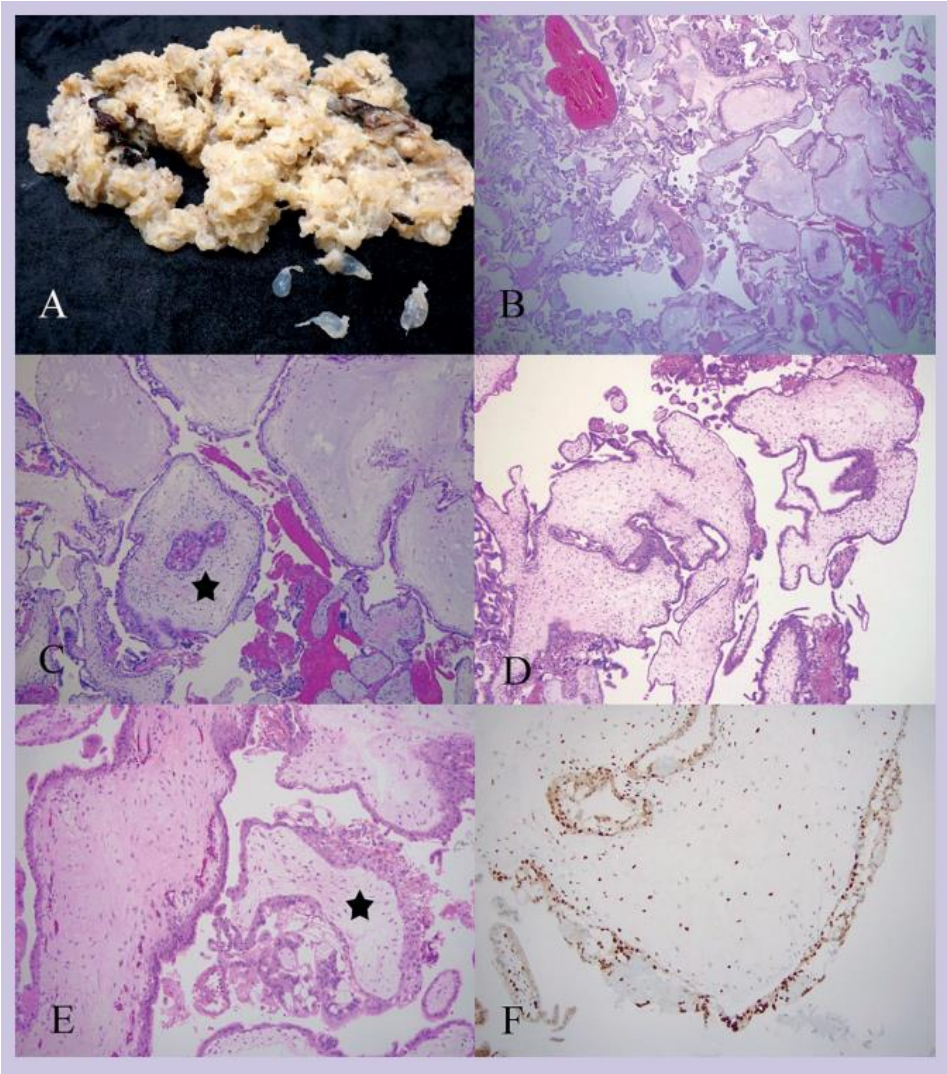

Figure 2. 4: Partial Hydatidiform Mole.
A) In this gross image of a PHM, focal vesicle formation is evident with some vesicles separating from the specimen at bottom right. B) This low power view shows a mixture of villous types with a group of hydropic villi seen on the right and smaller, more normal sized villi on the left. C) The villus with a star has a trophoblast pseudoinclusion. D) The PHM villi are markedly irregular with fjord-like indentations. E) There is focal non-polar trophoblast hyperplasia affecting the villus with the star while the surrounding villi show little or no hyperplasia. F) p57 immunohistochemistry is normal.

Mimics of molar pregnancy show one or more of the above features and thus need to be distinguished from hydatidiform mole based on their more limited morphological features of mole or use of ancillary techniques. Hydropic abortions are characterised by a diffuse population of hydropic villi but they are generally regular in outline and show no significant trophoblast hyperplasia. Non-molar POCs that show morphological abnormalities termed “abnormal villous morphology” (AVM) may mimic molar pregnancy and may have some of the features of a mole such as small trophoblast pseudoinclusions or irregular villous outlines or focal abnormal villous trophoblast hyperplasia. Some specimens with AVM may result from genetic abnormalities such as aneuploidy (Figure 2.5).(216)

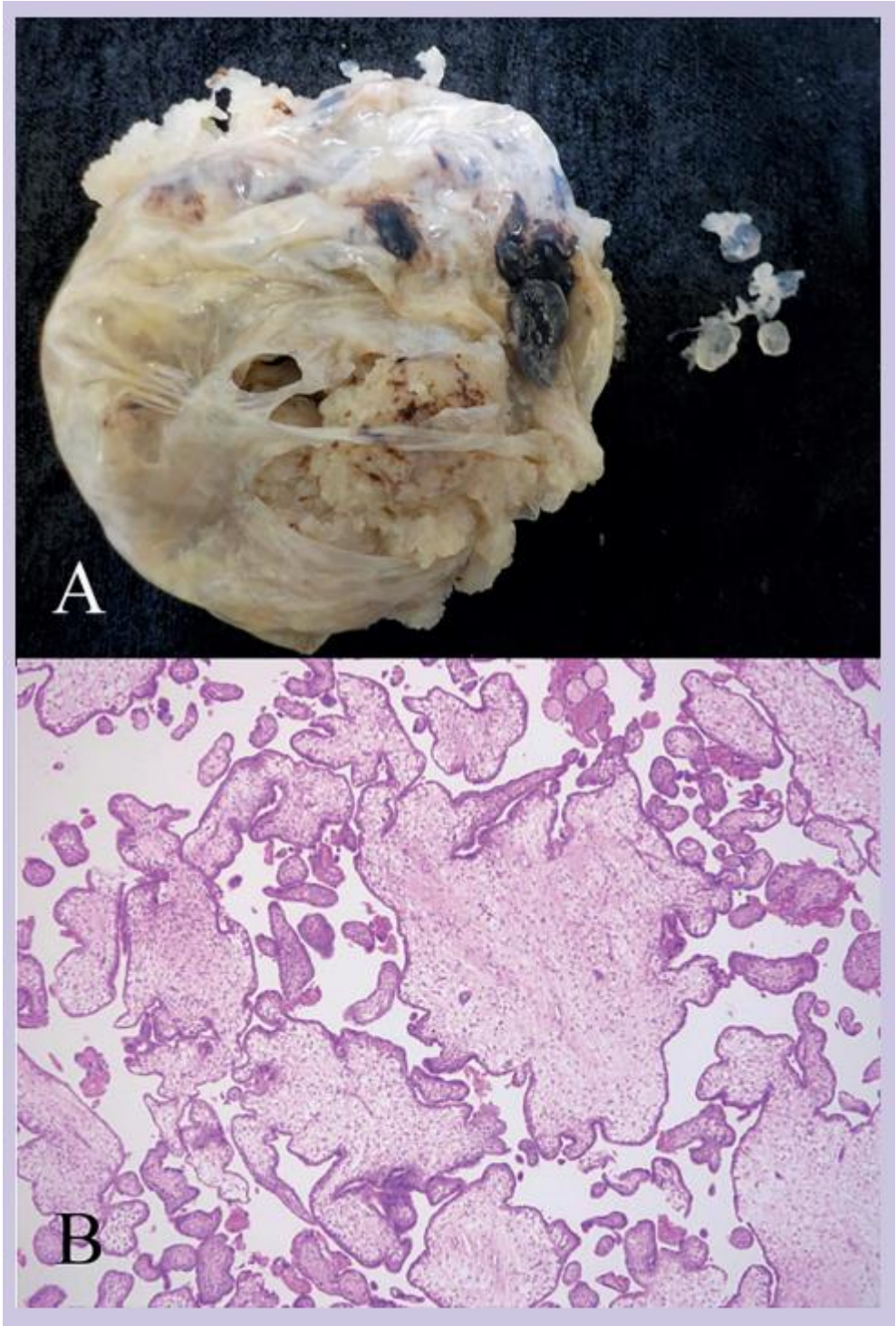

Figure 2. 5: Abnormal villous morphology.
A) This second trimester placenta has a number of clear vesicles becoming detached from the specimen. B) On microscopy of this placenta there was abnormal villous morphology with villous enlargement, some irregularity in villous outlines and small pseudoinclusions. This case was confirmed on microarray of fresh tissue to be an example of mosaicism for monosomy X (Turner syndrome).

Immunohistochemistry (IHC), ploidy analysis using cytogenetics, flow cytometry, or fluorescent in-situ hybridisation (FISH) and molecular genotyping can help to distinguish moles from the above mimics and improve the accuracy of GTD diagnosis. Ploidy analysis using cytogenetics may also identify aneuploidies which can help explain a particular pregnancy loss.

2.8.1 p57 Immunohistochemistry

The protein p57 is a cyclin-dependent kinase inhibitor encoded by the gene *CDKN1C* ($p57^{kip2}$) on chromosome 11p15.5. This gene is paternally imprinted and maternally expressed. When the maternal genome is present (non-molar POC or PHM), p57 is expressed in placental villi and stromal cells and cytotrophoblast stain positively. In complete moles (diploid diandric), p57 is not expressed in cytotrophoblast and villous stroma and staining in these cells is lost. Staining is however preserved in the decidua and extravillous trophoblast which act as internal controls for p57 IHC. (57) As a readily available test, p57 immunostaining can support the morphological diagnosis of CHM in routine practice.

Rarely other challenges in the interpretation of p57 IHC arise (e.g. when a maternal chromosome 11 is retained in a CHM and it continues to expresses p57 or a maternal chromosome 11 is lost in a PHM and p57 immunostaining is lost.(46,69) Further ancillary testing and close correlation with morphology is required to prevent misdiagnosis in these cases.

One category of morphologically abnormal POC in the differential diagnosis of molar pregnancy results from androgenetic/biparental mosaicism which may be recognised by their unusual patterns of p57 staining.(61,71) In p57 discordant villi the villous stroma cells are p57 negative but the cytotrophoblast are p57 positive (Figure 2.6). Inverted p57 discordant villi have the reverse pattern where the cytotrophoblast are negative and stromal cells are positive. Staining may also be divergent where two or more populations of villi exist with different staining patterns.(71)

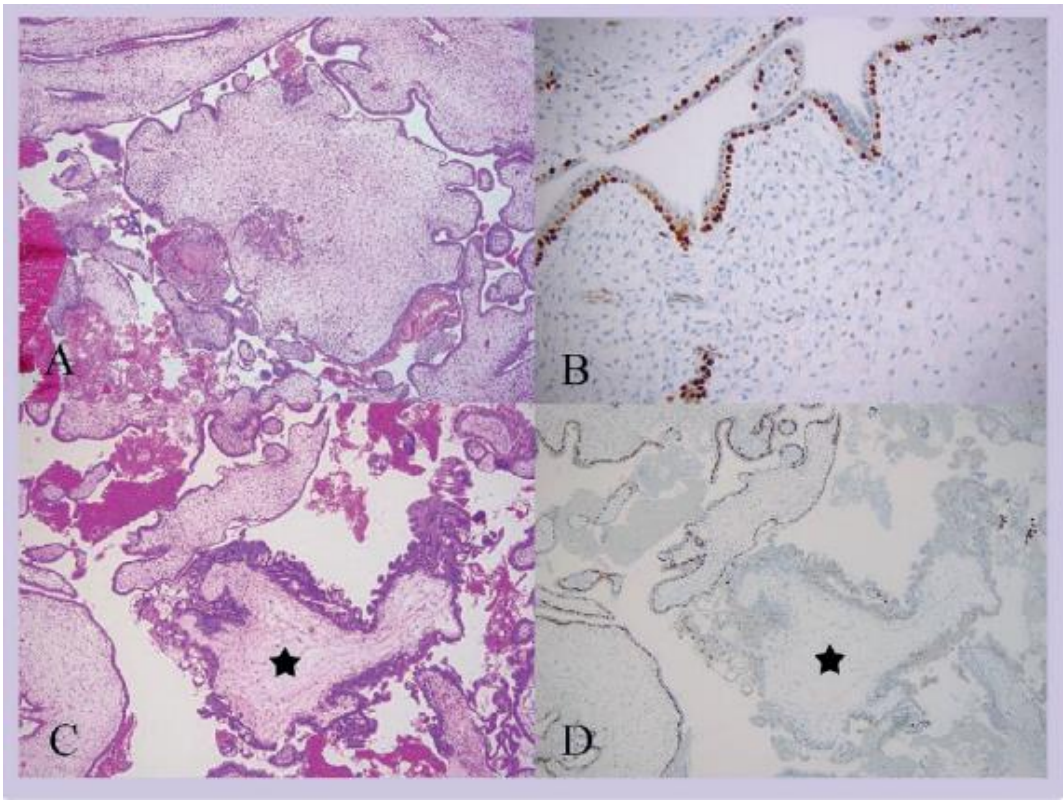

Figure 2. 6: p57 discordant villa
A) The low power view shows abnormal villous morphology with enlarged, hypercellular villi and a trophoblast pseudoinclusion but no trophoblast hyperplasia. B) Immunohistochemistry for p57 shows a discordant pattern with positive cytotrophoblast and negative stromal cells. C) Careful examination of the specimen identified a small focus of villi with trophoblast hyperplasia (star). D) This focus of villi showed absence of P57 staining in stroma and cytotrophoblast in keeping with a component of CHM.

While they are uncommon, it is important to be aware of these types of POC as they may harbour a component villous population that is purely androgenetic and therefore a CHM and thus require the appropriate clinical follow-up.(71) In addition to their representation in early pregnancy POC samples, these patterns may be seen in the second and third trimester in placental mesenchymal dysplasia (p57 discordant villi)(217,218) and as a focal finding in otherwise normal placentas (inverted p57 discordant villi).(219)

While p57 assists in the differential diagnosis of CHM, ploidy analysis is frequently used to support morphology and confirm or exclude a triploid conceptus in the differential diagnosis of PHM. However, it will not distinguish between triploidy with two paternal (diandric) or two maternal (digynic) contributions to the genome and thus must be used in combination with morphological assessment.

2.8.2 Molecular Genotyping

Molecular genotyping is considered the gold standard for HM as it can establish ploidy and identify the parental origin of the molar tissue. It can also help differentiate complete and partial moles from GTD mimics. Analysis of polymorphic short tandem repeat (STR) DNA sequences on multiple chromosomes in the human genome are used to determine genotype.(60) The STR profiles of the molar tissue are compared to those obtained from the maternal DNA from decidua to classify the mole. The clinical accuracy of STR genotyping to refine the diagnosis of GTD has been confirmed by a number of studies.(220,221) Some pitfalls in the use of molecular genotyping include the analysis of FRHMs which have a diploid biparental genome and may be misinterpreted as a non-molar gestation. In addition, an egg donor pregnancy which does not carry alleles from the recipient mother may be misinterpreted as a diandric complete mole. Close correlation of morphology, p57 immunostaining and genotyping is required to ensure the correct diagnosis is reached in all cases.(56)

2.9 Biochemical diagnosis

hCG is a member of the glycoprotein hormone family which includes three pituitary hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). These hormones are heterodimers which share a common alpha (α) subunit but have a unique hormone-specific beta (β) subunit. hCG is a heterogenous molecule with many different isoforms produced by complex post-translational modification. In early pregnancy, hyperglycosylated hCG is produced by the syncytiotrophoblasts of the placenta to promote growth and differentiation.(222) The hCG variants found in serum or urine include intact hCG (α and β) along with fragments of nicked hCG (hCGn), nicked hCGβ (hCGnβ) and hCG beta core fragment (hCGβcf).(96) These five biologically active hCG isoforms are clinically relevant in gestational trophoblastic disease.(90)

2.9.1 hCG assays

Most commercial immunoassays were developed for the detection of hCG in early pregnancy. Total hCG assays detect intact and free β-subunit (β-hCG) but will not necessarily detect all isoforms secreted in GTD.(75,79) There is no international standardised hCG assay approved for GTD. Ideally hCG should be monitored with an assay

that detects all hCG isoforms in equimolar amounts.(77,82) GTD reference centres should have access to at least two hCG assays, one for primary analysis and another for confirmatory diagnosis. Several factors contribute to the variability in hCG assays including assay calibration, analyte specificity and antibody heterogeneity. In the absence of centralised hCG monitoring, hCG should be measured using the same assay and analytical platform throughout follow-up to avoid inter-assay variability.(79)

In molar pregnancy, hCG is monitored post-operatively until normalization is achieved. Despite EOTTD consensus on what constitutes "normalization", the decision thresholds used to define normality are assay dependent and influenced by the functional sensitivity of the assay and confidence intervals applied.(100) Thus, the surveillance period may be prolonged in women monitored in a centre using a cut-off <1 IU/L as opposed to the commonly quoted cut-off <5 IU/L for pre-menopausal (non-pregnant) women.

2.9.2 Immunoassay Interference

All tumour markers measured by immunoassays are subject to false positive and false negative results from analytical or biological interference. Analytical interference due to the presence of heterophilic antibodies (e.g., anti-mouse antibodies) in serum usually produces false positive results. A paired urine hCG measurement will not be subject to interference as heterophilic antibodies are retained in the kidney. Non-linear dilution of serum samples may also confirm antibody interference.(155) Heterophilic blocking tubes or polyethylene glycol (PEG) precipitation may be used to remove antibody interference in serum.(98,223) Another common phenomenon in immunoassays called the "high dose hook effect" occurs when very high levels of hCG (>500,000 IU/L) generate a falsely low result.(224) This occurs when excess analyte (hCG) saturates the assay antibodies and results in underestimation of analyte concentration. Dilution of the patient's serum will bring the analyte concentration back into the measuring range of the assay and allow accurate quantitation. Clinicians and scientists need to be aware of the limitations of their hCG assay and ensure that suspicious results not fitting the clinical picture are discussed. Further investigations may involve using an alternate assay or sending the sample for reanalysis to a GTD reference centre.

2.9.3 Persistent low-level elevated hCG

Persistent low levels of elevated hCG may indicate disease recurrence or lack of response to treatment. In addition, some women with quiescent GTD have persistently low levels of hCG without clinical or radiological evidence of disease.(99) Diagnostic interpretation may also be complicated by a pregnancy or the presence of pituitary derived-hCG in post-menopausal women. hCG may be normally elevated up to 14 U/L in women ≥55 years of age.(225) Snyder et al. provide an algorithm for investigating pituitary-derived hCG and the European Organisation for the Treatment of Trophoblastic Disease (EOTTD) practical clinical guidelines suggest further ways to investigate persistent low level hCG elevations.(86,100) Familial hCG, a rare inherited form of persistent elevated non-functional hCG may also complicate hCG monitoring.(101)

Inaccurate serum hCG results can have serious adverse consequences for patient management. Cole et al. reported a series of twelve women diagnosed with gestational choriocarcinoma due to false positive hCG results.(226) Seven of these women had either major surgery or chemotherapy and five had procedures which resulted in loss of fertility. Concurrent testing of serum and urine hCG in a GTD reference centre resolved all cases with false positives results due to circulating heterophilic antibodies.(226)

2.10 Clinical guidelines

In the absence of randomised controlled trial data for this rare disease, systematic reviews and consensus expert opinion have informed the development of international clinical GTD guidelines.(122,137) Clinical practice guidelines have been generated by a number of organisations including the Royal College of Obstetrics and Gynaecologists (RCOG), European Society for Medical Oncology (ESMO) and EOTTD.(27,91,100)

The EOTTD clinical working party recently published practical clinical guidelines for GTD in an effort to harmonise practice across Europe.(100) This guideline advises when women should be referred to specialist GTD centres and where expert advice is available. They recommend a centralised model of care with central hCG monitoring and pathology review as there is evidence that centralised pathology review alters the diagnosis in 40% of cases.(227) Management of women with GTD by physicians experienced in trophoblastic disease has also resulted in better survival outcomes.(123,228)

A recent update to the RCOG clinical guidelines for GTD recommends registration of all affected women with a GTD centre as a minimum standard of care.(27) The Irish clinical guidelines endorse this recommendation and advise centralisation of hCG measurement in a quality assured laboratory using an oncology approved assay.(108) All guidelines recommend hCG monitoring of women post-molar pregnancy to enable early detection of disease persistence.

A large retrospective UK study of 20,000 women who had hCG monitoring post-molar pregnancy found the risk of GTN after hCG normalisation was 0.25% for CHM and 0.03% for PHM.(109) A systematic review reported a slightly higher risk of GTN (0.35%) following hCG normalisation in CHM.(229) Another study found that prolonged hCG surveillance particularly after PHM is not cost-effective given the rarity of GTN.(230) Consequently, the RCOG guidelines recommend a shorter hCG surveillance period after PHM with monitoring complete once two normal hCG values are obtained, one-month apart. Complete moles have a longer surveillance period reflecting their higher risk of GTN. In CHM cases where hCG normalisation occurs within 56 days (8 weeks), women have hCG monitoring for a total of 6 months post uterine evacuation. However, when hCG normalisation occurs beyond 56 days, women have hCG monitoring for 6 months post normalisation. (Figure 2.7).(27,108,109) Another update in the RCOG guideline concerns women who did not require chemotherapy post-molar pregnancy. These women no longer require a post-pregnancy hCG test or histopathological examination of their placental tissue after future normal pregnancies.(231)

Women with invasive mole or choriocarcinoma are stratified into low or high risk GTN based on the Federation of Gynaecology and Obstetrics (FIGO) staging and modified WHO prognostic scoring system (Table 2.2).

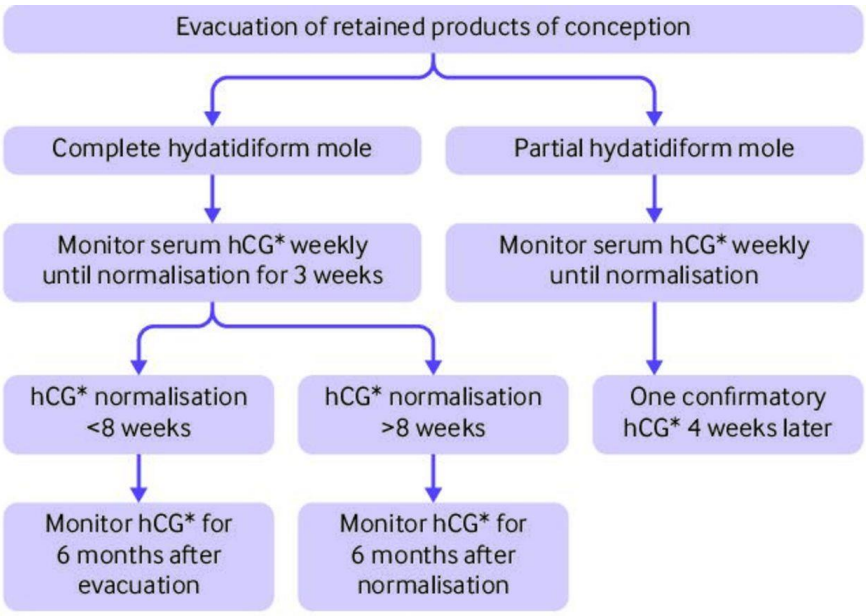

Figure 2. 7: hCG Monitoring Protocol for Complete and Partial Hydatidiform Moles.
*hCG monitoring is performed on the same analytical platform throughout follow-up. Adapted from RCOG guidelines.(27) Reproduced with permission.(232)

Table 2.2: FIGO staging and WHO modified prognostic scoring system for GTN

FIGO stage	Description			
I	Gestational trophoblastic tumours strictly confined to the uterine corpus			
II	Gestational trophoblastic tumours extending to the adnexa or to the vagina, but limited to the genital structures			
III	Gestational trophoblastic tumours extending to the lungs with or without genital tract involvement			
IV	All other metastatic sites			
Risk scores	**0**	**1**	**2**	**4**
Age (years)	<40	>40	-	-
Antecedent Pregnancy	Mole	Abortion	Term	
Interval from index pregnancy, months	<4	4-6	7-12	>12
Pretreatment hCG mIU/ml	$<10^3$	$>10^3$-10^4	$>10^4$-10^5	$>10^5$
Largest tumour size including uterus[a], cm	-	3-4	≥5	-
Site of metastases including uterus	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

To stage and allot a risk factor score, a patient's diagnosis is allocated to a stage as represented by a Roman numeral I, II, III and IV. This is then separated by a colon from the sum of all the actual risk factor scores expressed in Arabic numerals, e.g. Stage I:4, Stage IV:9. This stage and score will be allotted for each patient. [a]Size of the tumour in the uterus. Reproduced with permission(233)

The FIGO scoring system is endorsed by all international GTD guidelines and revolves around three main measures: post-evacuation hCG concentration, presence of metastatic disease and histopathological diagnosis. A doppler pelvic ultrasound should be performed to confirm the absence of a pregnancy and ascertain the size of any intrauterine tumour.

Chest X-ray (CXR) as opposed to computed tomography (CT) is the preferred imaging modality for detection of pulmonary metastases.(91) Some centres consider a hCG concentration ≥20,000 U/L four weeks after uterine evacuation as an indication for immediate chemotherapy but this has not been adopted by FIGO.(234) Genomics may also be incorporated into future updates to the FIGO scoring system to reflect its critical role in identifying the antecedent pregnancy for GTN.

Women with low-risk disease (score ≤6) without metastatic disease are offered single agent chemotherapy or hysterectomy. While those with high-risk disease (score ≥7) are offered multi-agent chemotherapy, ideally under the supervision of an expert in GTD management. In the event of drug resistance or disease-related complications, surgery may be considered. Women with ultra-high-risk disease (score >12) generally present with liver or brain metastases and require specialist multidisciplinary care. An adjustment to the FIGO scoring system is required to identify “low risk” women who become resistant to single-agent chemotherapy to enable them be treated at the outset with multi-agent chemotherapy.(177)

Women treated with chemotherapy post-molar pregnancy are advised to avoid pregnancy for at least a year when the risk of relapse is greatest (3%) and a rising hCG may prevent early detection of disease recurrence.(235) Advice on safe contraception after a molar pregnancy can be found in national fertility guidelines.(236) A systematic review found no evidence for an association between oral contraceptive use during post-molar follow up and the incidence of GTN. (237) Moreover, a Brazilian study found no association between hormonal contraception use during molar pregnancy follow-up or GTN treatment and the risk or severity of GTN, nor did it postpone the normalisation of hCG levels.(238) In a retrospective review of 1,532 women treated with chemotherapy, 230 became pregnant within 12-months of finishing chemotherapy and 5 of these relapsed. However, the relapse rate in women post-chemotherapy was not higher in women who became pregnant. The study also reported that single-agent chemotherapy had no effect on fetal outcomes but that multi-agent chemotherapy may have a transient effect on fertility and increase the risk of miscarriage.(239)

Importantly, all guidelines recommend that GTN be considered in the differential diagnosis of women who present with irregular vaginal bleeding post pregnancy and that serum hCG be performed as part of the diagnostic work-up.(16)

2.11 Advances in diagnostics and therapeutics

Molecular genotyping now plays a central role in establishing the genomic origin of trophoblastic tumours and informs prognosis and treatment options. When tumour tissue is inaccessible, liquid biopsy can provide a non-invasive method of analysing circulating tumour DNA (ctDNA) in maternal blood to confirm the genetic origin of choriocarcinoma.(172) In a study of 20 women with GTN, STR analysis of ctDNA provided a genetic diagnosis in all but three cases. The cases without a diagnosis had low levels of ctDNA and concurrent low hCG levels reflecting a low tumour burden. Digital droplet PCR combined with single nucleotide polymorphism (SNP) analysis may provide a more sensitive technique for such cases.(170)

The use of circulating gestational trophoblasts (cGTs) to establish the genetic origin of trophoblastic disease shows promise. A comparative study of ctDNA with cGTs found the latter superior in confirming a diploid androgenetic conceptus.(173) Use of cGTs has the advantage of using single cells to allow better discrimination of mosaicism and lacks maternal DNA which simplifies result interpretation.

There is evidence that matrix metalloproteinases (MMPs) which facilitate extra-cellular matrix degradation may allow malignant trophoblast cells invade the maternal uterus.(240) The high expression of MMPs and low expression of their inhibitors in choriocarcinoma may explain its invasiveness and malignant potential.(7,241) Similarly, the wingless (Wnt) signalling pathway which regulates placental cell migration may be implicated in trophoblastic disease. Methylation-based silencing of Wnt signalling inhibitors could enable Wnt hyperactivation and facilitate trophoblast invasion seen in CHM and choriocarcinoma.(242) Drugs that inhibit MMP or downregulate Wnt signalling may provide useful future therapeutic options.

High levels of the angiogenic factor BMP-9 (bone morphogenetic Protein-9) have been associated with chemoresistance to primary methotrexate therapy. The combined use of serum BMP-9 levels with an ultrasound biomarker for tumour vascularity (uterine

pulsatile index) shows promise in helping predict which women will develop methotrexate resistance.(157) Algorithms using hCG regression nomograms to predict early chemoresistance may prove useful but they are assay specific and not widely adopted.(162)

In recent years, use of checkpoint inhibitor immunotherapy (e.g. Pembrolizumab) for chemo-resistant ultra-high risk GTN has had some success.(243) Immunotherapy targets the T-cell receptor, PD-L1 which is highly expressed on normal trophoblasts and all forms of GTN.(177,243) The presence of tumour infiltrating lymphocytes (TILs) may serve as a biomarker to select women who may respond to Pembrolizumab.(201) Another PD-L1 inhibitor, Avelumab is safe and effective in GTN cases resistant to single-agent chemotherapy.(244) An alternative salvage therapy for chemo resistant GTN involves use of Camrelizumab plus apatinib.(148) Current clinical trials are evaluating the use of checkpoint inhibitors alone or in combination with chemotherapy to target choriocarcinoma.(122)

Women with low-risk GTN (FIGO score 5-6) who have a high chance of resistance to first line therapy (Methotrexate or Actinomycin-D) could be risk stratified to combination therapy based on prognostic factors (pre-treatment hCG, metastatic disease status and choriocarcinoma histopathology).(144) *In vitro* and animal studies in methotrexate resistant choriocarcinoma cell lines identified cell cycle (CDK4) and DNA damage repair (ATR) pathways which could be targeted in women with methotrexate resistance using ATR or CDK4/6 inhibitors.(159)

2.12 Impact of diagnosis on women and their families

Despite excellent cure rates, the psychosocial consequences of GTD are complex and clinicians need to be mindful of the need for counselling and psychological support for these women. Cancer-specific distress, future fertility fears, mood and sexual disturbances can persist for years in affected women.(7,245) A systematic review of health-related quality of life outcomes in women with GTD found clinically significant levels of anxiety, depression, sexual dysfunction and fertility-related distress, especially in women treated for GTN.(116) In a recent prospective study of the psychological impact of GTD on women, 47% reported feeling anxious and 70% reported feeling distressed

during the surveillance period.(115) Unfortunately, this study did not record prior mental health status of the participants.(246,247) A survey of women on the Irish national GTD registry found that women experienced feelings of intense sadness at the time of diagnosis and needed psychological support to help them deal with the pregnancy loss. The need for information leaflets, psychological counselling, bereavement care guidance and peer support was greatest in the first year after diagnosis.(232) A fear of disease recurrence and concerns about future pregnancies accounted for some of the psychological distress experienced by these women.(116,248,249). Healthcare professionals treating affected women need to be mindful of the psychological wellbeing of women and their partners as a consequence of GTD and be alert to their need for supportive services.

2.13 Conclusion

The prognosis for women following molar pregnancy is excellent but there is still some uncertainty around the aetiology of GTD, risk factors that cause malignant transformation and the optimum period of surveillance monitoring. The increased use of molecular genotyping has improved the diagnostic accuracy of GTD classification which is critical for prognostic stratification. Further work is needed to standardise hCG assays and identify those most appropriate for use in oncology. At this time, there is no effective prognostic biomarker to specifically identify those few women who will develop malignancy after molar pregnancy and require chemotherapy.

There have been significant improvements in the treatment options for GTN over the last decade with most women now cured and salvage treatment pathways available for those who develop chemoresistance. As our understanding of GTD evolves, we may identify more sensitive biomarkers to detect disease progression earlier and reduce the current lengthy surveillance period which impacts future pregnancy planning. Early identification of women with GTD remains of paramount importance as this disorder is highly curable.

Contributors: CJ developed the review outline, performed the literature review and wrote all sections of the manuscript. KOD developed the review outline, reviewed the manuscript and is supervising CJ for her PhD. BF provided the pathology images and

critically reviewed the manuscript. TMC reviewed and edited the manuscript and is supervising CJ for her PhD. JC reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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Chapter 3 – Experience of women on the Irish National Gestational Trophoblastic Disease Registry

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Chapter 3 - Experience of women on the Irish National Gestational Trophoblastic Disease Registry

3.1 Abstract

Objective: Gestational trophoblastic disease (GTD) is a rare pregnancy related disorder and the most curable of all gynaecological malignancies. GTD comprises the premalignant conditions of complete or partial hydatidiform mole known as molar pregnancy and a spectrum of malignant disorders termed gestational trophoblastic neoplasia. Clinical management and treatment in specialist centres is essential to achieve high cure rates and clinical guidelines recommend registration with a GTD centre as a minimum standard of care. National GTD registries are valuable repositories of epidemiological data and facilitate clinical audit, centralised pathology review and human chorionic gonadotropin (hCG) monitoring. This study sought the opinion of women enrolled on the Irish National GTD registry to inform future service development and establish a knowledge base for molar pregnancy in Ireland.

Study Design: A cross-sectional survey using an anonymised questionnaire was distributed by post to all women on the GTD registry. The questionnaire was designed by a multidisciplinary team and consisted of twenty-five closed-ended questions and two open-ended questions to facilitate feedback. Data collected in the survey included information on the patient experience of registration, knowledge of molar pregnancy, diagnosis at their local hospital, hCG monitoring and overall satisfaction with the service.

Results: The survey had a successful participation rate of 42.6% (215/504). Forty-nine percent (n=106) of respondents rated a rapid hCG result as their top priority. Forty percent (n= 84) of women had concerns about future pregnancies but acknowledged that these were largely addressed by the GTD specialist nurses. A quarter of respondents reported that other medical professionals with whom they interacted during follow-up treatment did not understand their condition. Many women commented on the emotional stress of attending their local maternity unit for phlebotomy while dealing with pregnancy loss.

Conclusion: This study is unique in being the first survey of women on the Irish National GTD registry. It highlights the specific needs of women with molar pregnancy in terms of psychological support, bereavement counselling and peer support groups. It reveals a

knowledge gap in molar pregnancy amongst healthcare professionals which should be considered in future planning of medical and nursing curricula.

Keywords: Pregnancy loss, Gestational Trophoblastic Disease, Molar Pregnancy, Human Chorionic Gonadotrophin

3.2 Introduction

Gestational Trophoblastic Disease (GTD) describes a group of rare gynaecological disorders characterised by abnormal proliferation of placental trophoblasts. GTD comprises the premalignant partial (PHM) and complete hydatidiform mole (CHM) known as molar pregnancy and a group of malignant disorders consisting of invasive mole, choriocarcinoma, placental site trophoblastic tumours (PSTT) and epithelioid trophoblastic tumours (ETT) termed gestational trophoblastic neoplasia (GTN). Incidence of GTD varies worldwide with a higher incidence in women of Asian descent.(181) The United Kingdom (UK) incidence of GTD is 1:714 live births.(22,27) In Ireland, the incidence of hydatidiform mole is 1:512 pregnancies (CHM 1:1945; PHM 1:695).(28) GTD affects women at the extremes of their reproductive age (<20 and >40 years) but reproductive function can be preserved with specialist care.(16,180,250,251)

A molar pregnancy often presents with vaginal bleeding in early pregnancy and is frequently suspected on ultrasound and supported by abnormally high concentrations of human chorionic gonadotrophin (hCG).(95) Following miscarriage or surgical removal of the products of conception, a molar pregnancy is confirmed by histological examination of the placenta.(27) Disease surveillance is performed using the biomarker hCG and a raised or persistently elevated hCG concentration can indicate progression to GTN.(252) During follow-up treatment, women attend scheduled phlebotomy to monitor hCG levels until normalisation is achieved as outlined in the International Federation of Gynaecology and Obstetrics (FIGO) Cancer Report 2018 (Figure 3.1).(100,253)

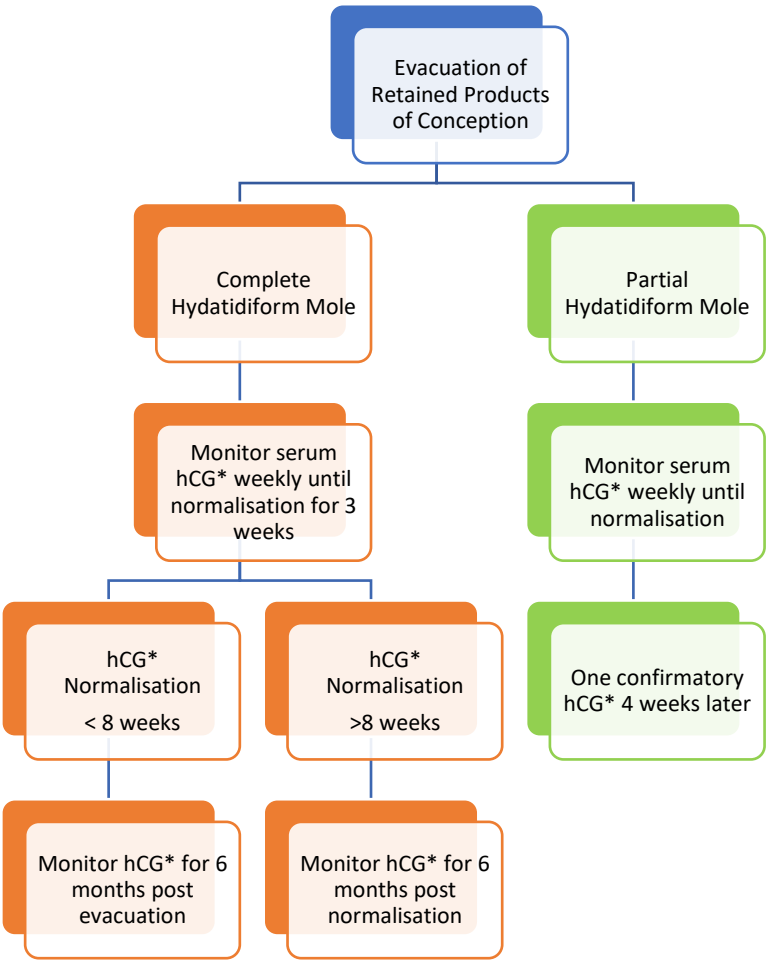

Figure 3. 1: hCG monitoring protocol for complete and partial hydatidiform mole, adapted from the FIGO guidelines.
*hCG monitoring is performed on the same analytical platform throughout follow-up.
hCG = Human Chorionic Gonadotrophin; FIGO = International Federation of Gynaecology and Obstetrics

GTN is a rare malignancy requiring expert care to ensure optimal clinical management and maximum cure rates.(16,124) Studies have shown that outcomes are better for women receiving clinical care from GTD centres.(27) Research shows that countries without centralised care have lower survival rates.(123) Clinical guidelines further support patient management in specialised centres with epidemiological data collected in registries to facilitate clinical audit.(21) The Royal College of Obstetricians and Gynaecologists (RCOG) clinical guideline for GTD management recommends registration of all affected women with a GTD centre, as a minimum standard of care.(27) The UK National GTD service

provides a central registry with multidisciplinary care, pathology review and hCG monitoring services spread across three designated centres: Dundee, Sheffield and Charing Cross.(254) In Ireland, the national clinical guideline for GTD recommends registration of affected women with a GTD centre.(255) The Irish National GTD Registry was established in 2017 as a monitoring and advisory centre. It co-ordinates patient care along with local clinicians and was notified of 130 molar pregnancies in 2020.(256) The GTD nurses organise regular hCG follow-up at local hospitals and contact patients with their results.

In the area of pregnancy loss, there is a paucity of information on the experiences of women with molar pregnancy. Previous surveys typically focused on the experience of women with miscarriage (257,258) and ectopic pregnancies.(259) This study evaluated the experience of Irish women enrolled on the national GTD registry to identify their specific needs and guide service development.

3.3 Materials and Methods

3.3.1 Questionnaire design

A 27-item questionnaire was developed by a multidisciplinary team consisting of a clinical biochemist, an obstetrician gynaecologist, a GTD specialist nurse, and the clinical director of the national GTD centre. Closed- ended questions containing checkboxes, rated, ranked and multiple-choice questions were provided with two open-ended questions for patient feedback (Appendix I). Registrants on the GTD centre are from different educational and socioeconomic backgrounds so questions were designed to be readily understood with minimum use of medical terminology. A patient representative from the national GTD guideline development group evaluated and advised on the questionnaire prior to finalisation which added further clarity. The questionnaire sought information on the following issues:

A. Registration with a GTD centre
B. Prior knowledge of Molar Pregnancy
C. Diagnosis of GTD at local hospital
D. Monitoring hCG levels
E. Patient experience

3.3.2 Study Participants

A letter from the clinical director was sent to all women on the GTD registry inviting them to partake and explaining the purpose of the study. Participation in the survey was voluntary and women consented to participate by completing the questionnaire and returning it in a stamped addressed envelope within six weeks of receipt.

3.3.3 Data Collection

Data from the questionnaire was recorded anonymously. A total of 429 questionnaires were distributed in November 2020 with a further 75 distributed to new registrants in July 2021 providing 504 potential respondents.

3.3.4 Statistical Analysis

Data was recorded and analysed using SPSS Statistics v28 (IBM Corp, Armonk, NY, USA). Descriptive analysis was performed using counts and percentages. “Valid percent” recorded in SPSS excluded missing data from unanswered questions in percentage calculations. Patient feedback in the open-ended questions was reviewed for content and responses were grouped into categories.

3.4 Results

3.4.1 Study Population

Characteristics of the study population are presented in Table 3.1. Over a ten-month study period, 504 questionnaires were successfully distributed by post and completed by 215 women giving a response rate of 42.6%.

Table 3. 1: Characteristics of the study population

Study characteristics	n	%
Questionnaires		
Distributed (undelivered)	518 (14)	
Delivered	504	
Completed	215	42.6
Age of women on GTD registry		
<20y	2	0.4
20-40y	366	70.6
>40y	150	28.9
Length of time since diagnosis		
<1 year	92	42.9
1-3 year	95	44.4
>3 years	27	12.6
Partial Hydatidiform Mole	351	67.7
Complete Hydatidiform Mole	157	30.3
Choriocarcinoma	5	1.0
Atypical Placental nodule	2	0.4
Placental site nodule	3	0.6

n= numbers; % = percent; y=years

3.4.2 Questionnaire Response

A. Registration with GTD centre

Study participants were largely informed about the national GTD centre by their attending clinician (90%, n=193) or midwife (7.9%, n=17) while others accessed information on the HSE website or social media. Most women were enrolled by their clinician with 3.8% (n=8) instigating registration themselves. Almost half of the study participants (43%, n=92) were diagnosed with GTD in the preceding twelve months and 93% (n=196) were registered with the GTD centre within a year of their diagnosis.

B. Prior Knowledge of Molar Pregnancy

Many participants 78% (n=165) were unaware of molar pregnancy prior to their diagnosis and received a patient information leaflet upon registration. One fifth of study participants (21.6%, n=45) were not aware of the name of the clinician responsible for their care at the local hospital. Some participants (40%, n=81) had concerns about the management of future pregnancies and acknowledged that these were addressed by staff at the GTD centre.

C. Diagnosis of GTD at local hospital

Participants scored three statements relating to their diagnosis at the local hospital on a rating scale (Figure 3.2). Most women were satisfied with how they received their diagnosis and had adequate time to ask questions. Over a fifth of respondents (23.6%, n=48) did not agree that other medical professionals with whom they interacted throughout their treatment understood GTD and 14% (n=30) were unsure. In a later question on factors impacting phlebotomy attendance, 26.5% (n=57) of participants reported having to explain to healthcare professionals why they needed hCG monitoring.

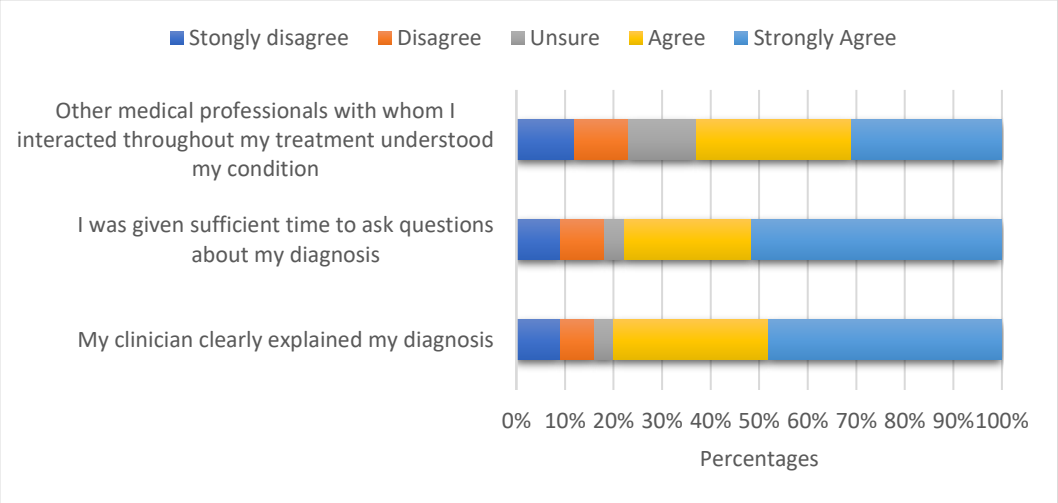

Figure 3. 2: Diagnosis of GTD at local hospital

D. Monitoring hCG levels

Many women (59.6%, n=127) attended the early pregnancy clinic (EPC) at their local hospital for phlebotomy (Figure 3.3). While most respondents (80%, n=167) agreed that hCG measurement should be performed by experienced scientists in a centralised quality assured laboratory, all women reported timely communication of hCG results. Study participants were impacted by multiple factors when providing blood samples for hCG monitoring (Figure 3.4). A significant number of respondents (34%, n=67) would opt for urine hCG monitoring if this were available. Reflecting a move to virtual clinics and digital communication during the SARS-COV-2 pandemic, participants were asked their preferred mode of result communication. Participants favoured multimedia formats of communication with a preference for telephone and text message as opposed to emails and letters.

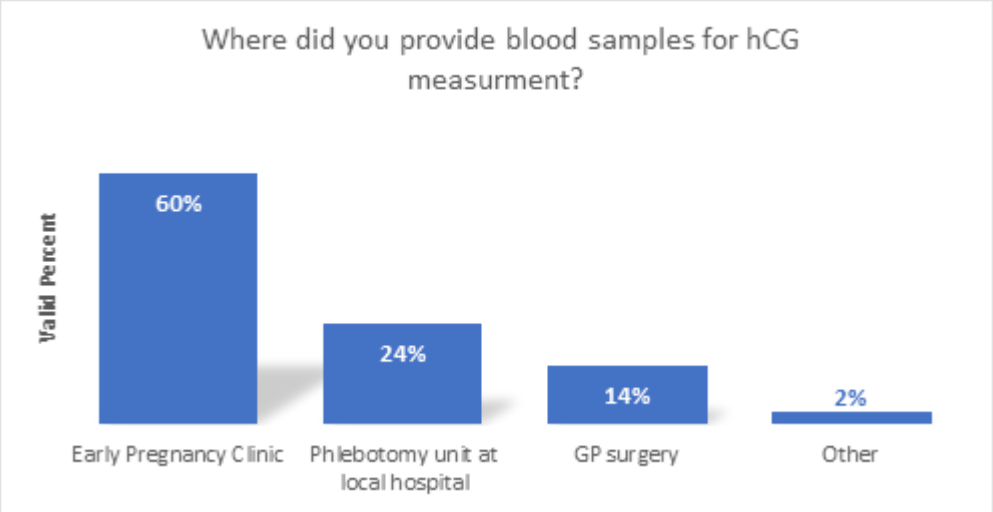

Figure 3. 3: Provision of blood samples for hCG measurement

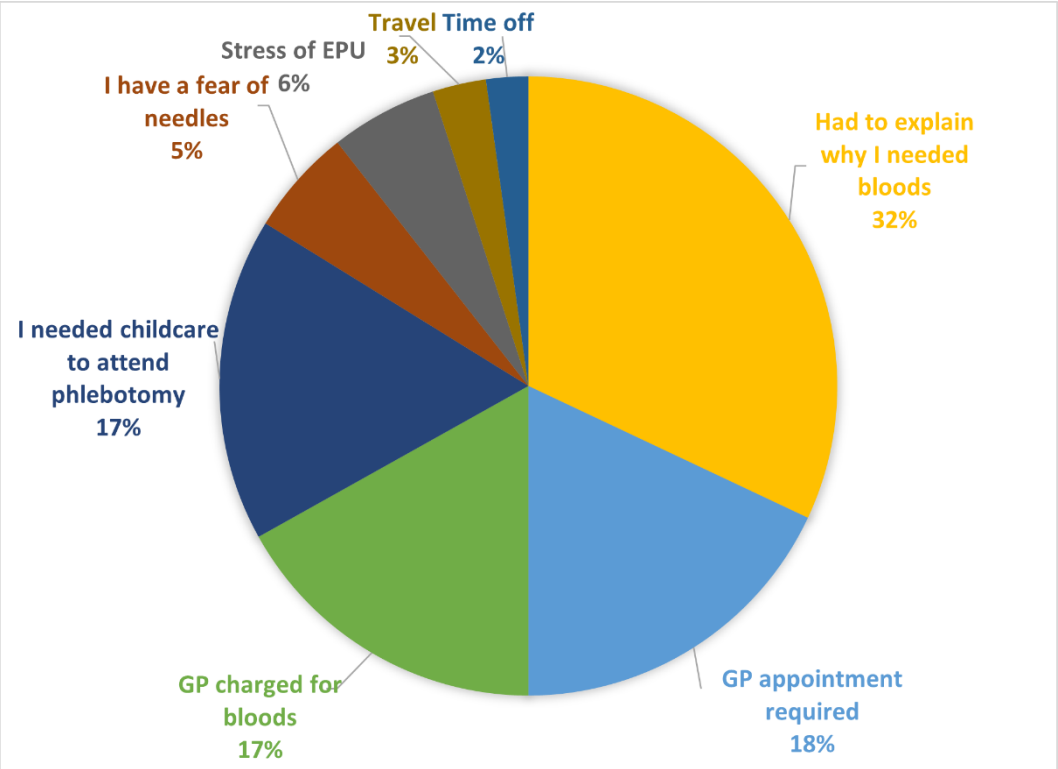

Figure 3. 4: Factors impacting attendance for phlebotomy
GP = general practitioner; EPU = Early Pregnancy Unit

E. Patient experience

Study participants ranked their priorities for services from the GTD centre (Figure 3.5). Most respondents (49%, n=106) ranked a rapid hCG result as the top priority with some women giving equal priority to all options provided.

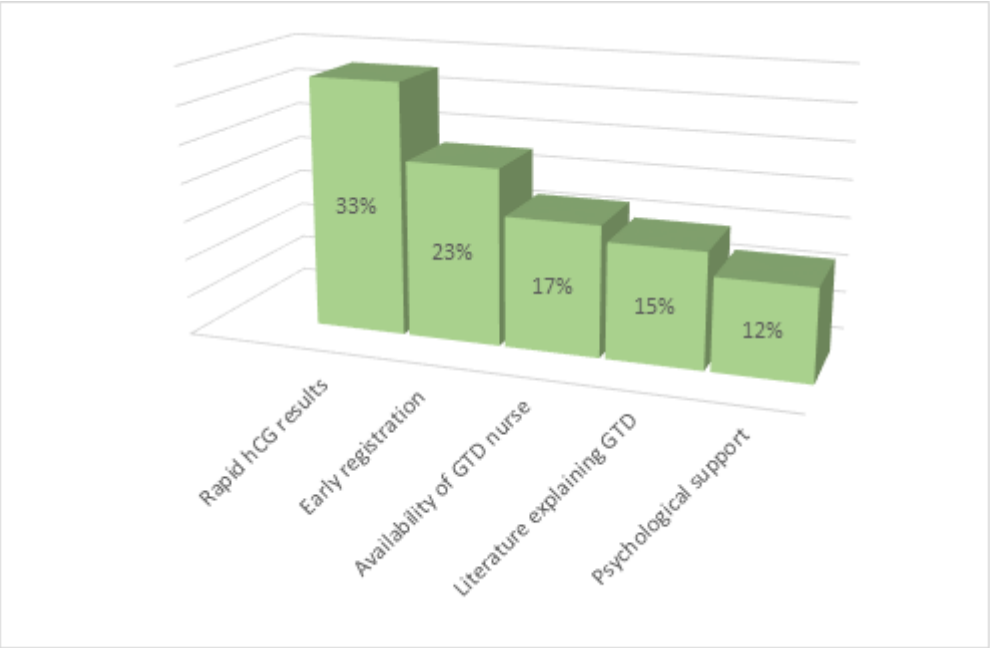

Figure 3. 5: Patient priorities based on their experience on the GTD Registry

All participants were satisfied with the quality of care provided by the National GTD Centre and this is reflected in the illustrative quotes in Table 3.2. In the open-ended questions, respondents requested psychological counselling for woman and their partners, as well as peer support groups.

Table 3. 2: Illustrative quotes from "other" options and open-ended questions

Section of Questionnaire	Quotes
Registration	*"Cannot compliment the GTD registry highly enough. I felt in good hands in world class care at all times."*
Diagnosis at local hospital	*"Wasn't explained as clearly as I needed it to be, more brushed over. Contacted by the GTD nurses who are very good."*
Factors impacting Phlebotomy	*"I had to travel 40mins to hospital every Monday for 6 months."*
	"I had to leave work to attend appointments."
	"EPC is a difficult place to be. I have been there too many times over the last number of years to count. It is difficult when you have had a miscarriage to keep going back there for blood tests and sitting with people who are having positive scans and making their next appointments at the desk of the very small waiting room…. It certainly adds to the trauma of being there in the first place."
Additional Resources:	*"Mental health support/counselling services due to the uncertainty of the illness and the fact that it is often following a pregnancy loss."*
	"Options for peer support. Perhaps an online support group"

3.5 Discussion

This study evaluated the experience of women with molar pregnancy at diagnosis and during follow-up management at a national GTD centre. Most survey respondents were satisfied with how they received their diagnosis at the local hospital, but some had insufficient time to question their diagnosis. This emphasises the importance of medical professionals allocating appropriate time for women to ask questions, discuss their concerns and comprehend their diagnosis. One fifth of study participants did not know the name of the local clinician responsible for their care. A recall bias may contribute to this finding as almost half of the respondents were diagnosed more than 12 months prior to completing the questionnaire.

The study highlights a knowledge gap in molar pregnancy amongst healthcare professionals. Approximately one-third of study participants were not confident that other medical professionals with whom they interacted throughout their treatment

understood their medical condition. This emphasises a need for further education on molar pregnancy which could be addressed in undergraduate medical and nursing/midwifery curricula and by promoting its inclusion at international conferences, national study days and postgraduate modules.

Currently, registration with the national GTD centre is voluntary and this study found that 3.8% of respondents had to instigate registration themselves. Our findings suggest consideration be given to mandatory registration of women with GTD to ensure optimal clinical management and maximum cure rates for this rare disorder. Overall, there was a high level of satisfaction with services provided by the national GTD centre with many women commenting on the excellent care received from the specialist nurses.

The top priority for women was a rapid hCG result and all participants received their hCG results in a timely manner. Most women favoured use of multimedia formats to receive their hCG results. Some women elected to receive results initially by telephone particularly when a critical result was awaited and by text messages at all other times. A few participants reported receiving telephone calls informing them of their hCG result or histological diagnosis at an inappropriate time during their working day which added to their distress. These women voiced a preference for an advance text message to arrange an appropriate time for a follow up telephone call. This feedback will help inform service development and improve communication with users.

Multiple factors impacted women attending phlebotomy for weekly hCG monitoring Predominant among these was childcare, travel time and charges for appointments with a general practitioner (GP). An extension of the Irish maternity and infant care scheme to include free GP care for women with molar pregnancy could help eliminate phlebotomy costs and travel time. Some participants remarked on the emotional stress of attending their maternity unit on a weekly basis while dealing with pregnancy loss and some reported feeling *"in the way"*. A common thread in feedback received was the need for a separate phlebotomy service for women with molar pregnancy. This could be addressed by offering early morning appointments at the EPC or providing a separate phlebotomy pathway through use of a pregnancy loss alert symbol sticker on the patient chart.

Some women experienced feelings of intense sadness on receiving a diagnosis of molar pregnancy and many reported a need for psychological support at the time of pregnancy

loss. A recent study on the psychological impact of GTD reported similar levels of distress at diagnosis.(115) Healthcare professionals interacting with these women need bereavement care training to ensure all aspects of care are provided. Appropriate time is required during clinic visits to acknowledge the woman's loss and provide psychological support. Previous studies identified a fear of disease recurrence and concerns about future pregnancies as a cause of psychological distress.(116,248,249) Women with GTN are known to have higher depression scores than women with hydatidiform mole.(249) Knowledge of the patient experience has become a key factor in optimising outcome in medicine.(260) A study examining the concepts of empathy, knowledge and kindness to women during their reproductive life found that healthcare professionals with an emotional awareness of the woman's situation, can contribute greatly towards the woman's recovery and satisfaction with care.(261) An appointment for a follow up call by the GTD nurse specialist could provide an opportunity for support and referral for counselling as needed and aid greatly in the patient's recovery.(262) The model of psychosocial care for patients with cancer and their families recently launched by the Irish national cancer control programme could be a useful model to explore for women with GTD.(119)

Participants in this study highlighted the need for information leaflets for partners and family members. Women had difficulty coping with the diagnosis themselves and did not feel able to share their diagnosis with others or explain it to family members. Provision of additional information leaflets for family members deserves consideration by GTD centres.

The desire for "real" or "virtual" patient support groups was expressed by many participants. The internet provides opportunities for individuals living with rare disease to communicate with each other via online support groups. These groups offer many unique advantages, such as convenience, anonymity, and access to a diverse range of group members.(263) Recounting personal experiences in the confines of a closed social media support group may help other women and this shared understanding between patients may be difficult to replicate in information leaflets and website testimonials.(255) Healthcare professionals should consider the formation of new partnerships of collaboration and advocacy with these groups. This approach is best exemplified in the

Brazilian Facebook GTD page, led by a network of expert physicians working in GTD reference centres. Online forums provide an opportunity for medical professionals to respond to patient queries, correct misinformation and provide a valuable resource for medical education.(264)

Our study results should be viewed in the context of some limitations. The survey provides the view of responders and assumes that non-responders will have a similar view. A recent review of 717 annual patient satisfaction surveys provided response rates ranging from 16-80% (pooled average 49.8%).(265) This survey had a response rate of 42.6% which is close to the expected average. However, the response rate may have been influenced by the sensitivity of the subject area and the ability of the survey to provoke unpleasant memories. Our findings may also be impacted by completion bias and might not represent the majority view of women on the GTD registry. Previous studies have shown that the most satisfied patients and to a lesser extent, the least satisfied patients are most likely to participate in a hospital satisfaction survey.(265) Furthermore, the questionnaire was only produced in English which may have deterred non-nationals from completing the survey. As with all surveys, the study findings are limited by the questions asked and feedback from the open-ended questions suggested additional questions of relevance to these women. Finally, the views of the partners of women experiencing a molar pregnancy were not included in this survey but their views are important and should be included in future surveys.

Despite these limitations, this study is the first of its kind to evaluate the experience of women on the Irish national GTD registry. The anonymous survey gave women an opportunity to provide unbiased feedback and provides valuable information for optimising the care of women with molar pregnancy.

3.6 Conclusion

This study is the first survey of women on the Irish national GTD Registry. Women with molar pregnancy have not been included in previous pregnancy loss surveys but deserve inclusion as their experience of pregnancy loss is equally relevant. The survey highlights the educational needs of healthcare professions caring for women with rare disease. It informs the service needs and psychological challenges of women with GTD and establishes a knowledge base for future service development.

Author Contributions

KOD and CJ: survey concept and design. CJ, JC, CK, TMcC and KOD: survey questionnaire review. CJ: survey conduct, protocol development. JC: patient recruitment and survey oversight. CK: patient liaison and survey collection. CJ: data acquisition, statistical analysis and data integrity. CJ, JC, TMcC and KOD critically reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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Ethical Consent

This research study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork, Ireland (ECM 4(d) 20/10/2020).

Chapter 4 – A novel scoring system provides high separation of diploidy and triploidy to aid partial hydatidiform mole diagnosis: an adaption of HER2 D-DISH for ploidy analysis

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Chapter 4 – A novel scoring system provides high separation of diploidy and triploidy to aid partial hydatidiform mole diagnosis: an adaption of *HER2* D-DISH for ploidy analysis

4.1 Abstract

Aims: Diagnosis of hydatidiform mole or molar pregnancy based on morphology alone can be challenging, particularly in early gestation, necessitating the use of ancillary techniques for accurate diagnosis. This study aimed to adapt the VENTANA® *HER2* dual-colour dual-hapten in-situ hybridisation (D-DISH) assay by using the internal chromosome 17 enumeration probe (CEP-17) to determine ploidy status.

Methods: We selected twenty-five products of conception (POCs), consisting of molar and non-molar cases to validate the *HER2* D-DISH assay. These cases had prior morphological assessment by a perinatal pathologist and ploidy analysis using molecular cytogenetics. Three independent observers, blinded to the original histopathological and genetic diagnosis, scored 10 representative areas on each slide. Interobserver variability was assessed by comparing the total scores assigned by each observer using analysis of variance (ANOVA).

Results: Our ploidy scoring system accurately determined the correct number of diploid and triploid conceptuses, demonstrating complete concordance with pre-existing ploidy status and the initial diagnosis. ANOVA analysis revealed robust inter-observer agreement between the three independent scorers ($p=0.36$). We achieved clear separation of average nuclear signals for diploid and triploid conceptuses, which was statistically significant ($p<0.05$) using the Wilcoxon test. Employing our innovative scoring system, known as the "rule of 5", we established ploidy decision thresholds for all 25 cases.

Conclusions: Our modified HER2 D-DISH ploidy assay simplifies the process of ploidy determination and improves the accuracy of morphological diagnosis of molar pregnancy. The *HER2* D-DISH assay was selected for ploidy analysis due to the widespread availability of in-situ hybridisation in pathology laboratories.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The *HER2* D-DISH assay is used extensively in pathology laboratories to determine *HER2* amplification status, primarily in breast and gastric cancers.

WHAT THIS STUDY ADDS

⇒ This study demonstrates how an adaption of the *HER2* D-DISH assay, which focuses on nuclei with the highest numbers of nuclear signals from chromosome 17 centromeric probes, facilitates ploidy assessment. This adapted *HER2* D-DISH assay offers an accurate, reliable adjunct to morphology for partial hydatidiform mole diagnosis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The *HER2* D-DISH ploidy scoring system achieves high separation between diploidy and triploidy, helping pathologists achieve more accurate hydatidiform mole diagnosis. This will decrease diagnostic errors, reduce patient anxiety and lower hCG surveillance costs. Furthermore, it will facilitate the collection of more reliable incidence data, influencing future research, clinical practice and service development.

4.2 Introduction

Gestational trophoblastic disease (GTD) covers a wide spectrum of disorders from the pre-malignant conditions of hydatidiform mole (HM) to the malignant disorders of choriocarcinoma, invasive hydatidiform mole, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). HMs may be further classified into complete (CHM) or partial (PHM) based on their characteristic morphological features and genome complement.(266). CHMs mostly have a diploid (2n) androgenetic genome whereas PHMs mostly have a triploid (3n) diandric monogynic genome.

Diagnosis of PHM and CHM can often be made on morphology alone when the characteristic morphological features of HM are present. It is important to accurately classify HMs as the risk of progression to gestational trophoblastic neoplasia (GTN) is lower for PHM (0.5-1%) than for CHM (13-16%).(129) However, there are some reported cases of PHM progressing to GTN.(185,267,268)

Advances in ultrasonography have resulted in earlier detection of HMs when typical morphological features may be subtle or absent making the pathological diagnosis more challenging.(105) Early pregnancy loss tissue specimens may exhibit atypical villous morphology (AVM), sometimes due to aneuploidy, which can mimic HM, making it difficult to distinguish PHM from hydropic non-molar miscarriage.(66,212) Diagnosis of PHM on morphology alone has an error rate of at least 20%.(220) Consequently, ancillary techniques have diagnostic utility in differential diagnosis when morphology is subtle or atypical and can help distinguish triploid PHM from diploid and aneuploid gestations. This can allow women with confirmed non-molar miscarriage avoid human chorionic gonadotrophin (hCG) monitoring and attempt a new pregnancy without delay.

p57 immunohistochemistry is frequently used as a diagnostic tool to confirm CHMs as in these cases abnormal absence of staining for p57 in stromal and cytotrophoblast cell nuclei supports the diagnosis.(68) When p57 staining is normal, (i.e. cytotrophoblast and stromal nuclear staining is maintained), and/or where morphology assessment raises a differential diagnosis of PHM, ploidy analysis can identify a diploid or triploid conceptus and help distinguish PHM from non-molar miscarriage. Ploidy analysis can be determined using flow cytometry, fluorescent in-situ hybridisation (FISH) or chromogenic in-situ hybridisation (CISH). Galea and colleagues have used silver in-situ hybridisation (SISH) for

the determination of ploidy in suspected molar pregnancy and others have adapted HER2 chromogenic in-situ hybridisation assays to determine DNA ploidy.(269–271) The *HER2* dual colour dual hapten ISH (D-DISH) assay is readily available in many pathology laboratories for determining *HER2* gene amplification status in women with breast cancer. This assay incorporates a chromosome 17 enumeration probe (CEP17) which can be used to provide ploidy status.

Molecular genotyping can infer ploidy while also elucidating the genetic origin of the additional chromosomal complement in the triploid conceptus. It can also inform the differential diagnosis of a twin pregnancy with HM, mosaics and discordant p57 immunohistochemistry.(61,66,69) Furthermore, it can help identify familial recurrent hydatidiform mole (FRHM), a rare autosomal recessive disorder due to pathogenic variants in various genes (*NLRP7*, *KHDC3L* and *PAD16*).(186,208,272)

Triploidy is a common chromosome anomaly occurring in 1-2% of all conceptions.(273,274) In our clinical practice, we routinely send samples of all placentas from second-trimester pregnancy loss and selected recurrent first trimester pregnancy loss products of conception (POC), for genetic analysis to identify aneuploidies and genomic imbalances. The impetus for this study arose from an incidental finding of triploidy in some POCs following cytogenetic analysis, where subtle morphological features of PHM had led to the initial underdiagnosis of PHM. This led to the search for an ancillary technique that would be accessible in our pathology laboratory to aid diagnosis in POCs with features suspicious for PHM.

In this study, we sought to adapt the VENTANA® *HER2* dual-colour dual-hapten ISH (D-DISH) assay by using the internal chromosome 17 enumeration probes (CEP-17) for ploidy analysis.

4.3 Materials and methods

4.3.1 Study design

Twenty-five atypical POCs whose cytogenetic results were available, were randomly selected for verification of the HER2 D-DISH assay. These POCs consisted of a mixture of CHMs, PHMs and non-molar pregnancies. All cases had morphological assessment by a perinatal pathologist and confirmation of ploidy status by prior genetic analysis. The HER-2 D-DISH assay was adapted for ploidy analysis by using the internal chromosome 17 enumeration probe to determine ploidy status. Misinterpreting trisomy 17 as triploidy was considered, and deemed highly improbable given the rarity of trisomy 17 in pregnancy loss specimens (1 in 1,000 miscarriages).(275,276) The study formed part of a quality assurance activity in our pathology laboratory and ethical approval was not required.

4.3.2 Confirmatory genetic analysis

We send POCs from selected first and second-trimester pregnancy loss to an external laboratory for comprehensive genetic analysis. During this process, evaluation of aneuploidy was conducted by quantitative fluorescent polymerase chain reaction (QF-PCR) targeting chromosomes 13,15,16,18,21,22, and the sex chromosomes. Genotyping of 42 polymorphic genetic markers known as short tandem repeats (strs), and two non-polymorphic sex chromosome markers, namely SRY and Amelogenin was performed using the Devyser Extend kit v2 (Devyser AB, Sweden). Genomic imbalances, including deletions and duplications were detected through multiplex ligation-dependent probe amplification (MLPA) analysis, using the sub-telomeric kit SALSA P036-E1 (MRC Holland).

4.3.3 Chromosome 17 *HER2* D-DISH analysis

The VENTANA® *HER2* D-DISH assay was performed using 4µm tissue sections from selected formalin-fixed paraffin-embedded (FFPE) blocks derived from the 25 POC cases. This analysis was performed on the Benchmark Ultra-Advanced automated staining system (Roche™ Diagnostics). The *HER2* D-DISH assay is a closed system incorporating two DNA probes, each employing distinct chromogenic detection systems. The silver ISH dinitrophenyl (DNP) probe detects *HER*2 while the red ISH digoxigenin (DIG) probe binds to the centromeric region of chromosome 17, as illustrated in Figure 4.1. The procedure was performed according to manufacturer instructions and slides were counterstained

with haemotoxylin for interpretation by conventional bright-field light microscopy.(277) The *HER2* D-DISH-stained slides were reviewed for staining adequacy and the staining quality was monitored by internal quality assessment. The study was performed in a pathology laboratory accredited to ISO15189 international standards which participates in external quality assurance for *HER2* and ISH (UKNEQAS and Nordic).

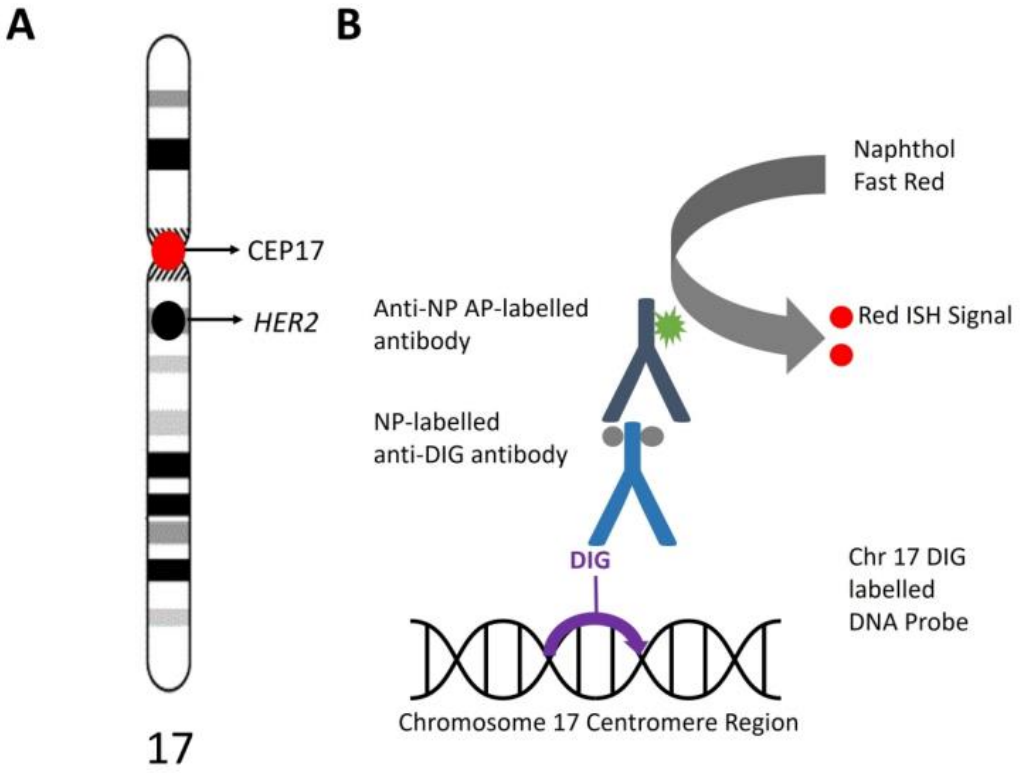

Figure 4. 1: Red ISH DIG-labelled Chromosome 17 detection system.
(A) Ideogram of chromosome 17 showing the HER2 locus and Chromosome 17 centromeric locus which binds the enumeration probe (CEP17). (B) Illustration of the Red ISH DIG detection system. A chromosome 17 DNA probe labelled with digoxigenin (DIG) targets the centromeric region to provide information on ploidy. An anti-DIG antibody labelled with Nitro Pyrazole (NP) is detected by a secondary antibody conjugated with alkaline phosphatase (AP) which cleaves the substrate (Naphthol Fast Red) to produce a red signal. Adapted from the Roche VENTANA™ HER2 Dual ISH Method Sheet (2020-07-27, Rev A)

A ploidy score was derived by counting the red ISH signals within the nucleus of villous stromal cells. For the nuclei to be eligible for scoring, they had to meet specific criteria: nuclei must not overlap, and red signals must be contained within the nucleus and must be separate from one another. During the signal counting process, we deliberately chose villi that met certain criteria: villi were selected to avoid excessive cellularity that could

lead to nuclei overlapping, and highly hydropic villi were excluded as the stroma would be too paucicellular. Consequently, villi with an intermediate level of cellularity were selected for assessment in each POC.

Scoring was performed by three independent observers: a perinatal pathologist, a gynaecological pathologist and a medical scientist. An average of the three scores was taken for each case. All observers were blinded to the original histological and genetic diagnosis. Before formal scoring, a preliminary evaluation of the slides was performed to identify areas of the slide with the cleanest CEP17 signals (x400 magnification) to facilitate accurate analysis.

4.3.4 Development of a Ploidy Scoring System

When we initially adapted the HER2 D-DISH method for counting the CEP17 signals, it became evident that a considerable number of nuclei within a field of view exhibited either zero or just one signal. This reduced the average nuclear signals in both diploid and triploid POCs, blurring the distinction between diploidy and triploidy. For instance, using this counting method, a triploid mole (Figure 4.2A) with 54 nuclei (whole or partial) and 86 red signals resulted in an average nuclear signal of 1.59. In contrast, a diploid tissue (Figure 4.2B) with 53 nuclei (whole or partial) and 58 red signals resulted in an average nuclear signal of 0.91. This counting method did not intuitively reflect the number of genomes present. One would expect diploid (2n) and triploid (3n) conceptuses to yield scores closer to 2 and 3, respectively. As a result, we decided to establish our own scoring system.

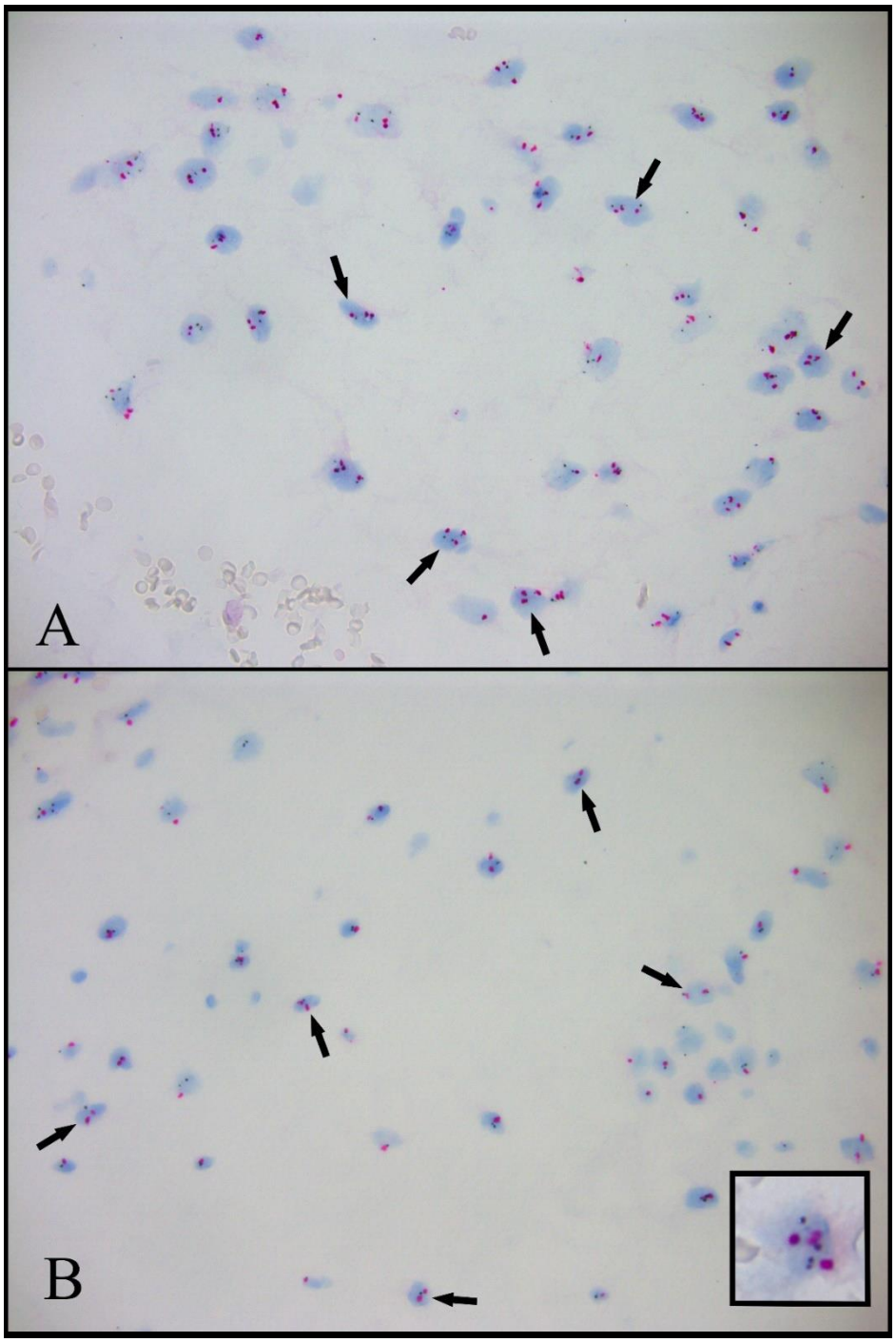

Figure 4. 2: HER2/CEP17 D-DISH immunostaining with black arrows showing.
(A) three red signals in some but not all villous stromal nuclei in a triploid conceptus and (B) two red signals in some but not all villous stromal nuclei in a diploid conceptus (nuclei with three signals are rare in this situation). In B the inset shows an example of a nucleus with three red signals in a genetically confirmed diploid conceptus. As 4 black Her2 signals are present this example likely represents two overlapping nuclei that could not be recognised as separate (these black signals although present are not used in the ploidy counting methodology)

We hypothesised that by focusing on a subset of nuclei with the highest nuclear signal counts within the high-power fields (hpf) under evaluation, we would consistently identify and count nuclei with two CEP17 signals in diploid POCs and three CEP17 signals in triploid POCs. To validate this approach, we obtained the average of the highest nuclear signal count obtained from *5* nuclei, in *5* adjacent high-power fields in *5* different areas – the "rule of 5" (Figures 4.3 and 4.4). To ensure this total number of nuclei was sufficient for scoring, we initially compared this method across 10 and 5 representative areas. Applying our newly established system to the *HER2* D-DISH example illustrated in Figure 4.2, we found that a triploid mole (2A) had 5 nuclei with three signals, resulting in an average nuclear signal of three. In contrast, a diploid POC (2B) had 5 nuclei with two signals, resulting in an average nuclear signal of two. Hence, this scoring system aligns more intuitively with the evaluation of diploid and triploid status.

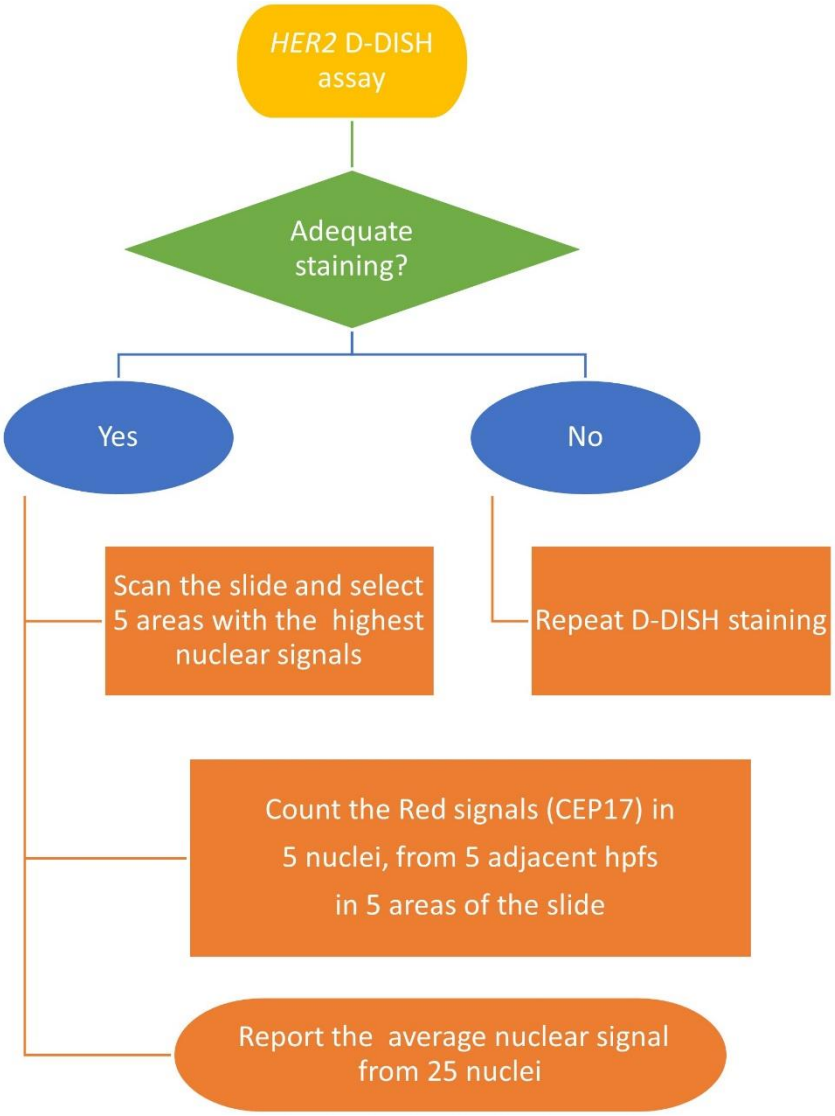

Figure 4. 3: Flow diagram illustrating steps in the performance of the adapted HER2 D-DISH assay for ploidy analysis and "Rule of 5" scoring system. CEP17: chromosome enumeration probe 17, hpf: high power field

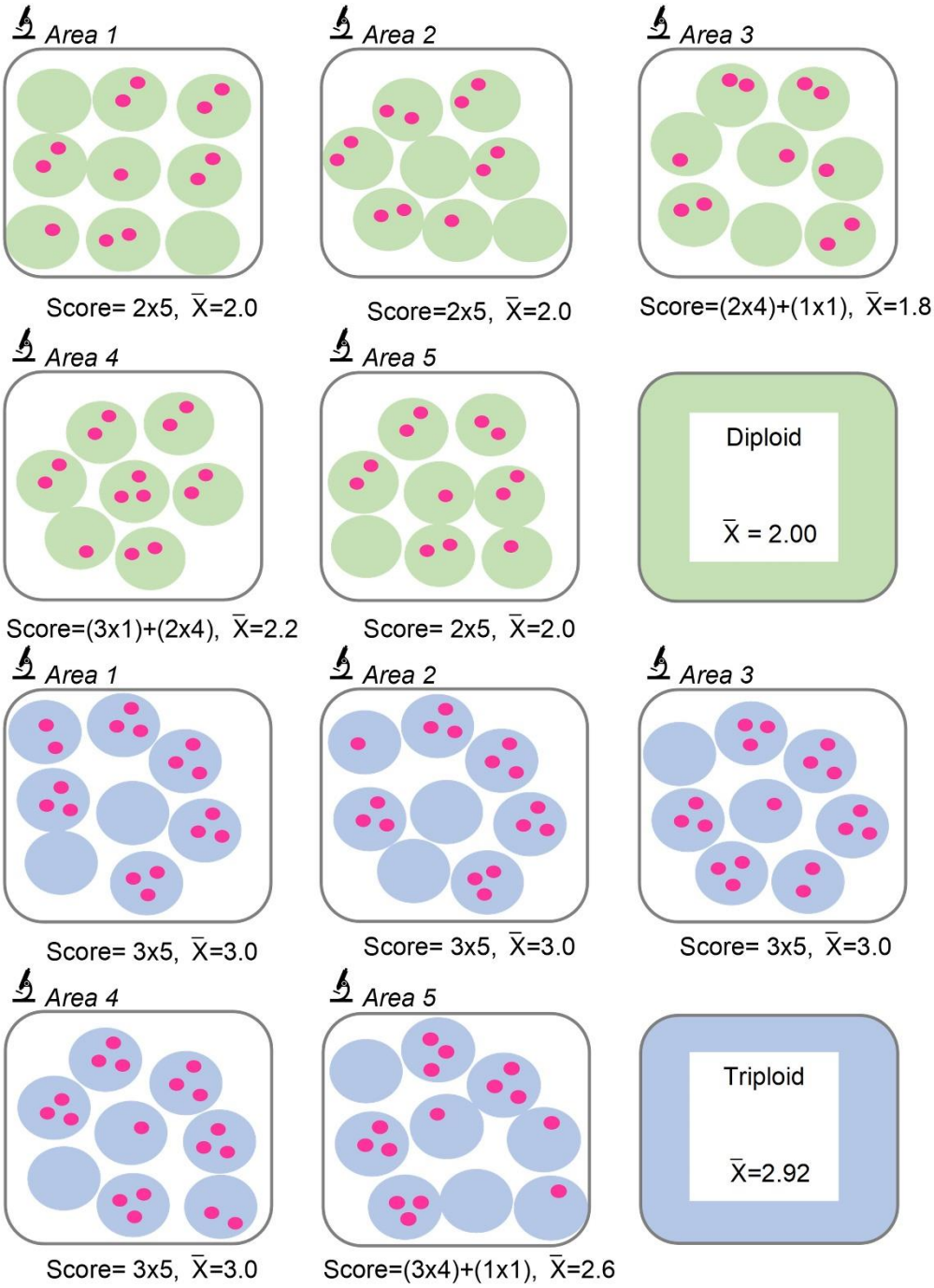

Figure 4. 4: "Rule of 5" scoring system.
The figure shows how the highest nuclear signals are selected. In each area, comprising 5 high power fields (hpfs) the 5 nuclei with the highest number of CEP17 signals are identified and the average nuclear signal is recorded. This process is then repeated in 5 different areas of the slide to give an average across 25 hpfs. The average nuclear signal for diploidy is close to 2 and for triploidy is close to 3. $\overline{\mathbf{X}}$: average nuclear signal

4.3.5 Statistical analysis

The “rule of 3” was used to estimate the power provided by our study sample size of 25 POCs. Applying this rule, we would have 95% confidence that the probability of an event occurring which is not seen in the validation cohort (i.e. Misclassification of diploidy or triploidy) is 12% (3/25) providing an estimated 88% power to determine study accuracy.(278)

The CEP17 nuclear signal counts were recorded in a scoring spreadsheet. Statistical analysis was performed using Microsoft Excel 2021 and Analyse-IT software. Average nuclear signals were counted in 10 representative areas on each slide. The total scores assigned by three independent observers were compared using analysis of variance (ANOVA). The Kappa statistic was used to assess inter-rater agreement across the three raters.

To determine the minimum number of areas required for scoring, a comparison was made between the average nuclear signal counts obtained from counting 5 versus 10 representative areas. Normality was assessed using the Shapiro-Wilk test. Parametric data were represented by mean (standard deviation) and non-parametric data by median (interquartile range) and a p value of <0.05 was deemed statistically significant.

4.3.6 Ploidy implementation audit

Application of the diagnostic threshold values established for ploidy status was audited for a 2-year period after implementation to assess its impact on diagnostics, its ease of implementation, clinical practice points and to determine the equivocal rates for PHM diagnoses.

4.4 Results

In this study, the 25 cases selected had prior ploidy status established by molecular cytogenetics which reported 17 cases of diploidy (15 NM and 2 CHM) and 8 cases of triploidy (PHM).

Using our *HER2* D-DISH ploidy scoring system, we demonstrated complete concordance with the original genetic diagnosis. We successfully identified the correct number of diploid and triploid conceptuses yielding a 95% CI for a maximum sensitivity of ≥88%.

Notably, the two diploid CHM cases identified by genetic analysis were diagnosed in our laboratory through morphological examination and ancillary p57 immunohistochemistry.

There was robust inter-observer agreement between the three independent raters, A (mean 2.27, SD 0.45), B (mean 2.28, SD 0.47) and C (mean 2.25, SD 0.48), as assessed by ANOVA (p=0.36). The inter-rater agreement across the three raters, as measured by the Kappa statistic was 0.812 (p-value<0.001). If we exclude the two cases categorised as equivocal due to suboptimal staining, the Kappa statistic is 0.927 (p-value<0.001). In keeping with Landis and Koch (1977) Kappa values of 0.81 or more may be interpreted as almost perfect agreement.(279) This highlights the need for good quality staining to ensure more accurate counts and better precision when applying this novel scoring system.

Average nuclear signal counts for diploid and triploid cases remained constant when we reduced the count from 10 to 5 representative areas, as depicted in Table 4.1. This consistency held true when we analysed all cases collectively, and when we excluded the two equivocal cases from our analysis. Consequently, we can confidently adopt a scoring system that relies on just 5 areas characterised by high signal intensity. This finding emphasises the reliability and robustness of our scoring system.

Table 4. 1: Average nuclear signal counts from 5 versus 10 representative areas

Cases	$\overline{X}_5$	SD	$\overline{X}_{10}$	SD	P value
Total (n=25)	2.27	0.39	2.26	0.39	0.4524
Total (n=23)*	2.27	0.40	2.26	0.40	0.3048
Diploid (n=17)	2.03	0.05	2.04	0.06	0.2334
Triploid (n=8)	2.78	0.28	2.77	0.26	0.9453

*Equivocal cases excluded, ($\overline{X}_5$) mean average nuclear signal count for 5 areas on each slide ($\overline{X}_{10}$) = mean average nuclear signal count for 10 areas on each slide, SD: standard deviation. P value: statistical significance (p<0.05).

4.4.1 Establishing decision thresholds

Ploidy decision cut-off values were determined by considering all 25 cases collectively and then reevaluating with the exclusion of the 2 cases ultimately deemed “equivocal”. Notably, there was a statistically significant separation between the average nuclear signal counts for diploid and triploid conceptuses, as demonstrated using the Wilcoxon test ($p<0.05$). This held true even when equivocal cases were included and validated the application of our innovative “Rule of 5” scoring system (Figure 4.5).

The differentiation between diploid from triploid conceptuses is visually depicted through box and whisker plots, illustrating the average nuclear signal counts for both groups (Figure 4.5). Final decision thresholds were established based on the analysis of 23 cases (Table 4.2). A conceptus was categorised as diploid (2n) if it exhibited an average nuclear signal ≤ 2.2 and triploid (3n) if it displayed an average nuclear signal of ≥ 2.8. Cases falling within the range of scores > 2.2 and < 2.8 were categorised as equivocal.

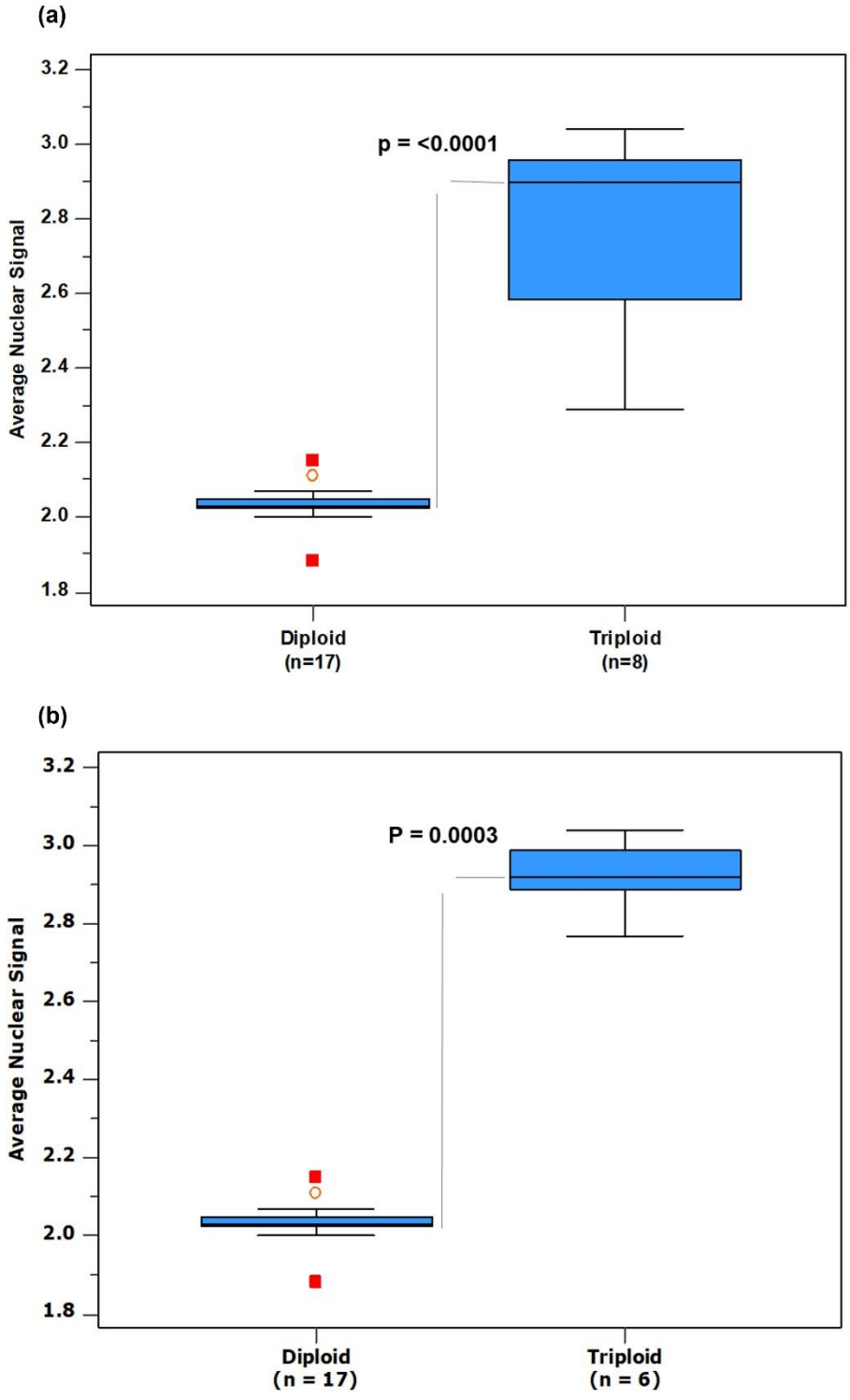

Figure 4. 5: Box and whisker plots
The graph is showing the average nuclear signal based on the "Rule of 5" scoring system. Scores are divided into diploid and triploid conceptuses to enable the establishment of ploidy decision thresholds. Figure (a) shows all 25 cases and (b) shows 23 cases

(equivocal cases excluded). The box portion of the plot includes 50% of the data, the lower, median (represented by a solid line) and upper quartile. The whiskers extend to the maximum and minimum values. Disconnected points are potential outliers. Grey lines show statistical significance ($p<0.05$) between diploid and triploid medians.

It's worth noting that two cases ultimately designated as having "equivocal" scores (cases 14 and 19) were indeed triploid but displayed scores slightly lower than the other triploid conceptuses, with scores of 2.29 and 2.40, respectively. Upon review, it became apparent that both cases had suboptimal staining quality, which likely accounted for the lower average nuclear signals observed. Importantly, neither of these cases exhibited characteristic morphological features of PHM and required ploidy analysis to establish the diagnosis. While their scores clearly distinguished them from the diploid cases (all of which had scores less than 2.15), it was decided to place these two cases in an equivocal category to maximise diagnostic confidence. This approach ensured a substantial gap between diploid and triploid categories (Figure 4.5b).

Table 4. 2: Ploidy decision thresholds for diploid and triploid genomes

Ploidy status	Genome Number (n)	"Rule of 5" based Average nuclear signal
Diploid	2n	≤2.2
Equivocal	2n-3n	>2.2 and < 2.8
Triploid	3n	≥2.8

4.4.2 Implementation Audit results

Following the implementation of our ploidy determination method with established decision thresholds, an audit of all POCs received in our pathology department over a 2-year period revealed that 7.7% (98/1264) of POCs underwent *HER2* D-DISH analysis to aid in the diagnosis or exclusion of PHM. Among those cases, 47% (46/98) were confirmed as triploid PHMs, with an equivocal rate of 1.02% (1/98). In collaboration with clinicians on the GTD steering committee, it was decided that all equivocal cases would be registered with the GTD centre for further clinical follow-up and hCG monitoring as potential PHMs. This decision was made due to the lack of molecular genotyping services nationally, which could have resolved the equivocal diagnoses, and the short surveillance period required for most PHMs.

During the practical implementation of this method, pathologists found that the scanning process, aimed at identifying nuclei with the highest signal count in each hpf, effectively evolved into a search for nuclei with three or more signals. This approach allowed for the rapid evaluation of a substantial number of nuclei within the villus stromal population, spanning a total of 25 hpfs. As a result, it enabled a more comprehensive assessment of the tissue than initially appreciated.

4.5 Discussion

Our validation study has demonstrated the accuracy of *HER2* D-DISH ploidy analysis in effectively distinguishing between diploid and triploid gestations. While the initial validation study identified two out of 25 cases (8%) as equivocal, continuous experience gained in morphological assessment and *HER2* D-DISH staining has substantially reduced the occurrence of equivocal cases over time. This reduction is exemplified by the lower equivocal rate (1.02%) observed in our implementation audit of the "Rule of 5" scoring system, confirming the diagnostic utility of this technique.

We established the sensitivity and specificity of our novel scoring system in a follow up evaluation audit using molecular genotyping. This audit aimed to assess the accuracy of the *HER2* D-DISH ploidy assay in identifying triploid PHM conceptuses (sensitivity) and diploid non-molar conceptuses (specificity) in a cohort of samples with equal numbers of diploid and triploid conceptuses. Application of the scoring system to determine ploidy did not yield any false positive or false negative triploid PHMs results. This audit achieved 100% sensitivity and specificity (95% CI:≥ 92%, n=36) confirming the accuracy of our novel scoring system.(275)

It is important to note that ploidy analysis, while valuable in distinguishing diploid from triploid conceptuses, does not identify the genomic origin of the additional chromosomal complement in a triploid conceptus. This raises concerns about the potential over-diagnosis of PHM based on ploidy alone, as there is a risk of mistakenly including digynic triploid conceptuses. Additionally, there are documented cases where digynic triploid non-molar pregnancies exhibit morphological features similar to PHM.(220) However, recent auditing of our service using molecular genotyping demonstrated that *HER2* D-DISH ploidy analysis did not lead to misdiagnosing PHM due to digynic triploidy.(275) This suggests that, from a practical viewpoint, laboratories using morphological

assessment supported by an accessible ploidy assay may (a) accurately exclude PHM by confirming diploid conceptus status and (b) confirm triploid status in suspected PHM cases without a substantial risk of over-diagnosis by miscategorising digynic triploid conceptuses.

Use of molecular genotyping for ploidy analysis has certain challenges, primarily due to the low quantity and quality of DNA extracted from FFPE tissue, which in turn, impacts the success rate of the analysis. The potential for contamination of trophoblastic villi with maternal decidua further complicates the interpretation of genotyping results. Furthermore, it's important to recognise that genotyping represents a more expensive approach when compared to *HER2* D-DISH (approximately €250 per test), principally due to the requirement for specialist equipment and the need for scientists proficient in interpreting genotyping data.

In our laboratory, we have a low threshold for requesting ploidy analysis to support PHM diagnosis. The key enabler of this approach is the ready availability of *HER2* D-DISH analysis, which has proven to be highly effective in our setting. We have successfully identified triploidy in approximately 47% of atypical conceptuses selected for testing, coupled with a low equivocal rate of 1.02%. The adapted *HER2* D-DISH assay offers many advantages, most notably it's speed, convenience, ready accessibility and compatibility with FFPE tissue. It has many benefits over molecular genotyping including the potential for a quicker turnaround time, broader accessibility within pathology laboratories and the absence of a requirement for specialist training in molecular genetics.

In laboratories that lack access to ancillary techniques for aiding PHM diagnosis, especially in cases where a definitive diagnosis cannot be reached and PHM cannot be excluded, adherence to national clinical guidelines is advisable. In Ireland, these guidelines recommend registering such cases with the National GTD centre for hCG surveillance, as hCG levels generally normalise within a two-month period.(108)

The finding that a relatively high proportion (7.7%) of POC received by our laboratory required the application of *HER2* D-DISH ploidy analysis to aid in PHM diagnosis or exclusion is noteworthy. Equally striking is the almost equal distribution between the identification of triploid (47%) and diploid (52%) cases. This collectively underscores the

complexity of HM diagnosis, emphasising the importance of having an accessible solution to address these challenges in clinical practice.

4.5.1 Limitation and Strengths

Future prospective larger studies are needed to validate this innovative scoring system before it can be recommended for routine use and especially as it extends to other pathology centres. Given the broader availability of in-situ hybridization (ISH) in pathology laboratories, this makes the integration of ISH into routine practice more feasible than molecular genotyping. Going forward, implementation of *HER2* D-DISH ploidy analysis holds the promise of reducing the number of cases with uncertain diagnoses, thereby alleviating emotional distress for patients and eliminating the costs associated with clinic attendance and hCG surveillance. Moreover, it has the potential to address an unmet clinical need by providing more accurate estimates of HM incidence rates.

4.6 Conclusion

Use of the adapted *HER2* D-DISH assay, using the innovative "rule of 5" scoring system, provides a reliable adjunct to morphological assessment for partial hydatidiform mole diagnosis. This ploidy assay offers a potentially more accessible alternative to molecular genotyping, particularly in pathology laboratories where resources are limited.

Acknowledgements:

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Author Contributions:

Study design: CMJ, BF and SD. BF, JD and NC scored the *HER2* D-DISH-stained slides. Statistics, tables, and figures; CMJ,PMOS and PC. Drafted manuscript: CMJ and BF. All authors approved the final manuscript and are accountable for the integrity of the work.

Chapter 5 - Morphology combined with HER2 D-DISH ploidy analysis to diagnose partial hydatidiform mole: an evaluation audit using molecular genotyping

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Chapter 5 - Morphology combined with *HER2* D-DISH ploidy analysis to diagnose partial hydatidiform mole: an evaluation audit using molecular genotyping

5.1 Abstract

Aims: A hydatidiform mole (HM) is classified as complete (CHM) or partial (PHM) based on its morphology and genomic composition. Ancillary techniques are often required to confirm a morphologically suspected PHM diagnosis. This study sought to evaluate the clinical accuracy of PHM diagnosis using morphological assessment supported by *HER2* dual-colour dual-hapten in-situ hybridisation (D-DISH) ploidy determination.

Methods: Over a 2-year period, out unit examined 1265 products of conception (POCs) from which 103 atypical POCs were diagnosed as PHM or non-molar conceptuses with the assistance of *HER2* D-DISH ploidy analysis. We retrospectively audited a sample of forty of these atypical POCs using short tandem repeat (STR) genotyping. DNA extracted from formalin-fixed paraffin-embedded tissue was genotyped using twenty-four polymorphic loci. Parental alleles in placental villi were identified by comparison to those in maternal decidua. To identify triploid PHM cases, we sought three alleles of equal peak height or two alleles with one allele peak twice the height of the other at each locus.

Results: Thirty-six of the forty cases (19 PHM and 17 non-molar) were successfully genotyped and demonstrated complete concordance with the original diagnosis. All PHMs were diandric triploid of dispermic origin. In two non-molar diploid cases, we identified suspected trisomies (13 and 18), which potentially explains the pregnancy loss in these cases.

Conclusions: This study validates the use of *HER2* D-DISH ploidy analysis to support the diagnosis of a morphologically suspected PHM in our practice.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Diagnosis of partial hydatidiform mole on morphology alone can be challenging and is prone to high error rates, often necessitating the use of ancillary techniques to supplement the morphological assessment.

WHAT THIS STUDY ADDS

⇒ Ploidy determination using an adapted *HER*2 D-DISH assay assists in the diagnosis of partial hydatidiform moles in our practice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ *HER2* D-DISH ploidy analysis could if correctly implemented improve the accuracy of HM diagnosis and reduce the reliance on centralised HM review. This approach will ensure timely access for all women to a correct diagnosis and ultimately provide more accurate HM incidence rates.

5.2 Introduction

Gestational trophoblastic disease (GTD) is a gynaecological condition associated with both benign and malignant entities. The most common form of GTD is a hydatidiform mole (HM) which can be a complete diploid diandric mole (CHM) or a partial triploid diandric monogynic mole (PHM). Malignant forms of GTD include invasive mole, choriocarcinoma, placental site trophoblastic tumour and epithelioid trophoblastic tumour, collectively known as gestational trophoblastic neoplasia (GTN). GTN may develop after any pregnancy, but the risk is greater after CHM than PHM with both HMs having a higher malignancy risk than a non-molar pregnancy. Use of ultrasound in early pregnancy has led to an earlier diagnosis of HM which has been shown to correctly diagnose 88% of complete moles but only 56% of partial moles.(106) The confirmation of HM diagnosis is made upon histopathological examination of the products of conception (POCs). Early HMs can be confused with non-molar pregnancies on ultrasound and under pathological examination due to similarities in their features.(280) It is important to establish the correct diagnosis (CHM, PHM or non-molar) as this will inform clinical management, the need for follow-up serum human chorionic gonadotrophin (hCG) monitoring and the risk of progression to GTN.(30)

Hydatidiform moles are characterised by excessive proliferation of syncytiotrophoblast and cytotrophoblast cells and stromal oedema.(281) The confirmatory diagnosis of complete and partial HM is made on the basis of morphology assessment aided by ancillary techniques, where required. A complete hydatidiform mole (CHM) is a diploid conceptus consisting solely of a paternal genome (i.e., androgenetic). In CHM, the absence of a maternal genome arrests embryonic development and allows paternally expressed genes to drive trophoblastic proliferation.(282) A diagnosis of CHM may be confirmed, if required, by the absence of immunostaining for p57, the product of a paternally imprinted gene (*CDKN1C*).(42) Although useful for CHMs, p57 immunostaining is not informative when distinguishing partial mole from non-molar conceptuses. A partial hydatidiform moles (PHM) is usually a triploid conceptus consisting of one maternal genome (monogynic) and two paternal genomes (diandric) but may rarely occur as a tetraploid conceptus. The excess paternal contribution increases trophoblastic proliferation and the presence of the maternal genome allows embryonic development to proceed, often to the second trimester.(273) It is important to classify HMs correctly as the risk of developing GTN is higher for CHM (15-20%) than for PHM (<1%) which leads to a longer hCG surveillance period for CHMs.(129,185,267,283,284) Conversely, the risk of developing GTN after a histologically confirmed non-molar pregnancy is very low (1 in 50,000) and hCG surveillance is not required so these women can start planning a new pregnancy immediately.(285)

Diagnosis of PHM in the first trimester can be challenging when classical morphological features are subtle or absent and ancillary techniques may be required to aid diagnosis.(66) The majority of PHMs are misdiagnosed on ultrasound as an incomplete or missed miscarriage in the first trimester.(191,286) Diagnosis of PHM on morphology alone has reported error rates of at least 20%.(220) Abnormal villous morphology from a hydropic non-molar placenta or conceptus with aneuploidy may mimic PHM microscopically.(216) Karyotyping can help identify numerical and structural abnormalities, but it cannot be used on fixed tissue. In such cases, ploidy analysis using flow cytometry or in-situ-hybridisation (ISH) can help distinguish a diploid (aneuploid or hydropic) conceptus from a triploid conceptus (PHM).(287–289) However, ploidy analysis

will not distinguish between a triploid conceptus with two paternal genomes (diandric PHM) and those with two maternal genomes (digynic non-molar pregnancy).

The distribution of diandric and digynic triploidy has been reported differently depending on gestational age.(274,290,291) Typically, digynic triploid conceptions do not display morphological features of molar pregnancy but on occasion can exhibit some focal dysmorphic features suggestive of a PHM.(273,292) A digynic triploid conceptus may manifest on ultrasound with a growth restricted fetus, with relative macrocephaly and a non-cystic placenta but these abnormalities can be subtle and may be missed in the first trimester.(291,293) In contrast, diandric triploid PHMs generally have an enlarged placenta with focal cystic spaces and may have an abnormal fetus.(266) Digynic triploids have been reported to have some overlapping morphological features with PHM and as such there is a theoretical risk of over-diagnosing PHM using morphology with ploidy analysis alone; consequently molecular genotyping has been recommended for definitive diagnosis.(269,294,295) Evidence for the potential overlap between the morphological appearance of placental tissue from diandric and digynic triploidy is however sparse. Unlike PHM, digynic triploid miscarriages do not require hCG monitoring, hence the importance of obtaining an accurate diagnosis.

Molecular analysis using STR genotyping can infer ploidy in PHMs and also identify the additional chromosomal complement.(296) However, genotyping is not routinely available in most pathology laboratories. In this study, we sought to evaluate the accuracy of PHM diagnosis using morphology assessment aided by an adapted *HER2* D-DISH based ploidy determination technique by evaluation with molecular genotyping.(297)

5.3 Materials and methods

5.3.1 Study design

We performed a retrospective review of a sample of forty atypical POCs diagnosed over a two-year period (2018-2019) in our pathology laboratory at Cork University hospital (CUH) which is co-located with a large tertiary maternity hospital, Cork University Maternity Hospital (CUMH). The audit evaluated the clinical accuracy of a combination of morphological assessment supported by *HER2* D-DISH ploidy analysis by subjecting a sample cohort of our previously diagnosed cases to STR genotyping. As we did not have access to molecular genotyping technology locally, we collaborated with colleagues in the United Kingdom (UK) and performed tissue microdissection, DNA extraction and microsatellite DNA genotyping in the Trophoblastic Tumour Screening and Treatment Centre in Imperial College London.

In our study period, 1265 POCs were received for pathological examination. Following initial morphological assessment, *HER2* D-DISH ploidy analysis was requested on 103 cases to assist with PHM diagnosis. Use of this technique helped diagnose almost equal numbers of triploid PHMs (50.5%, 52/103) and diploid non-molar conceptuses (49.5%, 51/103). Six PHMs were diagnosed without the assistance of ploidy determination. From these 103 atypical cases we selected 40 POCs, spread across both years, to include equal numbers of cases determined to be triploid PHM or diploid non-molar conceptuses with the support of the *HER2* D-DISH ploidy analysis technique (Figure 5.1).(297) Our sample cohort contained approximately one third (38.5%, 20/52) of the triploid PHMs diagnosed with morphology and ploidy analysis during the study period thus adequately representing the study period. Formalin-fixed paraffin-embedded (FFPE) blocks were retrieved for each case and haemotoxylin and eosin (H&E) stained (3 micron) section and five consecutive unstained (5 micron) sections were prepared on non-coated slides. All cases were anonymised in line with institutional ethical approval (ECM 4 (k) 09/03/2021).

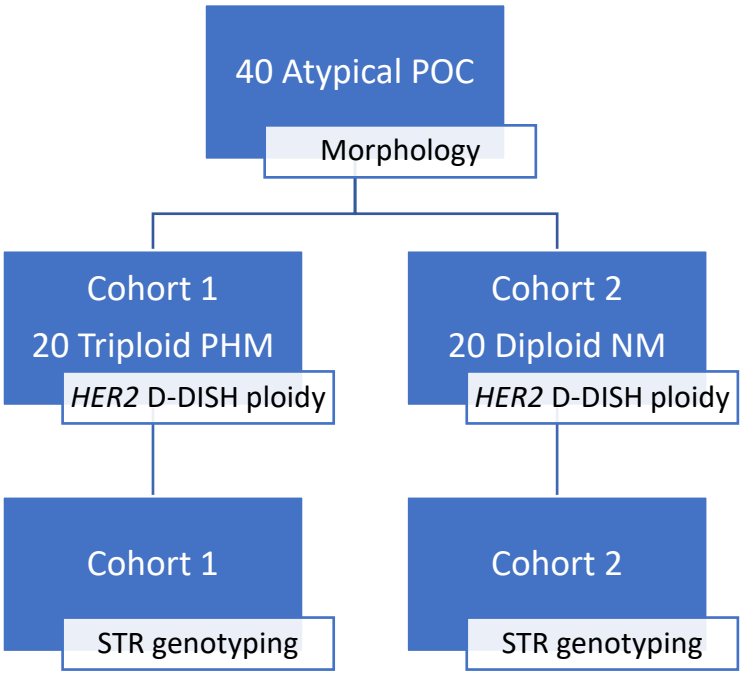

Figure 5. 1: Study Design, POCs: products of conception,
PHM: partial hydatidiform mole, NM: non-molar

5.3.1.1 HER2 D-DISH ploidy analysis

The VENTANA™ *HER2* dual ISH assay is a closed two probe system used to determine *HER2* gene amplification status in breast and gastric carcinoma. This assay uses two-colour chromogenic ISH to enable enumeration of the ratio of *HER2* to chromosome 17 nuclear signals. We adapted this method for ploidy analysis by using the chromosome 17 enumeration probe (CEP17) to help classify diploid and triploid conceptuses.(297)

5.3.1.2 Tissue Microdissection and DNA extraction

Tissue micro-dissection was performed using a Leica KL300 LED microscope (leicabiosystems.com) which filters out heat-generating infrared light and reduces the damage to heat sensitive specimens. Single-use sterile disposable surgical scalpels, blade No.21 (Swann-Morton Ltd) were used to separately dissect one area of trophoblastic villi and one area of maternal decidua from unstained slides. An adjacent H&E-stained slide pre-marked by a perinatal pathologist was used to guide the microdissection for each case (Figure 5.2). The dissected tissue was placed in a pre-labelled sterile eppendorf. Histochoice® clearing agent (Sigma-Aldrich, UK) was added to remove wax from the

micro-dissected tissue and DNA was extracted using the QIAamp® DNA FFPE Tissue kit (Qiagen, UK) according to the manufacturer's instructions.(298) DNA quantity was measured using the Qubit® Fluorometer (Thermo Fisher) as described in the product literature.(299)

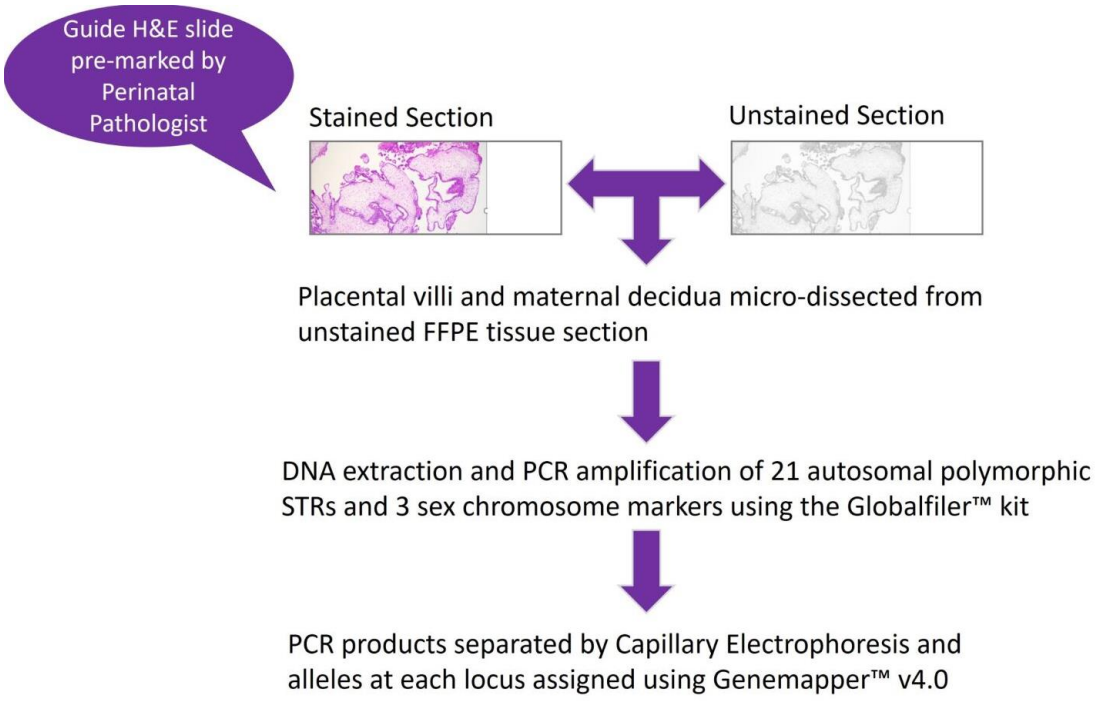

Figure 5. 2: Tissue microdissection and DNA genotyping of fixed tissue from products of conception to establish the genetic origin of hydatidiform moles.
H&E, Haemotoxylin and Eosin stain; FFPE, Formalin Fixed Paraffin-Embedded; PCR, Polymerase Chain Reaction, STR, Short Tandem Repeat.

5.3.1.3 Microsatellite DNA Genotyping

Following DNA extraction from villi and decidua, polymorphic microsatellites known as short tandem repeats (strs) were amplified from DNA (1-4 ng/µl) using the Globalfiler™ Multiplex PCR amplification kit (Applied Biosystems, UK) according to the manufacturer's protocol.(300) This microsatellite assay detects 21 autosomal short tandem repeat (STR) loci (D3S1358, vwa, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656,

D12S391, D2S1338). It also detects three STR's on the sex chromsomes; DYS391, an insertion/deletion polymorphic marker on the Y chromosome (Y indel) and Amelogenin (a sex determination marker) on both the X and Y chromosomes.

PCR amplicons generated from villi and decidua DNA were resolved by capillary gel electrophoresis using an ABI Prism 3500 Genetic Analyser (Applied Biosystems, UK). STR alleles were sized by comparison to a DNA ladder run adjacent to the PCR products during electrophoresis. STR alleles were assigned using genemapper™ Software v4.0 (Applied Biosystems, UK) according to the manufacturer's protocol. Non-maternal (paternal) alleles were identified in trophoblastic villi by comparison to maternal alleles in decidua. Parental allele number and/or peak height ratio in the villi were used to determine ploidy for each case. Triploid conceptuses were expected to have three alleles of equal height at informative loci or two alleles with one allele peak approximately twice the height of the other (2:1 ratio).(301,302) Diploid conceptuses were expected to have two biparental peaks of almost equal height at a locus. Preferential amplification of the shorter length allele is a feature of microsatellite genotyping and was considered when calculating allele dosage and biallelic ratios.(303) The STR genotyping analyst was blinded to the original histopathological diagnosis.

Where low level maternal DNA contamination was present in the villi, the genotype was inferred by adjusting peak heights to compensate for the additional maternal alleles. In such cases, the peak height of the smaller maternal allele provided a measure of the background contamination, and this was subtracted from the larger maternal peak in the contaminated sample.

5.3.2 Statistical analysis

The "rule of 3" power calculation was used to determine the minimum sample size required for the validation study to achieve an acceptable level of confidence in the accuracy of the results.(278) A cohort of 40 POCs were chosen consisting of equal numbers of triploid PHM and diploid non-molar cases to yield equal power estimates of sensitivity and specificity. The sample order for POC analysis was randomised to remove any potential bias in reporting.

The accuracy of the *HER2* D-DISH ploidy assay in identifying triploid PHM conceptuses (sensitivity) and diploid non-molar conceptuses (specificity) was determined by comparison to the reference standard, molecular genotyping. A confidence interval was provided based on the number of successfully genotyped diploid and triploid conceptuses. The overall test accuracy was calculated according to the validation of qualitative tests, by Mattocks *et al*.(278)

5.4 Results

In our study cohort of women with first trimester pregnancy loss, the median maternal age was 34 years (interquartile range, 31-39), and the median gestational age (confirmed by sonography) was 7.3 weeks (interquartile range, 6.4-8.2) (Table 5.1). The average DNA yield for the maternal decidua and trophoblastic villi was 4.0 and 3.5 ng/µl, respectively.

Table 5. 1: Clinical and pathological characteristics of the study population

Clinicopathological parameters	**Age**	**Cohort 1 (n=20)**	**Cohort 2 (n=20)**	**Total reported**
Maternal age† / years	34 (31-39)			
Gestational age† / weeks	7.3 (6.4-8.2)			
Histological Diagnosis: *Morphology + HER2 D-DISH*		PHM (n=20)	Non-Molar (n=20)	40
Genetic Diagnosis *STR genotyping*		PHM (n=19)	Non-Molar (n=17)	36
Genotype		P1P2M1	PM	
Inferred Ploidy		Triploid	Diploid	

† Median (interquartile range), STR: short tandem repeat, D-DISH: dual hapten dual probe in-situ hybridisation, P: paternal, M: maternal, n: number, P1P2: Diandric heterozygous, M1: Monogynic

Genotypes from placental villi were compared to genotypes from matched maternal decidua, for all POCs. Allele nomenclature was determined by DNA fragment size with the smallest sized fragment assigned the lowest allele number. Non-molar diploid POCs had biparental alleles at all informative loci. PHM triploid POCs had three alleles at some loci

and alleles in a 2:1 ratio of (inferred) paternal to maternal peak height at other loci (Figure 5.3).

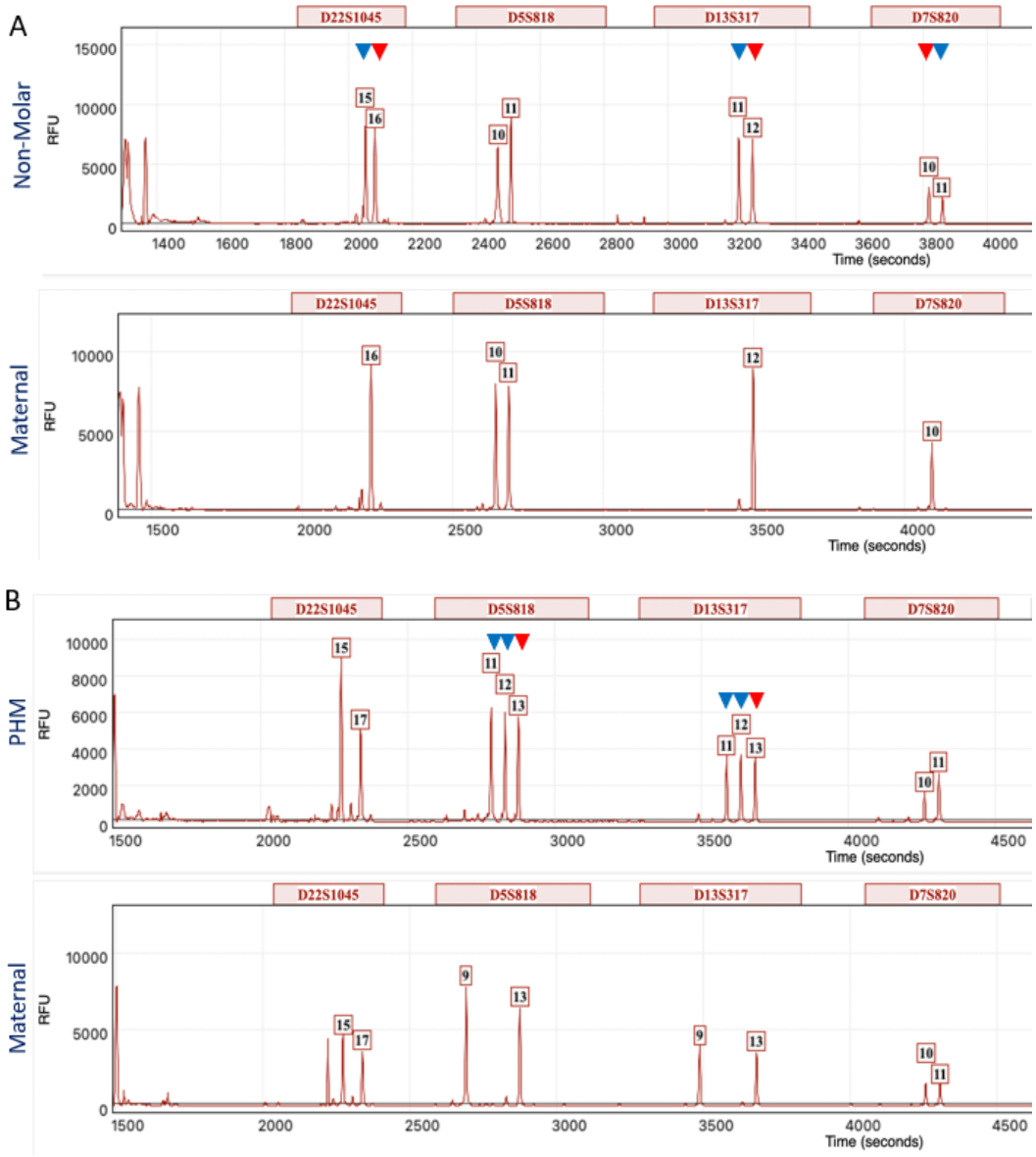

Figure 5. 3: Short tandem repeat (STR) genotyping
showing electropherograms for four loci in POCs. Genotypes from placental villi are shown in the top panel with genotypes from matched maternal decidua in the lower panel. (A) Case 38 showing a non-molar diploid POC with biparental alleles for three loci and one non-informative locus. (B) Case 18 showing a Triploid PHM containing three alleles at two loci (D5S818 and D13S317) and alleles in a 2:1 ratio of (inferred) paternal to maternal peak height for two loci, (D22S1045 and D7S820). A blue arrowhead is used to indicate paternal alleles and a red arrowhead is used to indicate maternal alleles for informative loci. Allele nomenclature is determined by DNA fragment size on the X-axis. The Y axis represents arbitrary units of fluorescence.

We successfully genotyped 36 of the 40 POCs with an STR genotype failure rate of 10% (4/40) (Figure 5.4). STR genotypes, showed complete concordance with the initial diagnosis (Table 5.2). All 19 PHM cases were diandric triploid of dispermic origin. Two non-molar diploid cases had three alleles at a single locus which was suspicious for trisomy 13 in case 1 (D13S317) and trisomy 18 in case 5 (D18S51).

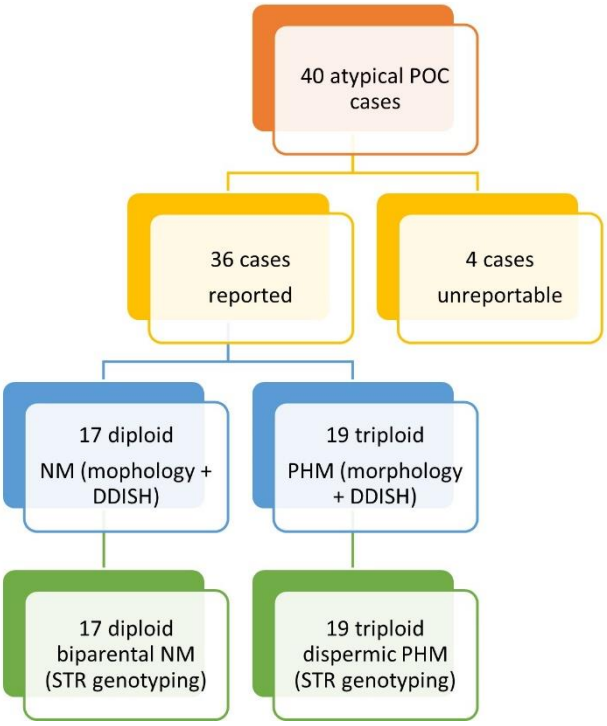

Figure 5. 4: Study results showing 100% concordance between D-DISH and STR genotyping.
NM: non-molar, PHM: partial hydatidiform mole, STR: short tandem repeats

Genotype failure occurred due to scanty villi or maternal decidua in the tissue sections provided. In this study, 5% of the POCs (2/40) had limited chorionic villi (cases 32 and 33) and 2.5% (1/40) had limited maternal decidua (case 20). The case with insufficient maternal tissue had villi with a triploid genotype (3 alleles at 3 loci) supporting a PHM classification. Additional tissue blocks were not available for these cases (following anonymisation) to allow repeat microdissection and DNA extraction. A fourth case (case 13) had very few informative markers and no marker with three alleles so DNA ploidy could not be accurately determined. The laboratory did not have access to an expanded STR genotyping assay which may have provided additional informative loci.

Our STR genotyping did not identify any false positive or false negative triploid PHM results. Accuracy and confidence intervals (CI) were calculated using the "rule of 3" estimate of statistical power for qualitative tests according to sample size.(278) Our *HER2* D-DISH ploidy assay analysed equal numbers of diploid and triploid conceptuses, resulting in 100% sensitivity and specificity (CI: 92%, n=36).

Table 5. 2: STR genotyping results for 40 atypical products of conception

Case	Parental Genotype & Inferred Ploidy	Original Diagnosis	Specific Loci information
2	Diandric Triploid	TRIPLOID PHM	Dispermy: D18S51, D12S91
4	Diandric Triploid	TRIPLOID PHM	Dispermy: FGA, D10S1248
8	Diandric Triploid	TRIPLOID PHM	Dispermy: D18S51, D5S818
9	Diandric Triploid†	TRIPLOID PHM	Dispermy: D18S51, D12S391
15	Diandric Triploid	TRIPLOID PHM	Dispermy: D21S11, D10S1248, D1S1656
16	Diandric Triploid	TRIPLOID PHM	Dispermy: D21S11, D22S1045
17	Diandric Triploid†	TRIPLOID PHM	Dispermy: D18S51, D22S1045
18	Diandric Triploid	TRIPLOID PHM	Dispermy: vwa, D8S1179, D5S818, D13S317, D10S1248, D1S1656
20	? Diandric Triploid*	TRIPLOID PHM	3 alleles at 3 loci but M1 fail
22	Diandric Triploid	TRIPLOID PHM	Dispermy: vwa, D12S391
23	Diandric Triploid	TRIPLOID PHM	Dispermy: D2S441, D16S539
24	Diandric Triploid	TRIPLOID PHM	Dispermy: DS21S11, FGA
26	Diandric Triploid	TRIPLOID PHM	Dispermy: DS21S11, D1S1656
28	Diandric Triploid	TRIPLOID PHM	Dispermy: D8S1179
29	Diandric Triploid	TRIPLOID PHM	Dispermy: FGA, D13S317
34	Diandric Triploid	TRIPLOID PHM	Dispermic: D2S441
35	Diandric Triploid	TRIPLOID PHM	Dispermy: vwa, CSF1PO, D22S1045
37	Diandric Triploid	TRIPLOID PHM	Dispermy: FGA, D13S317, D1S1656
39	Diandric Triploid	TRIPLOID PHM	Dispermy: D10S1248
40	Diandric Triploid	TRIPLOID PHM	Dispermy: D18S51, FGA, D1S1656
1	Biparental Diploid	DIPLOID NM	D13S317 (query Trisomy 13)

3	Biparental Diploid	DIPLOID NM	
5	Biparental Diploid†	DIPLOID NM	D18S51 (query Trisomy 18)
6	Biparental Diploid	DIPLOID NM	
7	Biparental Diploid	DIPLOID NM	
10	Biparental Diploid†	DIPLOID NM	
11	Biparental Diploid	DIPLOID NM	
12	Biparental Diploid	DIPLOID NM	
13	? Biparental Diploid†	DIPLOID NM	Very few informative loci
14	Biparental Diploid	DIPLOID NM	
19	Biparental Diploid	DIPLOID NM	
21	Biparental Diploid	DIPLOID NM	
25	Biparental Diploid†	DIPLOID NM	
27	Biparental Diploid	DIPLOID NM	
30	Biparental Diploid	DIPLOID NM	
31	Biparental Diploid	Diploid NM	
32	Failed Villi*	DIPLOID NM	
33	Failed Villi*	DIPLOID NM	
36	Biparental Diploid	DIPLOID NM	
38	Biparental Diploid	DIPLOID NM	

NM: non-molar, M1: maternal decidua, *Insufficient tissue/DNA, †Genotype results post-subtraction of maternal contamination.

5.5 Discussion

Accurate classification of hydatidiform moles is required for appropriate clinical management and hCG monitoring. It also dictates treatment pathways and helps predict the risk of malignant disease. Despite the availability of specific morphological criteria to guide GTD diagnosis, there is still up to 35% discordance in GTD reporting by both expert and non-expert pathologists. In a centralised pathology review, almost 95% of complete moles were confirmed but only 61% of partial moles, which highlights the need for ancillary techniques to aid diagnosis.(66,304) Fukunaga et al. 2005 found significant inter-observer and intra-observer variability amongst placental pathologists when histology alone was used for diagnosis with consensus reached in only 60% (30/50) of cases.(64) The differentiation of PHM from hydropic non-molar tissue proved the most challenging. The integration of ploidy analysis (flow cytometry) improved concordance amongst pathologists by 10% with consensus reached in 5 additional PHM cases. This review identified a need for more specific histological criteria, particularly for early HM lesions and highlights the importance of integrated ancillary techniques to improve the accuracy of HM diagnosis.(64,305–307) This leads to improved GTD detection rates which provides more accurate incidence rates.(308–310)

Ploidy analysis using our adapted *HER2* D-DISH assay will not identify the genetic origin of PHMs, therefore there was a slight theoretical risk that a digynic triploid non-molar miscarriage could be misdiagnosed as diandric triploid PHM. In a study of 251 cases referred to the gestational trophoblastic centre in Charing Cross, the authors found that in the triploid cases, the parental contribution was paternal in 84 and maternal in only a single case, suggesting that the pathology of digynic triploidy is sufficiently different from PHM that they are recognised as non-molar by experienced pathologists. (311) Additionally, our audit has shown that digynic triploid cases were not represented in our atypical POCs, originally diagnosed by perinatal pathologists as PHM (using morphology and ploidy analysis) and so this is not likely to be a practical concern for laboratories reporting routine specimens. Our finding of dispermy in all PHMs analysed confirmed their diandric origin and is consistent with the reported frequency of dispermic PHMs (98%) in the published literature.(69,312)

Although the risk of developing GTN after a triploid PHM is low, there have been reports of choriocarcinoma and metastatic trophoblastic disease arising after PHM.(185,267) Some earlier studies on the risk of GTN following PHM (0.5-1%) were based on morphology with and without ploidy analysis but these studies lacked confirmatory genotyping.(283,284,306,313,314) Recent publications using STR genotyping to confirm PHM suggest the risk of progression to malignancy may be much lower.(185,267,315) Scholz et al. Provide an extremely low risk (0%) of progression from PHM to GTN based on four cohort studies with 265 patients.(316) Clinical guidelines for GTD management therefore recommend a shorter hCG surveillance period after PHM.(100) Given the lack of consensus on the exact risk of GTN after PHM, patient preference should be considered when choosing chemotherapy, as some PHM cases with persistent disease have normalised without treatment.(317)

In our study of first trimester pregnancy loss POCs, the median gestational age was 7.3 weeks. At this early stage in gestation, some of the classical morphological features of PHM may be subtle or absent on pathology and use of adjunct tools to determine ploidy can greatly assist the diagnosis of PHM. Due to the widespread use of in-situ hybridisation in pathology laboratories, there should be easy access to ancillary techniques such as *HER2* D-DISH for atypical/early PHMs to confirm triploidy and exclude PHM mimics.

Given that *HER2* D-DISH nuclear count signals are quantified from chromosome 17 centromeric probes, there is a possibility that trisomy 17, although rarely encountered in pregnancy loss (318–320), could be mis-interpreted as triploidy if *HER2* D-DISH was used in isolation. Our study was limited by the fact that the STR amplification kit used did not include strs on chromosome 17.While, we were unable to exclude the presence of trisomy 17 in our study cohort, PHM were confirmed as triploid based on genotyping of other chromosomes, excluding the possibility of isolated trisomy 17. Trisomy 17 has never been observed in livebirths and is only found in 1 in 1,000 miscarriages.(276) Also, there is a lack of evidence that trisomy 17 may mimic PHM during initial morphological assessment. Given both of these latter factors, the theoretical risk of misinterpreting trisomy 17 as a PHM during routine practice would seem to be extremely low.

Another limitation of the study is that in-situ hybridisation may be affected by weak staining, potentially leading to under-counting of nuclear signals, and resulting in a missed

triploid conceptus. In our practice, cases with equivocal scores due to poor staining are re-stained to achieve better staining and are often then scored successfully.(297) Some centres use genotyping when weak or equivocal staining is encountered and this practice could be adopted in laboratories with access to STR genotyping. In our audit, all cases initially determined to be diploid based on *HER2* D-DISH were confirmed diploid by molecular genotyping yielding no false negatives in our study cohort.

Adoption of molecular genotyping in suspected cases of PHM to infer ploidy may also detect clinically relevant aneuploidy in non-molar pregnancies and thus provide an additional explanation for the pregnancy loss.(216,321) In our study, two non-molar diploid cases were identified with suspected trisomy (13 and 18). Trisomy 13 has some common features with HM on ultrasound and three cases mimicking PHM have been reported.(65,322,323)

While STR genotyping is an extremely valuable technique, there are some challenges to its application in pathology. Occasionally, scant villous tissue in the first trimester may preclude the use of genotyping to aid PHM diagnosis. Another challenge is the possibility of maternal DNA contamination in trophoblastic villi and vice versa. Dissection of villi for genotyping is technically difficult and contamination of villi with maternal decidua can complicate the interpretation of genotyping results. Clinical practice guidelines recommend dissecting two or more villous regions for each POC to exclude mosaicism (i.e. Two diploid cell lines) and improve the accuracy of genotype interpretation.(66,303) Another limiting factor is the quality of DNA from FFPE tissue which is often degraded due to chemical fixation and this can produce a low DNA yield as occurred in two of our cases. A further limitation is the lack of sufficient informative loci in a conceptus to establish parental origin. In practice, at least one fully informative locus (three different parental alleles) with other loci supporting a finding of triploidy (2:1 ratio of paternal to maternal peak heights) is desirable.(303)

While the financial costs of reagent purchase for in-situ hybridisation and molecular genotyping equipment are comparable, the need for additional scientific expertise to interpret genotyping data may restrict the adoption of genotyping in smaller pathology laboratories. In contrast, many laboratories have access to in-situ hybridisation. Therefore, implementation of the adapted *HER2* D-DISH assay and bespoke “rule of 5”

scoring system for ploidy analysis should not necessitate additional investment.(297) The integration of ploidy analysis into PHM diagnosis will help to improve the accuracy of diagnosis and reduce the reliance on centralised HM review. This approach will provide equity of access for all women to reliable diagnostic services and eliminate delays in the diagnosis of GTD, a highly curable gynaecological disorder.

5.6 Conclusion

It is sometimes stated that morphology with ploidy analysis alone will not definitively differentiate PHM. This is because ploidy determination does not identify the parental origin of the additional genome leading to a theoretical risk of over diagnosing digynic triploidy as PHM. In our practice, morphological assessment by an experienced perinatal pathologist supported by *HER2* D-DISH ploidy analysis does not yield false positive results due to digynic triploidy. Instead, it proves to be a reliable adjunct to HM morphological diagnosis, potentially contributing to more accurate incidence rates. Larger prospective studies from other units would be useful, to determine whether digynic triploidy causes morphological confusion with PHM in others' practice or whether this risk is mostly theoretical. Our study emphasises the importance of the initial morphological interpretation by the pathologist. Although morphological interpretation in HM diagnosis can be challenging, it is still the starting point for nearly all routine tissue diagnostics. In our practice such morphological assessment appears to screen out cases of digynic triploidy. Appropriate use of ploidy determination as an adjunct tool then provides an efficient method of assistance in confirming or excluding PHM. Such an approach should be achievable in many laboratories where it should serve to improve diagnostic confidence, particularly where access to molecular genotyping is limited.

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Contributors: BF, CJ, TMcC and KOD: study concept and design. SD: identified and collated the cases, prepared FFPE slides and anonymised the cases. BF: marked decidua and villi on the master slides. CJ: micro-dissected FFPE tissue, extracted DNA and

performed PCR analysis. GM: helped with microdissection and STR analysis. NS helped with PCR amplification. All authors critically reviewed, edited, and approved the final version of the manuscript and are accountable for the integrity of the work.

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Competing Interests: None declared.

Patient consent for publication: Not applicable.

Ethics approval: This research study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork, Ireland (ECM 4(k) 09/03/2021).

Chapter 6 - An appraisal of hydatidiform mole incidence and registration rates in the Republic of Ireland following the establishment of a National Gestational Trophoblastic Disease Registry

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Chapter 6 - An appraisal of hydatidiform mole incidence and registration rates in the Republic of Ireland following the establishment of a National Gestational Trophoblastic Disease Registry

6.1 Abstract

Aims: This study aimed to reevaluate the incidence of hydatidiform mole (HM) and determine gestational trophoblastic disease (GTD) registration rates in the Republic of Ireland following the establishment of the National GTD Registry in 2017.

Methods: We performed a 3-year retrospective audit of HM cases (January 2017 to December 2019) reported in our centre. In 2019, we surveyed Irish pathology laboratories to determine the number of HMs diagnosed nationally and compared this data to that recorded in the National GTD Registry. Additionally, we compared both local and national HM incidence rates to those reported internationally.

Results: In the 3-year local audit, we identified 87 HMs among 1,865 products of conception (POCs) providing a local HM incidence rate of 3.92 per 1000 births. The 1-year pathology survey recorded 170 HMs in 6008 POCs, yielding a national incidence rate of 2.86 per 1000 births. Importantly, the local HM incidence rate exceeded the national incidence rate by 37% and the local partial hydatidiform mole incidence (1 in 296 births) was 64% higher than the nationally incidence rate (1 in 484 births). Notably, 42% of the HM and atypical POCs diagnosed nationally were not reported to the National GTD registry.

Conclusions: Our study reveals increased HM incidence rates both locally and nationally compared to previous Irish studies. The higher local PHM incidence may reflect more limited access to ploidy analysis in other pathology laboratories nationally. Significantly, almost half of the women with diagnosed or suspected HM were not registered with the National GTD centre.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Access to accurate Hydatidiform Mole (HM) incidence rates is hindered by lack of standardised data collection, the absence of a universal denominator and limited national registration centres worldwide which makes comparative analysis of disease prevalence and treatment outcomes more challenging.

WHAT THIS STUDY ADDS

⇒ This study demonstrates how advances in pathological techniques have improved HM diagnosis, resulting in higher incidence rates in centres with access to ancillary diagnostic techniques. It also highlights the issue of low registration rates with our National Gestational Trophoblastic Disease centre, which compromises the reliability of registry data.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study emphasises the need to reevaluate HM incidence rates given recent advances in pathological diagnosis. It highlights the importance of adopting a universally accepted denominator for accurate HM incidence rates and advocates for mandatory registration to ensure equitable access for all women to expert clinical management of trophoblastic disease.

6.2 Introduction

Gestational trophoblastic disease (GTD) encompasses a range of conditions occurring during or after pregnancy. It is classified by the World Health Organisation (WHO) into two pre-malignant (complete hydatidiform mole, CHM and partial hydatidiform mole, PHM) and four malignant disorders (invasive hydatidiform mole, choriocarcinoma, placental site trophoblastic tumour, PSTT and epithelioid trophoblastic tumour, ETT).(8) A recently proposed trophoblastic lesion, atypical placental site nodule (APSN) is considered a precursor to ETT.(10,11) Accurate subclassification hinges on the identification of distinct pathological and genetic profiles, often necessitating histopathological examination complemented by ancillary techniques.(296) Early detection and accurate diagnosis of GTD coupled with effective, low toxicity chemotherapy affords cure rates approaching 100%.(324)

Hydatidiform mole (HM) represents the most common GTD manifestation and is classified into two genetically distinct entities: CHM characterised by two haploid sets of paternally derived genes (androgenetic diploid) and PHM characterised by one excess haploid set of paternal genes (diandric triploidy). (266,325,326) Ultrasonography in early pregnancy facilitates HM detection, often before 10 weeks gestation when classical histological features are less apparent.(105) In rare instances, a woman may experience more than one CHM due to familial recurrent CHM which is associated with a diploid biparental molar conceptus.(57) Following molar pregnancy, the risk of developing persistent GTD is estimated at 15-20% for CHM and 0.5-5% for PHM.(129) While most cases of persistent GTD occur within 12 weeks of diagnosis, exceptionally longer latency periods have been documented.(327)

Historically, GTD epidemiological data has been limited by inconsistencies in the application of diagnostic criteria, lack of access to specialist diagnostic services, the increasing availability of medical termination and non-standardized reporting of incidence rates.(22) Established risk factors include extreme maternal age and prior molar pregnancies, with additional risk factors potentially linked to diet and low socioeconomic status.(251) A distinct geographical distribution is observed, with the highest incidence in Southeast Asia (10 per 1,000 pregnancies) and lower rates in Western Europe and North America (1-2 per 1,000 pregnancies).(25,26)

Accurate subclassification of molar and non-molar specimens is essential for risk stratification and clinical management.(1,193) Even within specialist referral centers with experienced pathologists using consensus morphological diagnostic criteria, up to 20% of HMs are misclassified.(64) These diagnostic challenges likely influence the HM incidence rates reported. Use of ancillary techniques such as p57 immunohistochemistry (IHC), ploidy analysis (in situ hybridization, flow cytometry) and molecular genotyping, in conjunction with morphological assessment, has improved HM diagnostic accuracy.(220)

Use of p57 immunohistochemistry (IHC), an inexpensive and reproducible adjunct diagnostic test has greatly improved the histological discrimination of CHM from its mimics.(68) In androgenetic CHMs staining for p57 is lost in villous trophoblast and stromal cells. Rarely a mixed pattern of positive and negative staining is observed in villous trophoblasts and stromal cells in a phenomenon termed p57 discordant villi, typically associated with androgenetic/mosaic conceptions.(71) In addition, DNA ploidy analysis using in situ hybridization (ISH) or flow cytometry is integrated with morphological assessment to differentiate diploid (CHM or non-molar) from triploid (PHM) conceptions. Ploidy analysis will not differentiate between diandric PHM triploidy and digynic non-molar triploidy; however, this may not be a practical issue that leads to overdiagnosis of PHM.(275,328) Molecular genotyping using short tandem repeats (STR) will identify the parental genetic contribution and can infer ploidy allowing accurate classification of molar and non-molar pregnancies. Nevertheless, the cost and scientific experience required for genomic analysis has largely limited its widespread adoption in pathology laboratories.

While GTD is the most curable of all gynaecologic malignancies, its comparative rarity has led to inconsistencies in clinical management.(108) For low risk, non-metastatic cases, nearly all patients can expect to be cured and many with advanced disease can achieve remission with appropriate regimens without negatively affecting their reproductive status.(16) Studies have shown that once serum hCG levels normalize, the risk of developing gestational trophoblastic neoplasia (GTN) is very low.(329–331) International experience, most notably that of the UK, has shown that outcomes can be significantly improved with centralized disease registration and hCG monitoring.(27) This allows early specialist input, early detection of disease progression and enables use of effective, low toxicity chemotherapy regimens.(122–124)

In Ireland, the National Cancer Control Programme (NCCP) established a National GTD Registry, Monitoring and Advisory Centre in 2017 to provide multidisciplinary care for this rare disease.(232,332) Women with confirmed or suspected GTD are voluntarily registered with the centre for monitoring and follow-up care based on national clinical guidelines.(27,108)

This study aims to establish local rates of HM diagnosis, determine the number of GTD cases reported nationally, evaluate access to ancillary diagnostic techniques in Irish pathology laboratories, determine registration rates with the National GTD Centre and derive Irish national HM incidence rates.

6.3 Materials and methods

6.3.1 Study design

Two pathology studies were carried out to assess the incidence of HM in Ireland. The first was a local pathology audit at Cork University Hospital (CUH) from 2017-2019, and the second was a national pathology survey involving all 33 pathology laboratories in Ireland, focusing on cases diagnosed in 2019. The clinical audit formed part of a laboratory service evaluation and did not require ethical approval. In addition, the national pathology survey did not collect personal identifiable information, only aggregated laboratory data and was exempt from ethical approval.

6.3.1.1 Local Pathology Audit

We conducted a retrospective review of GTD cases reported in the pathology laboratory at Cork University Hospital (CUH) from January 2017 to December 2019. Study data was collected by interrogating the laboratory database to identify POCs examined, and HM cases reported during the study period. The pathology laboratory at CUH is co-located with a large tertiary maternity hospital (Cork University Maternity Hospital, CUMH) handling approximately 8,000 births annually. The laboratory has access to p57 immunohistochemistry and an adapted *HER2* DDISH assay for ploidy analysis, to aid in HM diagnosis.(297) The pathology laboratory at CUH is accredited to ISO15189 international standards by the Irish National Accreditation Board. During the study period, *HER2* DDISH

staining quality was monitored by internal quality assessment and by participation in two external quality assurance schemes (UK NEQAS and Nordic) and assay performance was deemed satisfactory in both.

6.3.1.2 National Pathology Survey

The national pathology survey was conducted using a questionnaire designed by a multi-disciplinary team comprising members of the Steering Committee of the National GTD Centre. The questionnaire contained 21 questions which sought to determine the number of POCs examined and GTD cases reported in 2019 (Appendix II). Distribution of the survey to laboratory managers in the 33 Irish pathology laboratories was co-ordinated by the NCCP. The survey sought information on POC case volume and rates of GTD diagnoses. Additionally, it sought information on indeterminate cases, where a pathology report indicated that a "hydatidiform mole could not be excluded" or where cases were "suspicious for hydatidiform mole" without a conclusive diagnosis of partial or complete mole. These cases are recorded as "Atypical POCs" in data collection spreadsheets. The survey also collected data on the number of cases with p57 discordant villi.

Respondents were asked to provide details of access to ancillary techniques (p57 immunostaining, DNA ploidy and STR genotyping) within their laboratories. Laboratories were also questioned about their adherence to guidance from the Faculty of Pathology of the Royal College of Physicians of Ireland (RCPI) on reporting GTD cases. The Faculty of Pathology recommends inclusion of the following comment on all GTD reports, "Patient registration with the National Gestational Trophoblastic Centre is recommended". This latter comment aims to promote patient registration in accordance with the National GTD clinical guidelines.(108)

6.3.1.3 GTD Registration and HM Incidence Rates.

Data provided in the questionnaire responses from Irish pathology laboratories was cross-referenced with cases reported to the National GTD Registry in 2019. This allowed us determine registration rates and identify potential gaps in the registration process. The data collected from our local pathology audit and the national pathology survey was compared to previously published Irish and International incidence rates to identify worldwide trends in the prevalence of GTD.

6.3.2 Statistical analysis:

Incidence rates in the local audit and national pathology survey were calculated per 1,000 births. We used "total births" as our denominator and define this as the number of livebirths and stillbirths per annum.(333) We referenced the South/Southwest Hospital Group Annual Reports to establish the total births in CUMH from 2017-2019.(334–336) The national Irish birthrate was taken from the Healthcare Pricing House report for 2019.(333) All data analyses were performed using Microsoft Excel 2021.

6.4 Results

6.4.1 Local Pathology Audit

The local CUH 3-year pathology audit identified 87 HMs in 1,856 POCs (Figure 6.1). This provided an incidence rate of 3.92/1000 births. In total, we identified 12 CHMs and 75 PHMs equating to 1/1,847 and 1/296 births, respectively. This gives a CHM:PHM ratio of 1:6.3 (Figure 6.2) In the local Cork audit (2017-2019), *HER2* D-DISH was requested on 151 POCs which confirmed triploidy in 66 (43.7%) and diploidy in 81 (53.6%) . Four cases were equivocal (2.6%) and nine PHMs were reported without *HER2* DDISH ploidy analysis, giving a total of 75 PHMs.

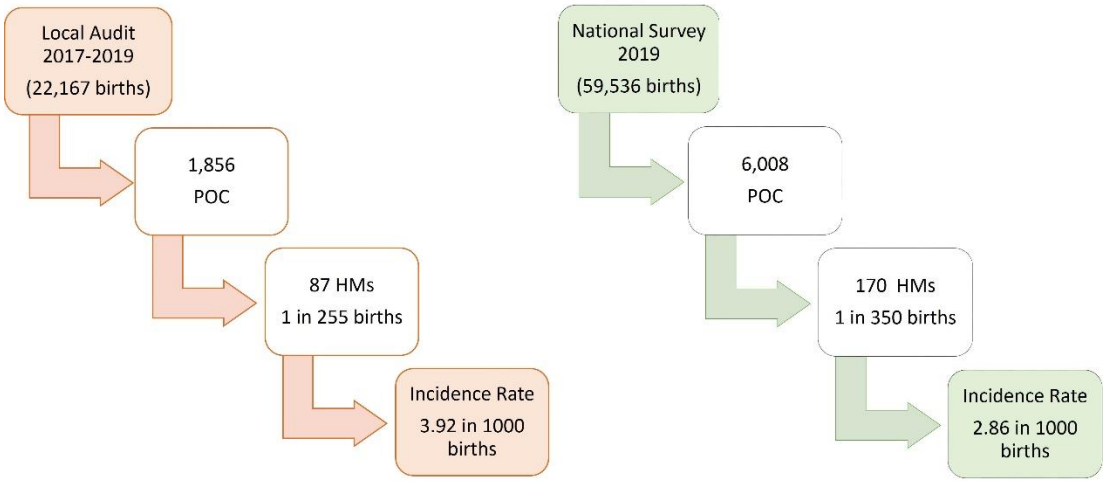


Figure 6. 1: Local and National hydatidiform mole incidence rates.
National birthrate data was sourced from the Healthcare Pricing Office. Local birthrate data was sourced from the South/Southwest Hospital Group annual reports 2017-2019. POC: product of conception, HM: hydatidiform mole, Births: total births (livebirth and stillbirth)

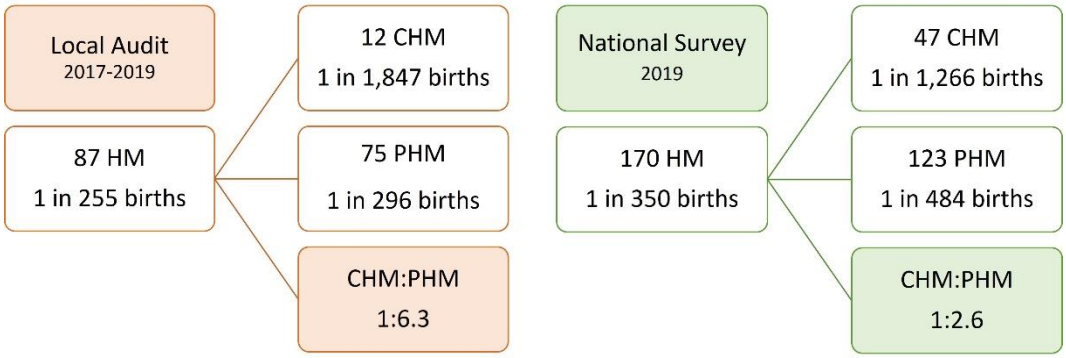


Figure 6. 2: Ratio of complete to partial hydatidiform mole in the local audit and national survey.
HM: hydatidiform mole, CHM: complete HM, PHM: partial HM

6.4.2 National Pathology Survey

The 2019 national pathology survey identified 170 HMs in 6,008 POCs. This provided a HM incidence rate of 2.86/1000 births. There were 47 CHMs and 123 PHMs identified during this period giving incidence rates of 1/1,266 and 1/484 births, respectively. This yielded a CHM to PHM ratio of 1:2.6 (Figure 6.2). Most laboratories (58%, 11/19) analysing POCs had access to p57 immunostaining, two laboratories had access to ploidy analysis (SISH & DDISH) and there was no laboratory with access to molecular genotyping (Table 6.1). Five hospital laboratories handled 62% (n= 3,705) of the workload and four of these hospitals had perinatal pathologists examining and reporting POCs. From the survey responses and follow-up phone calls, we determined that all histopathology laboratories who process POCs, responded to the survey giving a 100% response rate (19/19).

Table 6. 1: Results from the National Pathology Survey 2019

	Classification /n (%)	
GTD Diagnosis (Total POC = 6008)		
HM & atypical POCs	197 (3.3%)	
CMH	47 (0.8%)	
PHM	123 (2.0%)	
Atypical POCs (suspicious of HM)	27 (0.4%)	
APSN	1 (0.02%)	
p57 Discordant villi	1 (0.02%)	
Gestational Choriocarcinoma	3 (0.05%)	
PSTT/ETT	0 (0%)	
Access to Ancillary Techniques[†]	**Yes /n (%)**	**No /n (%)**
p57 IHC	11 (58%)	8 (42%)
Ploidy analysis	2 (11%)	17 (89%)
STR genotyping	0 (0%)	19 (100%)
Faculty of Pathology recommendation on reports[††]	3 (16%)	16 (84%)

[†]Responses from the 19 pathology laboratories who processed products of conception (POC).
[††]Faculty of Pathology recommended comment on GTD reports, "patient registration with the National GTD Centre is recommended".
n: number, %: percentage, CMH: complete hydatidiform mole, PHM: partial hydatidiform mole, APSN: atypical placental site nodule, PSTT: placental site trophoblastic tumour, ETT: epithelioid trophoblastic tumour, STR: short tandem repeat, IHC: immunohistochemistry.

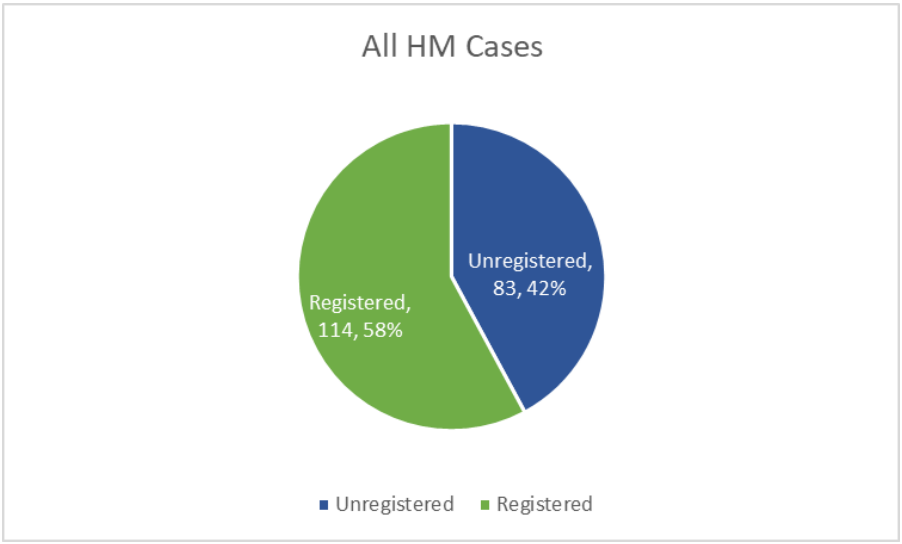
6.4.3 GTD Registration and HM Incidence Rates

A comparison of GTD cases reported in the national pathology survey to those registered with the National GTD Registry in 2019, revealed that 42% of diagnosed/suspected HM cases (83/197) were unregistered. All choriocarcinoma cases reported were registered but 34% of women with CHM (16/47) were unregistered. In addition, 34% of women with PHM (42/123) and 93% of women with "atypical" POCs (25/27) were unregistered (Figure 6.3 and Figure 6.4). A review of the GTD cases registered in 2019, found that 6.5% (2/31) of the CHMs and none of the PHMs required follow-up chemotherapy.

The local HM incidence rate is 33% higher (3.92/1000) than the rate reported in the national pathology survey (2.86/1000). While, the CHM incidence is consistent across

both studies, the local PHM incidence (1 in 296) surpasses the national rate (1 in 484). For a comprehensive comparison of incidence rates from this study with previous Irish studies, please refer to Table 6.2. Additionally, a comparison with international studies is detailed in Table 6.3.

A)



B)

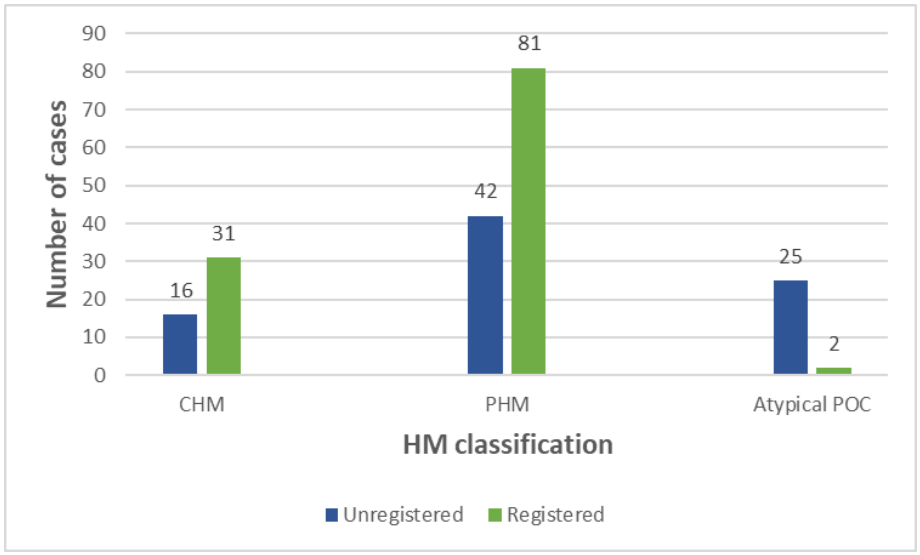


Figure 6. 3: Hydatidiform mole registration rates with the National GTD Registry in 2019

(A) Pie chart showing the total number of HM cases registered and (B) Bar chart showing the subcategories of hydatidiform mole registered and unregistered.

Table 6. 2: Comparison of all Irish HM Incidence Rates

Parameter	Dublin 3-yr audit* 1988-1990	Cork 3-yr audit 2017-2019	Dublin 10-yr audit† 1997-2006	National Pathology Survey 2019
Denominator	19,457 Pregnancies	22,167 Total births	66,296 Total births	59,536 Total births
POC	2,251	1,856	N/A	6008
HM	38	87	262	170
HM Incidence	1.95 in 1000 1 in 521	3.92 in 1000 1 in 255	3.95 in 1000 1 in 253	2.86 in 1000 1 in 350
CHM	10	12	60	47
CHM Incidence	1 in 1,945	1 in 1,846	1 in 1,105	1 in 1,266
PHM	28	75	202	123
PHM Incidence	1 in 695	1 in 296	1 in 328	1 in 484
CHM: PHM Ratio	1:2.8	1:6.3	1:3.4	1:2.6

POC, products of conception HM, hydatidiform mole CHM, complete HM PHM, partial HM yr, year, N/A, not available. *Jeffers *et al.* (28), †Purandare *et al.* (29)

Table 6. 3: Recent studies on HM Incidence Rates

Author	Country	Period /years	Incidence per 1000	Denominator	CHM:PHM ratio
Present study	Ireland	2019	2.86	Total Births	1:2.6
Present study	Cork	2017-2019	3.92	Total Births	1:6.3
Eysbouts(22)	Netherlands	1994-2013	1.67	Deliveries	1:1.3†
Colgan(308)	Canada	2013-2015	3.3	Deliveries	1:1.7
Lund(24)	Denmark	2007-2014	1.79 1.23	Deliveries Pregnancies	1:1
Tham(26)	North England North Wales	1991-1999	1.33	Livebirths	1:1.6
Savage(129)	England and Wales	2000-2009	1.65	Viable conceptions	1:1.3†
Joneborg(23)	Stockholm County Sweden	1991-2010	2.08 1.48	Deliveries Viable conceptions	1:0.8†
Matsui(337)	Japan	1985-2002	1.5	Viable conceptions	1:0.8
Yuk(338)	South Korea	2009-2015	1.10	Pregnancies	Not available

Adapted from Lund et al. 2020(24). †CHM:PHM ratio calculated from the study data provided in the research study.

6.5 Discussion

This is the first study to gather data on national HM incidence rates and GTD reporting practices following the establishment of the Irish National GTD Registry in 2017.

Our local 3-year pathology audit in Cork revealed an incidence rate of 1 in 255 births (3.92 in 1000) which is nearly double the rate reported in a similar 3-year Irish study in Dublin (1 in 512 pregnancies).(28) While both studies reported similar CHM rates, our study found a significantly higher PHM incidence rate at 1 in 296 births, almost 60% higher than the Dublin study. Both studies employed ploidy techniques to assist PHM diagnosis (flow cytometry in Dublin and *HER2* D-DISH analysis in Cork). However, the Dublin study used "pregnancy" as denominator without defining "pregnancy", which limits an accurate comparison between both studies.

Another 10-year Dublin-based study reported a HM incidence rate of 1 in 253 live births, which closely resembles our local rate (1 in 255 births) although denominators differed. In terms of CHM incidence rates, the Dublin study reported higher rates (1 in 1105 live births) compared to our local audit, but PHM incidence rates were similar (1 in 328 live births). Ancillary techniques (p57 IHC and flow cytometry in Dublin) were used to aid morphological diagnosis in both studies.(29) Our local audit reported a CHM:PHM ratio of 1:6.3, indicating a higher prevalence of PHM (relative to CHM). This is consistent with higher PHM rates reported in similar Irish and UK studies although at a lower proportion (Table 6.3).(26,28–30,198,339).

The National Pathology Survey yielded a HM incidence rate of 1 in 350 births (2.85 in 1,000), at least a third lower than our local incidence (1 in 255 births). In addition, the local incidence showed a higher prevalence of PHM (CHM:PHM ratio of 1:6.3) compared to the national survey (CHM:PHM ratio of 1:2.6). This discrepancy may reflect local reporting practice whereby experienced perinatal pathologists have access to *HER2* DDISH ploidy to aid PHM diagnosis.(297) The liberal use of ploidy analysis locally, where it was applied in the examination of 151 of 1856 (8.1%) of POCs and the lack of access to ploidy in all but one centre outside of Cork may contribute to the identification of a higher incidence of PHM in Cork. In the Cork audit (2017-2019) when the *HER2* DDISH assay was used to assist with PHM diagnosis, not too dissimilar numbers of diploid (53.6%, 81/151) and triploid (43.7%, 66/151) conceptuses were identified. This finding reflects and

emphasises the difficulty that exists when trying to diagnose PHM with morphology alone. Information on the pattern of use of ploidy analysis in the only other centre with access to it nationally was not available. Questions raised by variations in diagnostic rates in the survey suggest that data should be collected in future national surveys not just on technique availability but on patterns of use (such as numbers of tests performed as a percentage of POCs received or as an expression relative to the number of HM diagnosed). 27 cases of "atypical POC" were recorded in the national survey in comparison to 170 conclusive HM diagnoses. The National GTD centre can use this information, together with survey data on variation in reported incidence rates nationally, to inform discussions on how diagnostics can be improved and increasingly standardised nationally.

Variations in diagnostic rates may reflect differences in the application of morphologic criteria, access to adjunct diagnostic techniques, and the considerable morphological overlap in early gestation between CHM, PHM and non-molar pregnancies (especially those with aneuploidy).(66,321) Use of ancillary techniques to aid morphological examination has vastly improved the diagnosis of PHM and resulted in an increased incidence of PHM relative to CHM in recent years.(22) This highlights the importance of incorporating ploidy analysis into routine practice when evaluating POCs.

The low adherence to national clinical GTD guidelines and RCPI faculty of pathology guidance on patient registration with the National GTD centre and use of p57 IHC for POCs, highlights the need for improved standardisation of reporting practice.(27,108) Results from the national survey show that in laboratories processing greater than 10 POCs annually, 29% (4/14) did not have access to p57 IHC and 86% (12/14) did not recommend patient registration with the National GTD Centre.

Our gap analysis of the GTD registration process in Ireland revealed suboptimal registration rates with the National GTD Registry, with nearly half (42%, 83/197) of eligible women remaining unregistered. This lapse in registration likely poses clinical risks, especially since 34% (16/47) of women with CHM were unregistered, a condition associated with a higher risk of GTN, necessitating close clinical surveillance. Effective management of GTD relies on a multidisciplinary approach and the registration of patients with expert centres, which is increasingly seen to represent a minimum standard of care.(27,108) Notably, the UK has a nationally coordinated GTD program in place since

the 1970s, contributing to impressive long term survival outcomes.(27) Centralized registration offers opportunities to establish national incidence rates, conduct clinical audits and assess service quality. While voluntary registration may improve with heightened awareness and commitment from healthcare professionals, some expert centres advocate for mandatory registration due to challenges associated with voluntary compliance.(332)

The disparities in HM incidence rates worldwide are likely attributable to inconsistencies in data collection methods, the utilisation of different denominators for reporting, and variations in access to adjunct diagnostic techniques. Our study's findings of increased HM incidence rates are generally consistent with those reported in other European, Scandinavian and North America countries.(22,188,340) For instance, in the Netherlands, a population-based study spanning two decades revealed an increase in incidence rates during the first 10 years, particularly for PHM. Subsequently, the incidence rates stabilised, resulting in an overall HM incidence of 1.67 per 1000 deliveries. This trend was accompanied by a decrease in the number of atypical POCs reflecting advancements in diagnostic techniques.(22) In Canada, a hospital-based retrospective review of HM incidence over a 27-month period incorporating molecular genotyping to aid diagnosis, reported an incidence of 3.3 per 1000 deliveries. This figure corroborates our HM incidence rates, locally (3.92 per 1000 livebirths) and nationally (2.86 per 1000 livebirths).(308) Notably, the CHM:PHM ratio in the Canadian study (1:1.7) shows a lower PHM prevalence than ours but the Canadian study is a much smaller study with 49 HMs (18 CHM and 31 PHM).

Our study has shown that our local PHM incidence rate exceeds both national and international figures. While one might argue that this disparity could be attributed to an overdiagnosis of PHM in our local setting, it should be noted that our adapted *HER2* DDISH ploidy assay did not over-diagnose PHM when audited by molecular genotyping.(275) In terms of a population bias accounting for differences in incidence rates, we did not observe births to younger mothers in Ireland where the average maternal age was 32.5 years compared to the European average (30.6 years). In terms of ethnicity, the majority of babies (77%) were born to mothers of Irish nationality. The use of assisted reproductive techniques (ART) is unlikely to have affected HM incidence rates as ART only received

public funding in Ireland in 2022 and it's availability during the study period was limited to private fertility service providers. Of note, there is no national register of miscarriages in Ireland, so it is difficult to capture all fetal losses.(341) However, the national perinatal mortality report does not identify an increase in the stillbirth rate in our local population (4.0 per 1,000 births) compared to the national stillbirth rate (4.06 per 1000 births) when corrected for congenital anomalies.(336)(342)

Realistically, one would anticipate a higher prevalence of PHM compared to CHM, given that a PHM can occur regularly throughout a woman's reproductive life, whereas CHM is typically age-related.(129) Historically, CHM:PHM ratios of 1:3 have been reported in the United Kingdom.(339,343) Given our results, we suspect PHMs are underdiagnosed nationally and internationally. This raises the possibility that some instances of GTD, which follow seemingly "normal pregnancies," may be linked to undiagnosed PHM. This lends support to the notion that persistent GTD is a rare occurrence following a first-trimester pregnancy loss unrelated to molar pregnancy.(308,344) Furthermore, a recent systematic review found that the progression of PHM to GTN is an exceptionally rare event, occurring in less than 1% of cases.(229) Consequently, the potential underdiagnosis of PHM is unlikely to have a substantial impact on clinical outcomes given the low risk of malignant progression.

Our study has certain limitations, including our reliance on the proficiency of laboratories to extract cases from their databases and accurately interpret pathology coding. Additionally, patient characteristics such as ethnicity and maternal age, which could potentially influence incidence rates, were not collected. Moreover, pregnancy losses that did not undergo histopathological examination were not included in our study.

Accurate HM incidence rates are not readily available worldwide due to a lack of central registries and underreporting of cases to established registries.(126) The comparison of GTD incidence is further complicated by the use of different denominators.(25) While the "total number of conceptions" is the most appropriate denominator for reporting incidence, this is virtually impossible to establish with absolute certainty. Consequently, surrogate measures are employed, leading to variable reporting based on the total number of pregnancies (which, like conceptions, is difficult to define), deliveries, total births, or live births. Additionally, there is variable access to ancillary techniques to aid

HM differential diagnosis worldwide, with some centers relying solely on morphological criteria for diagnosis. This divergence in pathology reporting practices has resulted in inconsistent incidence rates. Given the advancements in obstetric management and pathological diagnosis over the last two decades, a re-evaluation of HM incidence rates is warranted worldwide.

6.6 Conclusion

This study provides the first audit of national GTD data in the Republic of Ireland. Our findings suggest a potential underdiagnosis of PHM nationally and internationally. They emphasise the need to adopt standardised diagnostic reporting of HMs using specific morphological criteria and ancillary techniques. Furthermore, this study advocates for mandatory registration to guarantee equitable access for all women to expert clinical care in a trophoblastic disease centre.

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Contributors: BF, CMJ, CD and CW: study concept and design. CW, SD, CMJ, and BF: preparation of national pathology survey. CD: Distribution of survey. SD and BF: interrogation of LIMS. CMJ, CW, and DCM: analysis of audit data. CK and JC: access to national registry data. CMJ, CW and BF drafted the manuscript. All authors critically reviewed, edited, and approved the final version of the manuscript and are accountable for the integrity of the work.

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Chapter 7 - Measurement of hCG in women with Gestational Trophoblastic Disease

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Chapter 7 - Measurement of hCG in women with Gestational Trophoblastic Disease

7.1 Abstract

Objectives: The objective of this study was to collect information on human Chorionic Gonadotrophin (hCG) laboratory testing and reporting in women with Gestational Trophoblastic Disease (GTD), to assess the associated challenges, and to offer perspectives on hCG testing harmonisation.

Design: Information was collected from laboratories by electronic survey (Survey Monkey®) using a questionnaire designed by members of the European Organisation for the Treatment of Trophoblastic Disease (EOTTD) hCG Working Party.

Methods: The questionnaire consisted of 5 main sections. These included methods used for hCG testing, quality procedures, reporting of results, laboratory operational aspects, and non-GTD testing capability. In addition to reporting these survey results, examples of case scenarios which illustrate the difficulties faced by laboratories providing hCG measurement for GTD patient management were described. The benefits and challenges of using centralised versus non-centralised hCG testing were discussed alongside the utilisation of regression curves for management of GTD patients.

Results: Information from the survey was collated and presented for each section and showed huge variability in responses across laboratories even for those using the same hCG testing platforms. An educational example was presented, highlighting the consequence of using inappropriate hCG assays on clinical patient management (Educational Example A), along with an example of biotin interference (Educational Example B) and an example of high-dose hook effect (Educational Example C), demonstrating the importance of knowing the limitations of hCG tests. The merits of centralised versus non-centralised hCG testing and use of hCG regression curves to aid patient management were discussed.

Limitations: To ensure the survey was completed by laboratories providing hCG testing for GTD management, the questionnaire was distributed by the EOTTD board. It was

assumed the EOTTD board held the correct laboratory contact, and that the questionnaire was completed by a scientist with in-depth knowledge of laboratory procedures.

Conclusions: The hCG survey highlighted a lack of harmonisation of hCG testing across laboratories. Healthcare professionals involved in the management of women with GTD should be aware of this limitation. Further work is needed to ensure an appropriate quality assured laboratory service is available for hCG monitoring in women with GTD.

7.2 Introduction

7.2.1 Challenges with laboratory measurement of trophoblast derived hCG

Hydatidiform Moles (HMs) are abnormal pregnancies which arise following errors in gametogenesis and/or fertilisation, resulting in an abnormal conception. They may exist as either a partial hydatidiform mole (PHM) or complete hydatidiform mole (CHM). The incidence of HM ranges from approximately 0.5 to 1/1000 pregnancies in Europe and North America to 1 to 2/1000 in Japan and China, to 12/1000 pregnancies in Indonesia, India and Turkey (22,345).

HMs belong to a larger family of rare diseases known collectively as Gestational Trophoblastic Disease (GTD) which, in addition to the pre-malignant HMs, also include the malignant conditions of invasive mole, choriocarcinoma, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). These latter malignant conditions may be referred to as gestational trophoblastic neoplasia (GTN).

HMs are characterised by abnormal trophoblastic proliferation, with marked hyperplasia and hydropic swelling of the chorionic villi. Whilst there may be fetal development in PHMs, the absence of a fetus is a well-known characteristic associated with CHMs. Both PHM and CHM have an associated risk of developing into GTN and therefore accurate diagnosis is very important to guide correct patient management. Whilst the risk of progression to malignant disease, at least in the United Kingdom (UK) population, is lower (0.5-1%) for PHM compared with CHM (15-20%) (30), monitoring after primary treatment, for progression to GTN is recommended regardless of molar phenotype (27).

Monitoring of women diagnosed with an HM is undertaken by serial measurement of hCG levels in blood or urine. In most cases, the follow-up period is uneventful, and women do not require any further clinical intervention. However, if there is persistent elevation of hCG levels, further surveillance is required to reduce the likelihood of progression to GTN. Treatment for GTN is generally by chemotherapy, of which low and high-risk treatment regimens are undertaken depending on the stage and classification of tumour determined by the International Federation of Gynaecology and Obstetrics (FIGO) scoring system (135).

Over the decades, several studies have explored the potency of different molecular biomarkers to enable early detection of GTN. These biomarkers have included, cell cycle regulators, cell adhesion molecules, heat shock proteins, anti-apoptotic-, oncogenic- and tumour suppressor genes (346–351). To date, hCG is still the biomarker of choice and is universally recognised as an integral component in the clinical management of patients diagnosed with GTD (100). The length of hCG follow-up after HM is dependent on the molar classification (PHM or CHM) and is in accordance with international guidelines. Of note, there is a longer surveillance period after hCG normalisation for those diagnosed with CHM due to the increased risk of developing GTN (30).

7.2.2 hCG and its molecular forms

hCG is expressed from the syncytiotrophoblast cells and is commonly known as the pregnancy hormone (352,353). hCG is composed of two non-covalently bound subunits (α- and β-). The α-subunit of hCG comprises 92 amino acids and is identical to the α-subunit of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Thyroid Stimulating Hormone (TSH). The hCG ß-subunit is composed of 145 amino acids and distinguishes hCG from other pituitary-produced glycoproteins. The hCG α- and ß-subunits are coded by different genes and synthesis is regulated independently during pregnancy to produce a subset of isoforms, which can vary during pregnancy progression (73,354). In GTD, the production and proportion of subunits is different to that of a normal viable pregnancy and greater variety of hCG molecular forms exist (355,356). The different variants include intact hCG (α- and ß- heterodimer), free hCG α-subunit (hCGα), free hCG β-subunit (hCGß), nicked free hCG β-subunit (hCG ßn), and hCG ß-subunit core fragment (hCG ßcf) (76,357). In an uneventful pregnancy, concentrations of intact hCG in blood double approximately every 2 days to reach a peak at 8-10 weeks of gestation. From week 10 to 20, these concentrations decline to levels comparable to those in early first trimester and from 20 weeks on they remain constantly low (358). Like many other polypeptide hormones hCG is heavily glycosylated (hyperglycosylated) with N-and O-linked sugar side chains attached to each hCG molecule. Hyperglycosylated hCG is produced by cytotrophoblast cells and enhances cytotrophoblast cell growth and cell invasion (74). Hyperglycosylated-hCG declines more rapidly than hCG in the first weeks after implantation, in an uneventful pregnancy (76,359). Hyperglycosylated hCG is also

produced by invasive or malignant trophoblast cells in choriocarcinoma, and this is the reason why hyperglycosylated hCG is referred to as Invasive Trophoblast Antigen (ITA) (360). ITA has been considered as one of the most effective biomarkers for the detection of invasive trophoblastic disease in patients undergoing GTD surveillance (360,361).

7.2.3 Quantification of hCG

Most diagnostic clinical laboratories measure hCG by immunoassay. Some clinical laboratories may choose to measure hCG using a laboratory developed test (LDT), whereas other laboratories rely on commercially available immunoassays on random access analysers or use a combination of both. The major laboratory challenge associated with hCG measurement in GTD patients is the detection of all hCG isoforms equally (82).
In 1956, Berson and Yalow introduced the concept of radioimmunoassay (RIA) for assessment of blood insulin levels (362). The principle of this technique is based on the competition of radioactive labelled analyte (tracer) with unlabelled analyte (sample) to bind an anti-analyte antibody. As the labelled and unlabelled analytes compete for a limited number of binding sites on the antibody, concentration of analyte in the sample can be determined by the amount of radioactivity bound by the antibody (shown in Figure 7.1A). To date, the UK-GTD centres (based in London, Sheffield, and Dundee) and the Dutch Central Registry for HM (based in Nijmegen) use RIA for detection of hCG in GTD patients. These assays utilise antisera specifically directed against the free hCG β-subunit (363). Although the UK and Dutch laboratories developed their assays separately with different hCGβ antisera, they both apply the same principle of using a primary hCGβ antibody to detect the major hCG isoforms. Both the Dutch and UK assays were developed in the 1970's and are still in use by their respective central hCG reference service laboratories today (364,365).

In the 1980s, the concept of a two-site 'sandwich'-type immunoassay was developed where two antibodies are used to bind the analyte (e.g., hCG). One antibody is immobilised on a solid surface (reaction tube, solid phase particle or plate wall) and captures the analyte, while the other antibody is labelled with a detection molecule (tracer) to generate a signal (shown in Figure 7.1B). Commercial hCG immunoassays mainly use the 'sandwich'-type hCG assay format which relies on the presence of 2 conserved epitopes. These assay methods are most notably suited to the application of

pregnancy detection in which circulating hCG variants are predominantly intact and exhibit the conserved epitopes. The hCG isoforms found in trophoblastic disease may not always be detectable in a 'sandwich'-type assay as these variants may not contain the conserved epitopes for efficient antibody capture (366).

The challenges associated with appropriate detection of hCG isoforms in GTD patients is well-documented and the recognition of hCG variants by diagnostic immunoassay has long been recognised (shown in Table 7.1) (82). To encourage standardisation, the International Federation of Clinical Chemistry (IFCC) preparations of hCG isoforms are available for use as WHO reference standards in hCG immunoassay platforms (77). Additionally, international collaborations and workshops studying the properties of hCG monoclonal antibodies commonly used in these immunoassays have provided calibration guidelines to improve comparability (367).

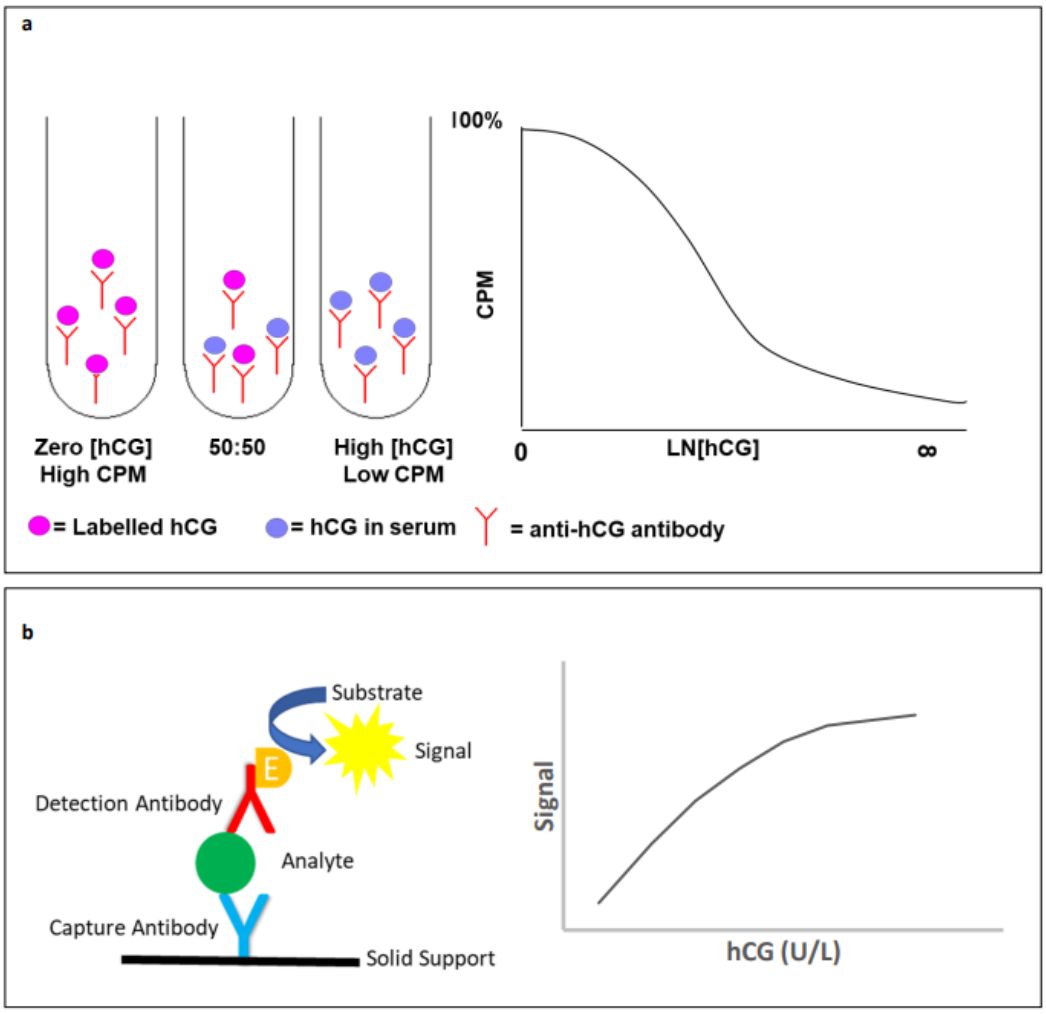


Figure 7. 1: hCG Immunoassay

Figure 7.1a. One-site competitive radioimmunoassay (RIA) showing the competition of unlabelled hCG in the patient's sample with radioactively labelled hCG. The anti-hCG antibody is raised to a single epitope (β1) which is common to all forms of hCG. Following equilibration and separation of components, the radioactivity in the patient sample is compared to a standard calibration curve. The signal obtained is inversely proportional to the concentration of hCG in the patient's sample as shown in the logarithmic (LN) curve. CPM = counts per minute

Figure 7.1b. Two-site enzyme immunometric assay showing the capture of analyte (hCG) by an immobilised antibody. A subsequent wash step removes unbound analyte and the detection antibody binds to a different epitope on the analyte to form a sandwich. The detection antibody is conjugated with an enzyme (E) which converts the substrate into a measurable product. The signal generated is directly proportional to the concentration of analyte (hCG) in the patient's serum as shown in the reaction curve.

Table 7. 1: hCG immunoassay parameters in commercially available assays and laboratory developed tests.

Manufacturer	Roche	Abbott	Beckman Coulter	Siemens	RIA
Analyser	Cobas® Elecsys	ARCHITECT	Access DxI	IMMULITE®/ Atellica®	UK LDT
Analyte measured	Total + free β-hCG	Total β-hCG	Total β-hCG	Total β-hCG	Total β-hCG
Urine hCG assay available, decision threshold	No	No	No	Yes, qualitative <30 IU/L	Yes user defined
Serum hCG assay for Oncology©	Yes	No	No	No	Yes
International Standard (IS)	4th IS (75/589)	4th IS (75/589)	5th IS (07/364)	3rd/4th IS (75/537) (75/589)	4th/5th IS (75/589) (07/364)
Serum hCG (U/L), decision threshold (95% CI) *Dutch RIA assay	<5.3 <8.3 pm	<5.0	<1.0 <8.0 pm	<2.2	<5.0 <2.0* ng/ml
Limit of detection (U/L)	<0.1	NA	<0.5	<0.4	0.39 0.37* ng/ml
High dose hook effect	>750,000	NA	>1,000,000	>2,000,000	NA
hCG isoform recognition†					
hCG nicked	+	+	+	+	+
hCG βeta	+	+	+	+	+
hCG βeta nicked	+	+	+	+	+
hCG βeta core fragment	+	-	-	+	+

†Adapted from Sturgeon et al., 2009 and Stenman et al., 2006 (82,96)

7.2.4 Monitoring of GTD-derived hCG

Early identification of women who develop GTN, even before evacuation of molar tissue is the ultimate goal. Currently there is limited clinical or scientific criteria to aid the prediction of GTN development at an early stage. Available studies are mostly retrospective and so far, none of these reach the top grade of evidence (level 1), which requires prospective multicentre studies (368). Furthermore, treatment strategies need to be optimized in order to obtain hCG normalisation in women with minimum chemotherapeutic side effects and at the earliest opportunity, as these women are often anxious to conceive as soon as possible.

Whilst there have been many publications acknowledging the diagnostic challenges associated with the measurement of GTD-derived hCG by immunoassay, this paper summarises the current issues involved in hCG measurement. It also offers practical advice on hCG result interpretation in the context of GTD, for both the scientific and clinical teams involved in the care of these women, including challenging or equivocal cases.

7.3 The EOTTD hCG survey

To gain further insight into laboratory hCG testing for GTD-patients, an in-depth hCG questionnaire was designed and distributed by the hCG working party of the European Organisation for Treatment of Trophoblastic Disease (EOTTD) to all member laboratories (Appendix III). The rationale for conducting this survey was to investigate how laboratories utilise hCG assays for the management of women with GTD.

The online structured questionnaire was distributed to approximately 200 EOTTD laboratory contacts. Twenty-one respondents from 13 countries, including The Netherlands, UK, Norway, Republic of Ireland (ROI), Portugal, Poland, Switzerland, Czech Republic, Slovakia, Japan, Belgium, Germany and Spain participated in this study. Unfortunately, five responses could not be used as the questionnaires were incomplete.

Participants were asked to complete 5 sections which focused on the methods used for hCG testing, quality procedures, reporting of results, laboratory operational aspects, and information on non-GTD sample testing. The questionnaire was distributed to the head of laboratory services in the field of GTD and therefore the answers provided are obtained

from the collective perspective of dedicated clinical chemists within this specialised area. Some of the participating laboratories have known dedicated GTD referral centres, including the UK, ROI, Switzerland, Norway and The Netherlands.

We obtained a response rate of 8% (16/200), and the questionnaire provides a valuable snapshot of current clinical laboratory practice in different countries. From participant feedback, it is clear further discussion on hCG measurement is needed across the wider scientific and clinical GTD community. There is also a desire for the development of harmonisation guidelines for hCG measurement in women with GTD.

7.4 Survey sections and results

7.4.1 Section 1 - Methods used for hCG analysis (shown in Figure 7.2)

To gain insight into the hCG testing methods used within laboratories, an investigation of the most common assays used for quantitative measurement of hCG in GTD patients was evaluated. Most users (56%, 9/16) undertook hCG testing in GTD patients using the Roche Cobas platforms. The remaining laboratories used either an in-house developed competitive Radioimmunoassay (RIA) (19%, 3/16), Siemens (19% (3/16) or Abbott (6%, 1/16) platforms. The only commercially available quantitative hCG assay approved to date, for use in oncology is the Roche Elecsys assay as noted in the accompanying instructions for use (IFU) literature (369). For those laboratories using LDTs all stated extensive validation had been performed on GTD patient samples. For those using the commercial kits of Siemens and Abbott, all stated they were aware the test was not validated for use in oncology, but half (2/4) of the laboratories had performed in-house validation on GTD samples.

All Roche users tested serum or plasma, but two laboratories also tested urine and cerebrospinal fluid (CSF). The Siemens Immulite user performed hCG testing on serum or plasma, but the Siemens Atellica user did additional testing on urine and CSF samples and the Siemens Centaur user tested plasma only. The Abbott Architect user tested serum only.

WHO International standards (IS) are routinely used as reference preparations for the manufacture of calibrators for commercial immunoassays. Preparation, analysis and evaluation of these standards are controlled by WHO guidelines and involve both bioassay

and immunoassay analysis by an "international collaborative study with expert laboratories". This analysis allows for the calibration of hCG therapeutic preparations used in bioassays, and the determination of immunoreactivity and suitability of standards as a calibrant for hCG immunoassays (370). Currently the 6th IS for hCG has been approved for use and shall replace the current 5th IS as supplies are close to exhaustion. Earlier IS preparations (3rd and 4th IS) were originally intended for bioassay rather than immunoassay use. In addition, impurities in these preparations and presence of nicked forms of hCG prompted preparation of new standards (371). The purer 5th IS was introduced in 2009 to allow hCG results be reported in molar units and allow easier comparison of the different hCG isoforms (372).

Results showed that most labs (69%, 11/16) used an assay calibrated to the 4th IS (9 Roche, 1 Siemens (Atellica) and 1 Abbott), 2 labs used an assay calibrated to the 5th IS (both in-house RIA), and the remaining labs used assays calibrated to the 3rd IS (Siemens Immulite and in-house RIA). The majority (94%, 15/16) of users reported values in IU/L or mIU/mL, with one user reporting values in ng/ml. We asked laboratories to provide the sensitivity value (limit of quantification (LOQ)) for their test, and values were reported from 0.1 IU/L to 6 IU/L. The differences in LOQ values may reflect the difference in assay used, however, even within the same group of users (eg. Roche Elecsys), different LOQ values were reported (0.1, 0.25, 0.4 and 0.5 IU/L). These results suggest there is a lack of consensus surrounding the LOQ adopted by laboratories using the same commercial immunoassay.

Roche hCG assay defines specificity for the "holo-hormone, "nicked" forms of hCG, the β-core fragment and the free β-subunit, however some users stated measurement of either "total hCG" or "hCG + beta". The Abbott Architect user stated the test was specific for intact hCG, nicked and beta-hCG, whilst it is stated in the IFU kit insert that the test is only suitable for intact hCG and free β-hCG. The three Siemens users did not clearly define hCG specificity. RIA users stated "all" hCG isoforms were detectable in their assays. Inevitably, results show the interpretation of the IFU by users may be different, regardless of the same manufacturer guidelines and highlights again the variability across laboratories when using the same assay platforms.

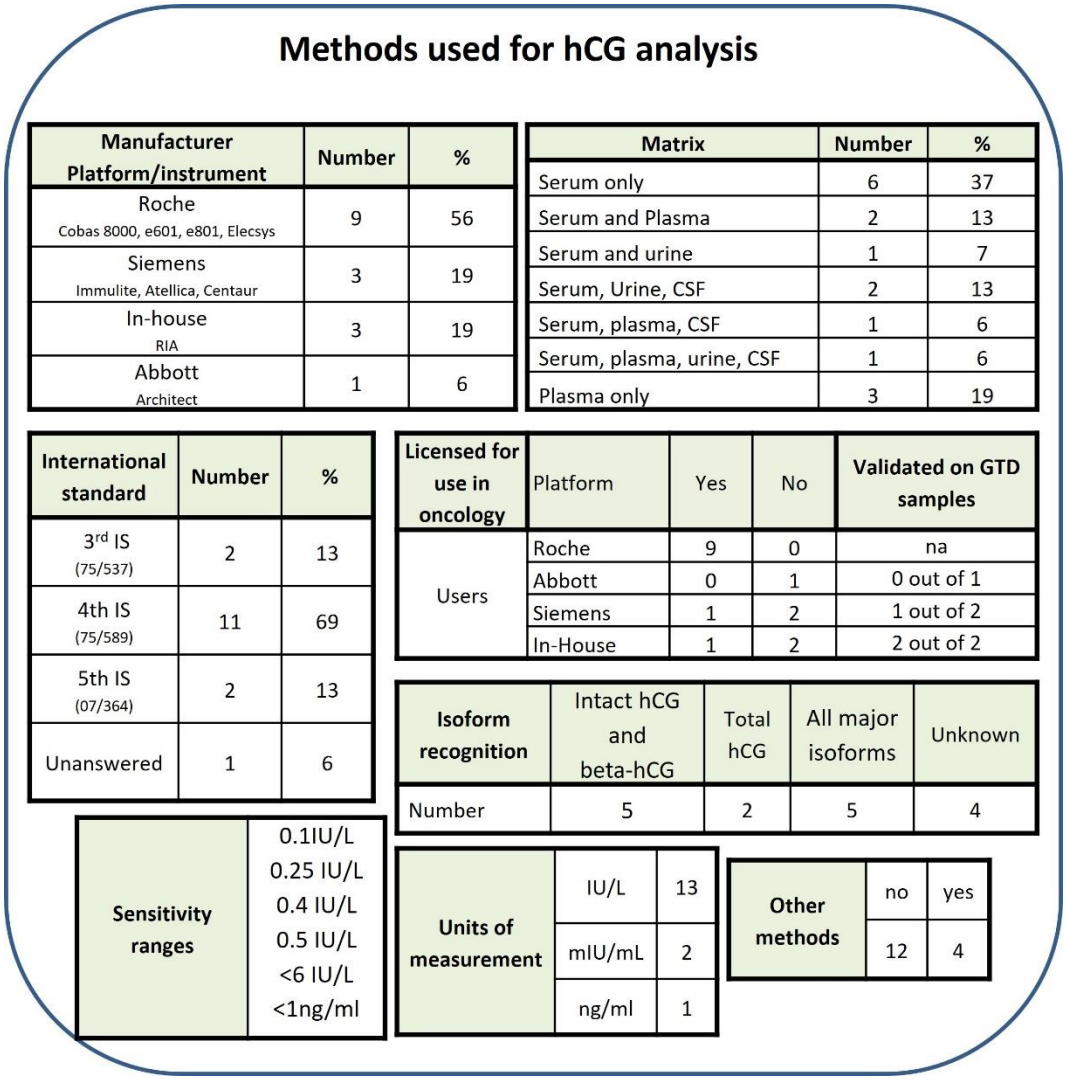


Figure 7. 2: Methods used for hCG analysis.
Users were asked to submit information regarding manufacturer/platform or instrument used; matrix tested; international standard (IS) used; if the hCG test used was licensed for oncology; the hCG isoforms recognised by the test used; the sensitivity of the hCG test (LOQ, limit of quantitation); reported units of measurement; and if other hCG test methods were used.

7.4.2 Section 2 - Quality aspects (shown in Figure 7.3)

Labs were asked to provide information on the quality control (QC) measures currently used for their tests including the type of internal controls, QC level ranges, or other parameters, and to indicate if they participated in any external quality assurance schemes for hCG testing or if their laboratory/department had gained nationally recognised accreditation from an external body.

Of those users who answered, the majority (70%, 9/13) used commercially available QC controls, two used in-house QC pools only (in-house RIA users) and two used both in-house and commercial QC's (in-house RIA user and Roche user).

A plethora of other quality parameters were reported including precision, coefficient of variation (CV) and acceptable (reporting) range. The most commonly used parameter in addition to QC controls, was %CV, either on its own, or in conjunction with precision profile or acceptable reporting range. No clear relationship existed between assay used and the quality parameters reported.

Of the 16 laboratories, 88% (14/16) participated in an external quality assessment scheme for hCG measurement and approximately half (56%, 9/16) were accredited to ISO15189:2012 standards with 2 laboratories accredited to ISO9001:2015, and the remaining 3 labs left this question unanswered.

External quality assessment (EQA) aims to ensure work is performed in an accurate and quality assured manner. Satisfactory EQA performance provides the clinician, and patient, with confidence and assurance that laboratory processes are monitored and measured for quality and consistency. For clinical diagnostic laboratories who report clinically relevant results, participation in EQA schemes is a key requirement for laboratory accreditation and clinical governance.

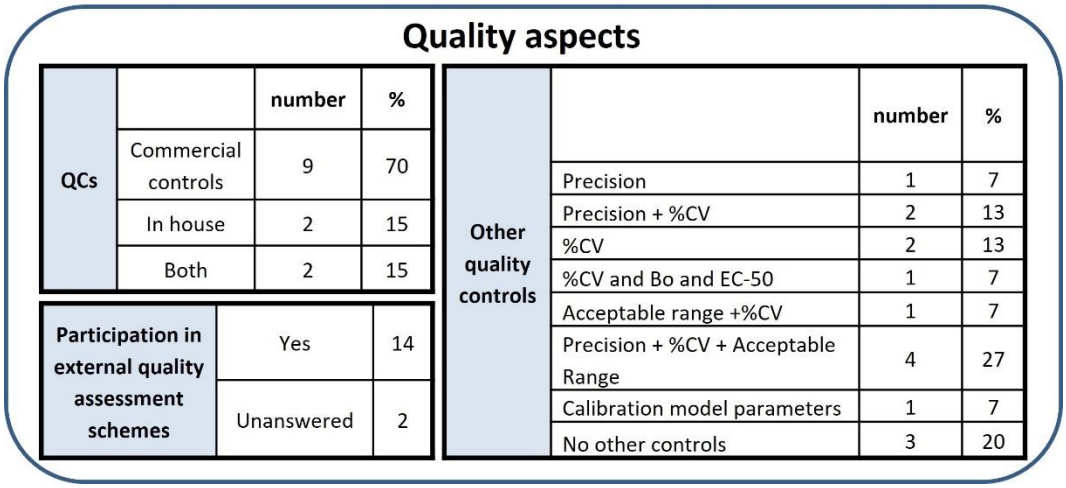


Figure 7. 3: Quality.
Users were asked to provide information on the source of quality controls (QCs) used; quality control parameters used for reporting hCG levels; and participation in external quality assessment schemes. CV=coefficient of variation. B0 = radioactivity bound (percentage) in absence of unlabelled analyte (i.e. maximal binding). Note not all responders answered every question.

7.4.3 Section 3 – Reporting (shown in Figue 7.4)

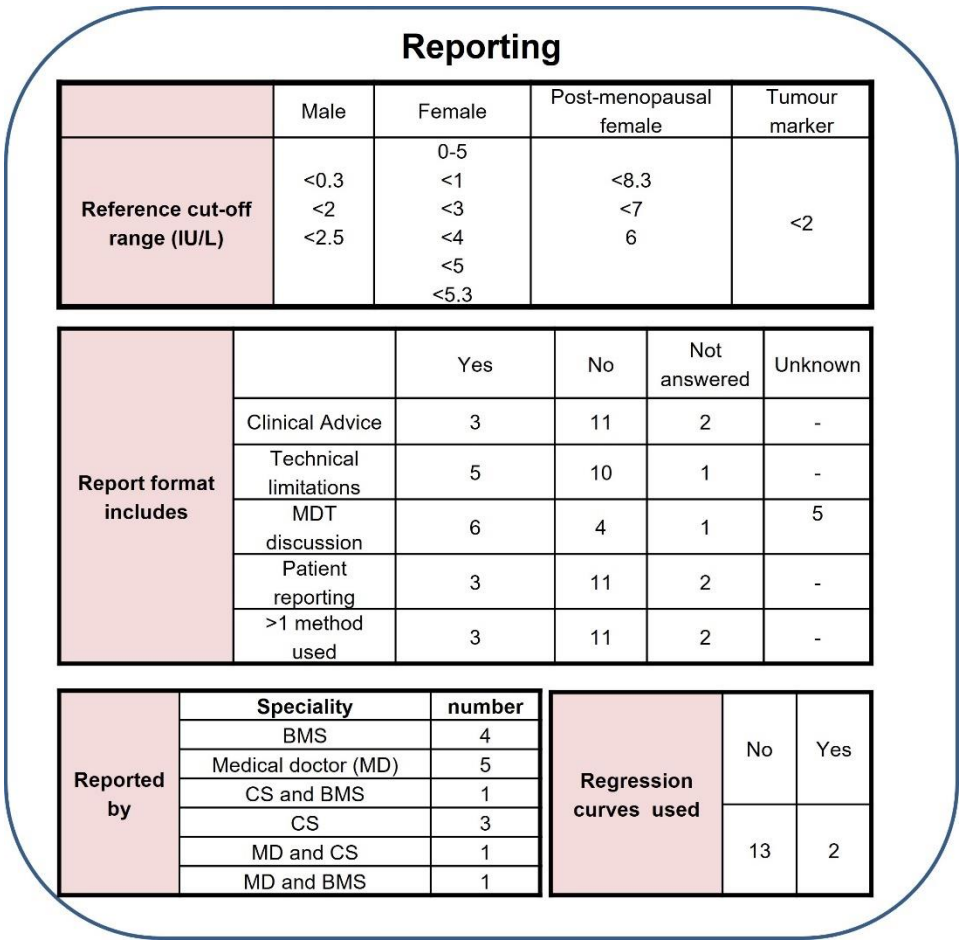
Information on reporting criteria were evaluated including type of raw data analysis, reference ranges and clinical cut-offs quoted, use of regression curves, persons responsible for reporting, multidisciplinary team (MDT) discussion and type of information included in the report. Reference ranges and clinical cut-offs used varied widely and did not always correlate with the IFU kit insert. Two responders out of 15 used regression curves (post-evacuation, post-methotrexate) to evaluate data, and of those who answered, three (21%, 3/14) laboratories displayed graphical results showing the longitudinal hCG levels over time.

Persons responsible for reporting hCG values to the referring clinician ranged from a medical doctor to registered scientists (clinical scientist (CS), biomedical scientist (BMS)) and this appeared to be institute dependent.

The report format tended to vary widely. Most reports did not offer clinical advice or interpretation (69%, 11/16), which may reflect the grade of staff who are authorised to report. The technical limitations of the assay were not included in the report in 63% (10/16) of cases. Only a small proportion of laboratories (38%, 6/16) mentioned their

reported hCG results are discussed at MDT meetings as part of patient care. Three laboratories reported results directly to patients. More than one hCG assay was used in 19% (3/16) of institutes, of which one laboratory reported both hCG results (Roche and Abbott, where the Abbott test serves as a rapid result with Roche serving as a confirmatory result) with the other 2 laboratories reporting in-house RIA results only.

Overall, reporting varied widely amongst laboratories and whilst we cannot explain these differences, many factors could contribute, including lack of centralised hCG testing, lack of specialised scientific personnel, or lack of similar or shared laboratory information systems. To address this, we plan to seek further information from laboratories in a future survey.



Reporting

	Male	Female	Post-menopausal female	Tumour marker
Reference cut-off range (IU/L)	<0.3 <2 <2.5	0-5 <1 <3 <4 <5 <5.3	<8.3 <7 6	<2

		Yes	No	Not answered	Unknown
Report format includes	Clinical Advice	3	11	2	-
	Technical limitations	5	10	1	-
	MDT discussion	6	4	1	5
	Patient reporting	3	11	2	-
	>1 method used	3	11	2	-

	Speciality	**number**
Reported by	BMS	4
	Medical doctor (MD)	5
	CS and BMS	1
	CS	3
	MD and CS	1
	MD and BMS	1

	No	Yes
Regression curves used	13	2

Figure 7. 4: Reporting of hCG results.
Users were asked to provide their reported reference range cut-offs for positive results; the information included in the report format; person/s responsible for reporting; and if regression curves were used and reported.

7.4.4 Section 4 – Laboratory Operational Aspects (shown in Figure 7.5)

Reporting times ranged from 30 minutes to 2 working days, most likely reflecting the nature of the assay platform in use, laboratory logistics and/or referral reason. For instance, for those GTD patients on routine follow-up the majority will not require a rapid hCG result. However, women who present with suspected GTN or ectopic pregnancy, may require a rapid hCG result to define diagnosis or guide treatment strategies.

The majority (88%, 14/16) of laboratories offered urgent hCG testing. Of the two laboratories which did not, one of these reported hCG results within 1.5 hour and the other laboratory did not offer urgent testing using their in-house RIA but had access to rapid hCG testing in their Clinical Chemistry department. The number of GTD hCG samples tested per annum ranged from 24 to 25000 and the number of GTD patients tested annually ranged from "I don't know" to up to 500, revealing that some centres test very few samples, whilst others have a much higher throughput.

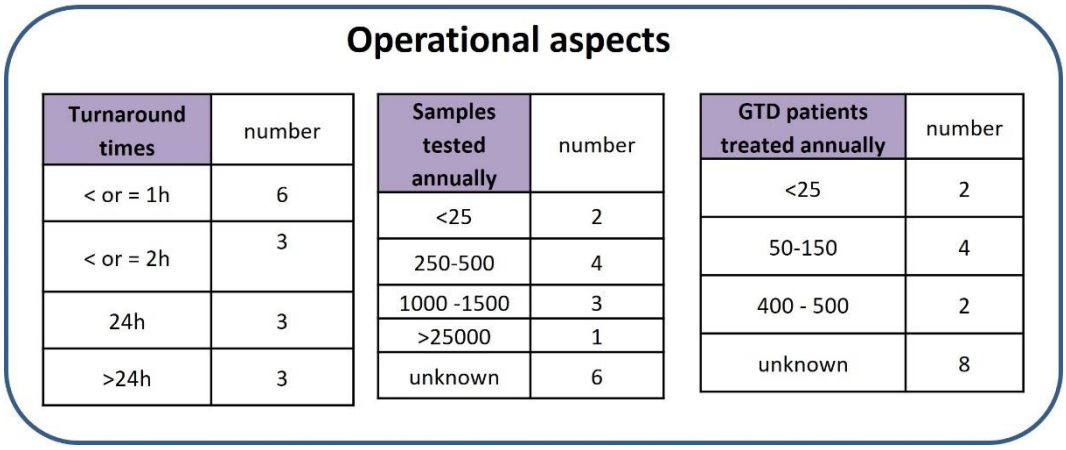


Figure 7. 5: Laboratory operational aspects.
Users were asked to report on test result turnaround times; how many hCG samples were tested annually; and how many GTD patients were treated annually.

7.4.5 Section 5 - Non-GTD sample information

Interestingly, 94% (15/16) of laboratories also tested hCG for non-GTD conditions which included early or ectopic pregnancy, germ cell tumours and testicular cancer. The majority (93%, 14/15) used the same assay platform for these tests. One respondent used a Roche platform for GTD samples, and Abbott Architect for non-GTD samples, specifically for

pregnancy monitoring. Siemens Immulite and Siemens Centaur users utilised these assay platforms for hCG testing associated with testicular and ovarian germ cell tumours.

7.4.6 Observations and conclusions from the EOTTD survey

Whilst we fully appreciate the number of responses were low (8%, 16/200), some valid issues were raised which should prompt further discussion across the GTD scientific community. It is apparent the challenges in measurement of GTD-derived hCG are long-standing. Although most labs are accredited to ISO15189, it does not mean the assays used for measurement of hCG in women with GTD are appropriate. The survey showed a lack of a harmonised approach (eg. LOQ, reference ranges, reporting criteria, QA) across laboratories providing hCG testing for GTD surveillance and highlights the need for centralised services.

Within today's modern healthcare, the standardisation of laboratory practice through laboratory accreditation, implementation of managed service contracts and high-throughput track systems in large clinical chemistry departments, plus adherence to healthcare policies and procedures associated with clinical governance has made most diagnostic laboratories dependent on commercial random access assay platforms. Commonly, departments are fixed into long-term service contracts for financial and economical utility, with limited access to esoteric assays under these contracts. Custom-made in-house assays or, assays which are only used for a subset of referrals, are becoming increasingly difficult to justify from both a legal (e.g., EU IVD-regulations) and clinical governance perspective (373). Whilst EOTTD practical clinical guidelines encourage women to be registered with a specialised GTD centre, they do not specify which hCG laboratory assay is most suitable other than the assay should recognise all major hCG isoforms (100). Therefore, some laboratories providing hCG results for women with GTD may use hCG assays which are not approved for use in oncology.

The responses collected from the present EOTTD survey clearly highlight variation in assessment of hCG levels in GTD patients across countries. We however recognise the limitations of the survey itself which include survey design and distribution where we intentionally only targeted EOTTD member laboratories, when in effect many other laboratories analyse GTD samples. We also recognise this is the first time to date a comprehensive survey has been performed. In particular, the survey did not ask any

questions relating to the knowledge of the participating laboratories surrounding national policies on hCG testing in GTD patients or if they were associated with a specialist GTD centre. Future work is needed to address these specific areas.

7.5 Educational examples

A possible solution to address the variation in hCG results observed across laboratories involved in the care pathway of GTD patients may be to acknowledge and outline the limitations of the hCG assay used for measurement of GTD-derived hCG. Reporting of hCG results from women with GTD could be standardised such that the technical specifications of the test are reported, and the results are interpreted in conjunction with the clinical picture and discussed in multidisciplinary team (MDT) meetings. A validated assay system should be used to ensure the assay can detect all relevant hCG isoforms. The potential limitations of hCG laboratory measurement could be highlighted to clinicians and scientists through specific education and training opportunities.

7.5.1 Educational example A -– discrepancies in hCG measurement between immunoassay platforms

Educational example A illustrates the importance of using the correct hCG assay for measurement of GTD-derived hCG as this can guide management of patients with persistent disease or GTN.

A 39-year-old woman was diagnosed with an HM which was evacuated at her local hospital. She subsequently underwent serum hCG monitoring locally where her results were reported as normal (ie. below 5 IU/L). The patient, however, was still clearly unwell with bleeding and nausea. Serum samples were sent to a regional centralised hCG service for further testing by another method (one-site competitive assay) where hCG levels measured 18 IU/L. The patient continued with further hCG monitoring at the regional hCG centre (shown in Figure 7.6). During surveillance she underwent chemotherapy for rising hCG levels, she relapsed, and eventually had a hysterectomy. Pathological examination of the specimen diagnosed choriocarcinoma in fundus. Although her hCG levels declined post-surgery, they remained slightly elevated (10 IU/L), suggesting residual disease elsewhere and she was treated for drug resistant choriocarcinoma.

During her surveillance the hCG was monitored by the one-site competitive immunoassay. All treatment decisions and clinical management were based on these results. On occasion, serum samples (red circles, shown in Figure 7.6) were also measured on a commercial two-site sandwich assay platform where hCG levels were non-detectable. Consequently, if only the commercial sandwich assay platform had been used for hCG surveillance, it would likely have resulted in a different treatment strategy for this patient which may have influenced the clinical outcome.

Whilst specificity may be lower in one-site competitive immunoassays (e.g. RIA) due to the use of a single antibody this can be advantageous for GTD management as more hCG isoforms are detected. The centres using RIA have accumulated vast amounts of data over a considerable length of time, where regression corridors and clinical decision limits have been well-defined based on the knowledge of these assays. This provides increased confidence in results and interpretation of the data. However, we do recognise RIA's may have a higher functional sensitivity cut-off value, where the lowest concentration of clinically useful results is higher compared with the cut-off values for 2-site immunoassays.

Figure 7. 6: hCG surveillance measured by Radioimmunoassay.

7.5.2 Educational example B – Biotin interference

In Educational example B (biotin interference) knowledge of the limitations of the assay in use is important to understand for both the laboratory personnel interpreting and reporting the results, as well as the receiving clinical team who are treating and managing the patient. Biotin interference in immunoassay is now well-documented and there are many examples where high levels of this vitamin can cause falsely lowered (non-competitive) and falsely elevated (competitive) results in immunoassays which utilise a biotin-streptavidin design (374).

As part of ongoing quality improvement at the UK-GTD centres, the effect of biotin interference in three different hCG testing platforms; one-site competitive immunoassay (Method A) and two non-competitive two-site sandwich immunoassays (Methods B and C) was evaluated (as shown in Table 7.2). Samples containing high hCG (206.6 IU/L) and low hCG (69.3 IU/L) were spiked with differing levels of biotin ranging from none (samples 1, 4), 750 µg/L (sample 2), 1500 µg/L (sample 3), 15 µg/L (sample 5), or 60 µg/L (sample 6).

Method A (competitive platform) measured hCG between 80% and 92% of the expected value of 206.6 U/L for samples 1, 2 and 3 which had been spiked with 0, 750 or 1500 µg/L biotin. One of the non-competitive platforms (Method C) measured hCG concentrations between 84% and 105%. Similarly, in samples 4, 5 and 6 spiked with 0, 15 or 60 µg/L biotin, both Method A and Method C showed no biotin interference.

Notably, measurement of hCG by Method B was most affected by high levels of biotin (samples 2 and 3), with only 7% to 38% (in red in table 7.2) of the expected hCG concentration detected. Of those samples spiked with lower levels of biotin (15 and 60 µg/L) hCG measurement was not affected, measuring between 95 and 100% of the target hCG value of 69.3U/L.

Biotin intake has become more popular in recent years and can be found in many multivitamin formulations. Whilst we understand the biotin-spiked samples used in this study have a higher concentration than that expected to occur physiologically in serum, laboratories using biotin-streptavidin based assays should be aware that high levels of exogenous biotin may cause interference. We note it is possible methods may have been

re-formulated since the time of this study, when measuring trophoblast-derived hCG, the reporting laboratory should be vigilant and correlate laboratory results with the clinical picture such that falsely lowered (or elevated) hCG values may be identified and dealt with appropriately.

Table 7. 2: Comparison of hCG measurement in biotin-spiked serum samples using 3 different Immunoassays methods.

Sample	1	2	3	4	5	6
Biotin spiked (µg/L)	none	750	1500	none	15	60
Mean hCG (µg/L)	206.6			69.3		
Recovery (%)						
Method A	92	80	84	93	88	92
Method B	90	38	7	95	100	100
Method C	105	84	93	85	91	85

Educational example C – High dose hook effect

In Educational example C, awareness of common laboratory pitfalls should be considered when results do not correlate with clinical presentation.

A 42-year-old woman, gravida 4, parity 3, presented at the emergency department 6 months post-partum with increased shoulder pain and feeling unwell. The patient was shown to have a positive pregnancy test (lateral flow chromatographic immunoassay), however, ultrasound scan showed an empty uterus and enlarged liver. Chest, abdomen and pelvis computerised tomography and liver MRI revealed right adnexal mass and lung and liver metastasis. Blood samples were sent to the local hospital laboratory for further testing using an immunometric immunoassay for the detection of β-hCG. Results showed a moderately increased hCG level of 2500 IU/L.

Whilst the patient was managed at her local hospital, guidance from a specialised GTD centre was sought and a further serum sample was sent to the centre for analysis using a competitive immunoassay platform for the measurement of GTD-derived hCG. Results showed an hCG concentration of 4.5million IU/L. Due to the patient's poor health, it was not possible to perform a liver biopsy to confirm a pathological diagnosis of a gestational tumour such as choriocarcinoma. However, as the patient presented with metastatic disease, raised hCG and history of previous pregnancy, she was clinically managed as having a gestational tumour and treatment was decided according to the FIGO 2000 guidelines (135).

The falsely lowered hCG results obtained from the local hospital immunometric assay (2500 IU/L) were most likely attributed to high-dose hook effect. This phenomenon can occur in two-site immunoassays when the amount of analyte is present at such high concentration the binding capacity of the antibodies become saturated. The formation of the immune complexes required to generate a signal cannot be formed, resulting in a falsely lowered signal and thus a falsely lowered result. High-dose hook effect is not an uncommon pitfall in hormone immunoassay, amongst other types of interference such as biotin (discussed above), macro-analytes, cross-reactivity, and heterophilic and anti-animal antibody interference (226,375,376). There are many reports in the literature where delayed diagnosis of GTD has occurred due to misleading hCG results, upon initial

investigation (377,378). Sequential dilution of the original serum sample may resolve such immunoassay artefacts and laboratories should be aware of these limitations in their assays. Of note, due to the artificially low hCG results initially obtained, the overall FIGO risk score for this patient may have scored lower, resulting in a different treatment strategy and outcome.

Where hCG results do not correlate with the clinical picture, the clinician should consult the specialist laboratory scientists, to allow further investigations.

7.6 Is centralized hCG testing the solution?

Clearly, there are many pitfalls and caveats involved in laboratory measurement of GTD-derived hCG and the results of our survey underscore this. This prompts the question; Is there a need for centralised hCG services in the future? Standardisation may arise by default if all laboratories ultimately use the same immunoassay and adopt the same clinical decision limits. However, the move to managed service contracts may preclude the use of a single hCG immunoassay for commercial reasons.

7.6.1 The benefits and challenges of centralized hCG testing

Most guidelines on the management of women with GTD advise that outcomes are better for women receiving clinical care in expert GTD centres (27). The basic elements of a reference GTD centre includes a national recommendation for centralised care, an expert clinical team, an experienced pathologist and a biochemistry laboratory with an oncology-specific hCG assay (122). The hCG immunoassay and analytical platform used for GTD surveillance should be one approved by the national GTD centre and ideally hCG should be measured in GTD reference laboratories co-located with reference GTD centres. (100). A GTD reference laboratory should have access to at least two hCG immunoassays and ideally one of these assays should be approved for use in oncology.(266) Centralization of hCG testing helps ensure equity of access for all women with GTD to an appropriate hCG assay performed in an accredited laboratory by scientists experienced in GTD management.

In GTD, patient treatment and management are dictated by the rate of decline in hCG levels post-evacuation of the products of conception. Normalisation is defined both by

the hCG assay and the reference range used. The reference range quoted by commercial hCG immunoassays can be dictated by variations in assay calibration (impure WHO standards), assay specificity for hCG isoforms (antibody heterogeneity) and the functional sensitivity of the assay. Generally, the cut-off used to establish normality in pre-menopausal (non-pregnant) women is <5 IU/L and this decision threshold is applied to GTD. However, in post-menopausal women the "age-dependent" increase in intact hCG (due to the influence of pituitary hCG) creates an additional challenge for the definition of normality. Ideally, each laboratory should establish their own population specific reference ranges for their specific hCG assay. However, variations in the decision thresholds used for different commercial hCG assays may affect both the duration and frequency of hCG monitoring and guide treatment decisions. For example, women having hCG measured in a lab with a hCG reference range of <1 IU/L will have a longer surveillance period than women whose hCG is measured in a lab with a reference range <5 IU/L. This leads to increased patient anxiety for these women, and it may also delay future pregnancies in women who are nearing the end of their reproductive years. The variation in reference ranges quoted by laboratories using the same commercial hCG assay is often due to their use of the 95^{th} or 99^{th} confidence interval (ie. <5 IU/L or <1 IU/L). The lower and upper limits of the reference range are commonly defined by the 95% confidence interval, but this practice requires standardisation. Centralisation of hCG monitoring would allow use of a standard decision threshold for normality.

A major advantage of a centralised hCG testing in a reference laboratory is the provision of serial hCG measurements using the same assay throughout patient treatment thereby eliminating the influence of assay bias and inter-assay analytical variation. It may also lead to improved hCG surveillance with less women lost to follow-up (379). Reference laboratories providing centralised hCG testing with the same assay accumulate data over many decades which allows them to establish their own reference range and develop hCG regression curves to guide patient management. Ideally, the hCG assay used for hCG monitoring should detect all isoforms in equal amounts. This reduces the risk of laboratories generating false negative results due to the limited detection of hCG isoforms by some commercial hCG assays. Commercial companies may also change antibodies at short notice which may alter the ability of assays to detect all isoforms and this makes earlier established reference intervals worthless. Centralised hCG testing may also reduce

the amount of time spent by healthcare professionals following-up hCG results generated by regional laboratories. The benefits and challenges of providing centralised and non-centralised hCG monitoring are outlined in Figure 7.7.

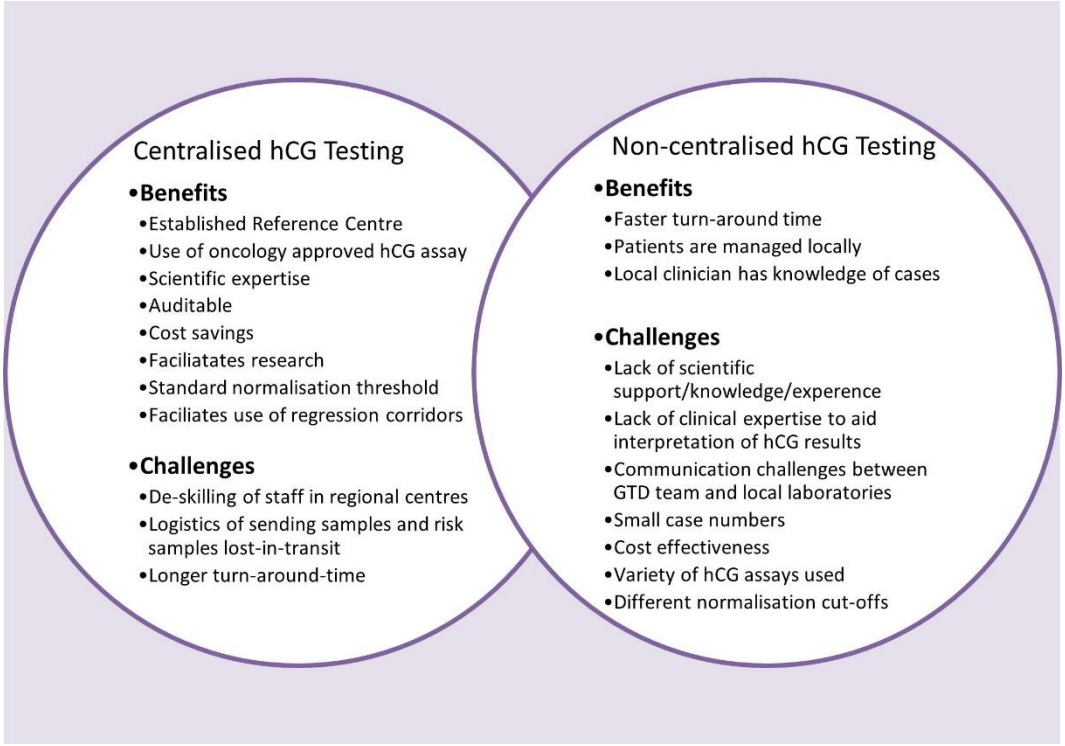


Figure 7. 7: Benefits and challenges of centralised versus non-centralised hCG testing for GTD management.

One of the drawbacks of centralised hCG testing and pathology review is the significant operational and administrative costs associated with setting up a reference GTD centre (126). The logistics of sending samples to a reference centre and the remote risk of losing precious samples during transportation may deter some countries from establishing centralised testing. However, a number of countries have successfully established centralisation (e.g., the UK has a GTD network in place since the 1970s).

Non-centralised hCG testing requires inter-laboratory connectivity supported by a national laboratory information management system (LIMS) to ensure timely communication of hCG results to the GTD centre. Lack of investment in LIMS may be a barrier to centralised testing as hCG results will need to be transmitted by post or telephone, potentially leading to a delay in reporting. In the absence of centralised hCG

monitoring, women with GTD may attend their local hospital, or GP surgery for hCG monitoring and each of these centres may use a different pathology service and/or hCG assay. A woman's hCG value can vary depending on the assay used and this introduces a risk that treatment is stopped too early or disease recurrence is missed (380). The use of rapid urine pregnancy tests (one-step immunoassay) for measurement of hCGβ in near-patient testing (e.g., emergency departments) introduces an additional risk of generating a false negative result and leading to delayed diagnosis and adverse patient outcomes (381).

Centralised hCG testing will enable harmonisation of decision thresholds and facilitate use of hCG regression curves which allows treatment to be stopped earlier, or additional treatment regimens be introduced. Creation of a centralised hCG testing centre for GTD will also benefit from the wealth of scientific knowledge built up over many years by dedicated clinicians and scientists working with this rare disease. They will be able to investigate unexpected rises in hCG concentrations or persistence of low level hCG elevation. Experienced scientists can trouble-shoot assay interference and for instance ensure appropriate dilutions are performed to detect extremely high hCG concentrations. Centralisation will also facilitate audit, provide incidence rates and support research into the development of new biomarkers or more sensitive hCG assays. The benefits of hCG centralisation would seem to outweigh the challenges in terms of improving patient outcomes for this rare disease.

7.7 Establishment of hCG Regression Curves and their use in clinical practice

HM's are evacuated using suction curettage (382). After evacuation, hCG is monitored on a weekly basis to detect potential malignant sequelae as soon as possible (383). Serum hCG concentration is reported to be normalized in 50% of patients between 6 and 14 weeks after molar evacuation (133,384–386). As shown in Figure 7.8, there is uneventful decline of serum hCG levels after evacuation, also referred to as hCG regression corridor/curve.

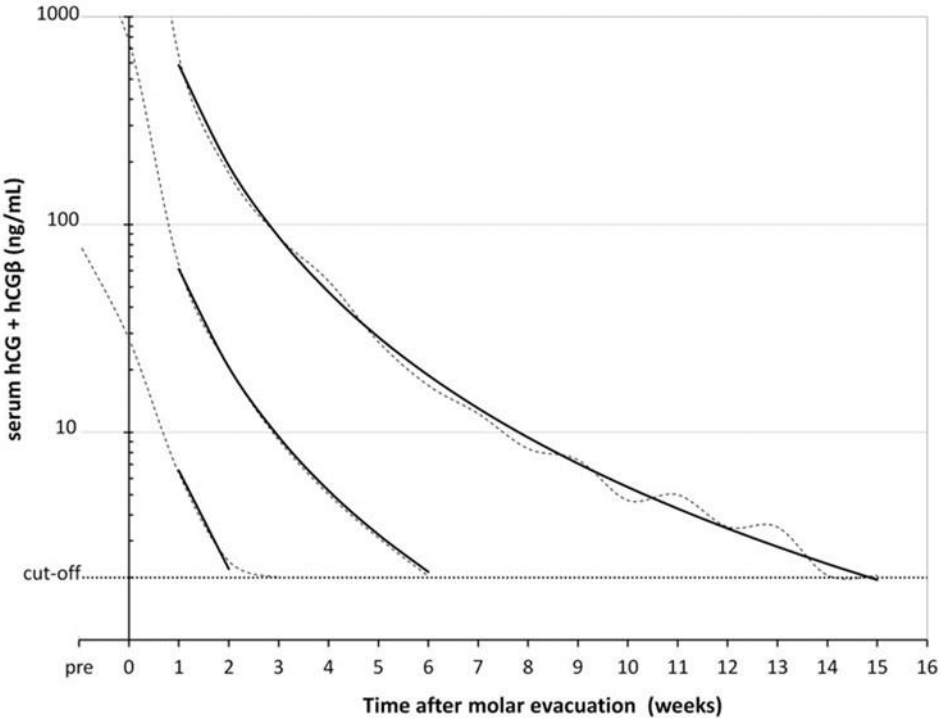


Figure 7. 8: Fifth, 50th and 95th percentile lines of serum hCG regression after evacuation of complete and partial HM.
The horizontal line represents the hCG cut-off concentration of 2 ng/mL as determined by Radioimmunoassay.

Whilst our survey showed that many of the laboratories did not use hCG regression curves, the reason for this, was not investigated further. Can we learn from laboratories which have implemented and established the use of regression curves in hCG monitoring to aid a more comprehensive surveillance program? Is this a decision tool that hCG laboratories can use to provide information to the clinician responsible for GTD patient management?

7.7.1 Persistent trophoblastic disease and hCG regression corridors

According to the FIGO guidelines, either a plateau in serum hCG concentration for three weeks, a hCG rise for two consecutive weeks, detection of hCG 6 months after evacuation, or the histological finding of a choriocarcinoma defines the presence of GTN (135)(387).

In 1993, Yedema et al. reported that 15% of patients with a plateau or rise for three weeks, but without a serum hCG measurement exceeding the 95th percentile (P95) of normal

regression will still have spontaneous serum hCG remission (133). These patients would therefore not require chemotherapy. The Dutch Society for Obstetrics and Gynaecology proposed an extended definition for GTN: a hCG plateau or rise for three consecutive weekly measurements, with at least one measurement above P95 of a normal regression curve (133,134). Using the Dutch criteria, GTN was diagnosed two weeks earlier than those cases derived from a plateau or rise of three consecutive measurements (385,386).

The clinical utility of hCG regression curves has been further demonstrated by Kerkmeijer et al. who compared uneventful CHMs diagnosed after introduction of routine first-trimester ultrasonography, with a historical cohort of complete mole patients diagnosed prior to ultrasonography (160). They examined gestational age, maternal age, pre-evacuation hCG concentration, serum hCG regression, and hCG disappearance time. Results showed gestational age, pre-evacuation hCG concentration, and hCG disappearance time were significantly lower in the recent CHM cohort compared with the historic series in which ninety-nine percent of the recent cohort achieved hCG normalization within 19 weeks after uterine evacuation compared with 25 weeks in the historic group.

This data was confirmed and extended by Eysbouts and coworkers who constructed new serum hCG regression curves for both uneventful complete and partial HMs (161). Serum hCG was measured by the same in-house-developed RIA as used by by Yedema et al. which has been in-use for all serum measurements sent to the Dutch Central Registry for Hydatidiform Moles since 1977. When compared with CHMs, patients with a PHM had significantly lower pre-evacuation serum hCG concentrations and earlier hCG normalization but higher gestational age. For both CHM and PHM, 95% of patients reached normal serum hCG concentrations within 14 weeks of uterine evacuation. The authors concluded that the small divergence in hCG regression curves between complete and partial HMs are not clinically significant as actual differences in regression encompass only days.

7.7.1.1 Resistance to first line chemotherapy and hCG regression corridors.

Predicting resistance to first line chemotherapy in GTD patients is of important clinical value. Low-risk GTN is treated with single-agent chemotherapy (MTX or Actinomycin-D).

If staged as high-risk GTN, multi-agent chemotherapy with EMA-CO (Etoposide, MTX, Actinomycin-D, Cyclophosphamide and Vincristine) is most widely used (388). Approximately 9-33% of patients treated with single agent therapy for low-risk GTN, will require multi-agent chemotherapy since they become resistant to the first-line drug or develop toxic side effects (389–392). Resistance to first-line chemotherapy may be defined as a plateau or rise in serum hCG and/or development of new metastases or a decrease of less than 10% in serum hCG concentrations within two weeks, or less than one log decrease of serum hCG within six weeks (see Table 1.3).

To identify patients not responding to single-agent chemotherapy at an early stage and prevent unnecessary multiagent chemotherapy, Van Trommel et al. constructed a normal serum hCG regression corridor for patients with low-risk GTN who were cured by single-agent methotrexate (MTX) chemotherapy (control group) (166). Another group of patients on single-agent chemotherapy, with low-risk GTN who needed additional alternative therapies (dactinomycin and multiagent chemotherapy) for failure of serum hCG to normalize were included (study group). Serum hCG measurement preceding the fourth and sixth single-agent chemotherapy course proved to have excellent diagnostic accuracy for identifying resistance to single-agent chemotherapy, with an area under the curve (AUC) for receiver operating characteristic (ROC) curve analysis of 0.949 and 0.975, respectively. At 97.5% specificity, serum hCG measurements after 7 weeks showed 50% sensitivity.

At high specificity, hCG levels in the first few courses of MTX can identify half the patients who are extremely likely to need alternative chemotherapy to cure their disease and for whom further treatment with single-agent chemotherapy will be ineffective. Kerkmeijer and colleagues validated their prediction of resistance to single-agent chemotherapy in an independent and larger cohort of GTN patients from Charing Cross Hospital, London, UK using a different hCG RIA (i.e. different antibody). ROC analysis identified an hCG cut-off value that enabled prediction of the subsequent development of single-agent chemotherapy resistance in 52% of patients before their fourth MTX course with 97.5% specificity (393). This would have enabled an earlier switch to combination chemotherapy reducing the MTX exposure by an average of 2.5 courses. This study confirmed that serum hCG cut-off levels predict resistance to single-agent therapy earlier than traditional

methods. Dekeister and colleagues proposed use of an online tool using mathematical modelling of hCG residual concentration (hCGres) to aid early prediction of the failure-risk in GTN patients during the first 50 days of MTX treatment. This website version of the NONMEM program® facilitated clinical interpretation of hCG decline curves in MTX-treated GTN patients (163).

These studies highlight the value of regression curves in aiding a multidisciplinary approach towards hCG surveillance in GTD patients where both scientific and clinical staff work together to achieve optimal patient care.

7.8 The future of hCG measurement within the clinical diagnostic laboratory

There is a need for a standardised hCG assay for use in women with GTD due to the wide variability between immunoassays. The prospect of using Mass spectrophotometry (MS) to measure ion mass and identify individual hCG isoforms holds some promise. Rapid advances in MS technology have facilitated the analysis of proteins with high specificity and sensitivity and facilitated their integration into workstreams in diagnostic laboratories (394). This provides a new tool for clinicians to monitor the most clinically relevant hCG isoform in their patients. However, measurement of hCG by MS is laborious and costly and many diagnostic hospital laboratories do not have access to MS instrumentation which will hinder its adoption in the shorter term. Moreover, the clinical relevance of each hCG isoform should be validated separately. There are several non-isotopic competitive hCG immunoassays in development which may replace RIA. Albeit proper analytical and clinical validation including development of cut-off values for these new assays is a prerequisite before implementation in a diagnostic laboratory. Examples of these include dissociation-enhanced lanthanide fluorescence (DELFIA) and chemiluminescence (394). Use of hyperglycosylated hCG assays as a potential biomarker for trophoblastic disease has not been broadly adopted into routine clinical practice (395).

In addition to the development of new hCG assays, short-term ambitions for the hCG scientific community (EOTTD hCG working party) include (a) development of appropriate practical guidelines for hCG measurement to address pre-analytical issues, sample dilution, quality control material used and use of defined reference ranges (b) development of sample exchange between European laboratories measuring GTD-

derived hCG or creation of a GTD-specific EQA program for centralised laboratories. The ultimate goal is to achieve harmonisation of data between institutes such that reporting and clinical cut-off limits can be agreed for all GTD patients regardless of assay used. Longer-term ambitions include the implementation of independent GTD reference laboratories. These institutes ideally would be able to measure a range of clinically relevant hCG isoforms using the most appropriate assay platforms, in an expert centre where there is greater access to clinical and scientific expertise in the field of GTD.

Guidelines for the use of tumour markers in clinical practice are a useful source of information for any laboratories involved in measuring biomarkers in clinical practice (396,397). In addition, we provide an abbreviated table of recommendations which laboratories may wish to consider when performing assays for measurement of hCG in GTD patients (Table 7.3).

Table 7. 3: Minimum requirements for a hCG assay

Minimum requirements for hCG assay	**Comment**
Assay validated for use in oncology and performed in an accredited laboratory	Most hCG assays are validated for use in early pregnancy only
Detects total and free beta-hCG	GTD-related hCG isoforms are detected
2-site immunometric assay	Wash step included to minimise false positive
Large measuring range	Manual dilutions kept to a minimum
Reference ranges representing the 95% confidence interval are used. ***Note:*** *reference ranges should be established in the testing laboratory to represent the local population bias.*	Provide decision thresholds for hCG normalisation Awareness of other sources of raised hCG. Examples include pituitary hCG, non-trophoblastic tumours and pregnancy
Quality control	Internal quality control (IQC): should be selected to represent the reporting range and clinical decision limits. Ideally third party IQC should be used (i.e. not produced by assay manufacturer) External quality assurance (EQA) participation in a scheme for hCG measurement to facilitate performance review and peer assessment. Sample exchange: GTD reference centres should consider sample exchange to enable comparison of hCG isoform detection.
Protocols should be available for investigation of assay interference	Examples include: -High-dose hook effect -Interfering antibodies (eg. heterophilic antibodies or human anti-mouse antibodies) -Rheumatoid factors -Gammopathies -Exogenous substances (eg. biotin)
Reporting	Results and cumulative reports should be authorised by an experienced GTD scientist

7.9 Conclusion

The EOTTD survey was devised and distributed with the aim of collating and evaluating aspects of hCG testing across laboratories involved in GTD surveillance. Whilst the response rate was lower than expected, it has provided invaluable insight into some of the laboratory procedures and processes currently in use. Generally, the survey highlighted the substantial variability between routine clinical diagnostic laboratories with respect to analytical and logistical issues associated with measurement of hCG in patients with trophoblastic disease. These included quality control used, reference ranges reported, use of regression curves, participation in EQA schemes and variation in reporting strategies. In addition, the lack of comparability between assay results obtained by different immunoassays for the quantitation of hCG in GTD patients can impair the commutability of research data. This may also create a barrier to the standardisation of clinical practice.

The EOTTD survey provided valuable insight into the processes underlying the provision of hCG testing for GTD patients in clinical laboratories. It also highlighted the need to develop laboratory guidance for hCG measurement, and to raise awareness of hCG assay variation within the GTD community. Ultimately, the lack of harmonisation across laboratories performing hCG testing is highlighted and underscores the value of working towards one principal objective; the quantification of all clinically relevant hCG isoforms in a correct, timely and efficient manner to help provide the most appropriate clinical care and treatment for GTD patients.

Acknowledgements

The authors thank Dr. Nick Unsworth (formerly Charing Cross GTD Service, now UK NEQAS [Edinburgh]) and Dr. Mark Busbridge (Charing Cross GTD Service) for their contribution to development and design of the Questionnaire; Ms Kamaljit Singh, Nurse Consultant (Sheffield GTD Service) for invaluable clinical advice and contribution to educational example C.3; and Dr. Cathie Sturgeon at UK NEQAS [Edinburgh] for providing biotin-spiked hCG samples.

Statement of Ethics

Ethical approval and consent were not required as this study was based on publicly available data and educational cases were anonymised in accordance with national guidelines.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

Study design: LMM, CMJ and FCGJS; Questionnaire Design: LMM, CMJ, LC, HM and FCGJS

Study data collection: LMM, CMJ and FCGJS

Figures and Tables: LMM and CMJ; Drafting manuscript: LMM, CMJ, LC, HM and FCGJS

Approval of final version of manuscript: LMM, CMJ, LC, HM, IJ and FCGJS

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Chapter 8 - Challenging Gestational Trophoblastic Disease Cases and Mimics: An Exemplar for the Management of Rare Tumours

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Chapter 8 - Challenging Gestational Trophoblastic Disease Cases and Mimics: An Exemplar for the Management of Rare Tumours

8.1 Abstract

Objective: Rare tumour management is challenging for clinicians as evidence bases are limited and clinical trials are difficult to conduct. It is even more difficult for patients where self-reliance alone is insufficient to overcome the challenges of navigating care which is often poorly evidence based. In Ireland, a national Gestational Trophoblastic Disease (GTD) service was established as one of 3 initiatives for rare tumours by the National Cancer Control Programme. The service has a national clinical lead, a dedicated supportive nursing service and a clinical biochemistry liaison team. This study sought to assess the impact of a GTD centre using national clinical guidelines and integrating and networking with European and International GTD groups on the clinical management of challenging GTD cases and to consider the application of this model of care to other rare tumour management.

Study Design: In this paper we analyse the impact of a national GTD service on five challenging cases, and review how the service affects management in this rare tumour type. These cases were selected from a cohort of patients who were voluntarily registered in the service based on the diagnostic management dilemma they posed.

Results: Case management was impacted by the identification of GTD mimics, the provision of lifesaving treatment of metastatic choriocarcinoma with brain metastasis, networking with international colleagues, the identification of early relapse, the use of genetics to differentiate treatment pathways and prognosis, and supportive supervision of treatment courses of up to 2 years of therapy in a cohort of patients starting or completing families.

Conclusion: The National GTD service could be an exemplar for the management of rare tumours (such as cholangiocarcinoma) in our jurisdiction which could benefit from a similar constellation of supports. Our study demonstrates the importance of a nominated national clinical lead, dedicated nurse navigator support, registration of cases and networking. The impact of our service would be greater if registration was mandatory rather than voluntary. Such a measure would also ensure equity of access for patients to

the service, assist in quantifying the need for resourcing and facilitate research to improve outcomes.

Keywords: Gestational Trophoblastic Disease, Rare Tumours, Molar Pregnancy, Ovarian Dysgerminoma, Choriocarcinoma, Case Report

8.2 Introduction

Rare cancers are defined as those with an annual incidence of less than 6 per 100,000 per year and account for 22% of all newly diagnosed cancers.(398–400) These rare cancers account for at least 5,200 annually diagnosed cancer cases in Ireland. Lack of information and guidelines on their management leads to more isolation and distress for patients and their families.(401) Challenges in the management of these rare cancers also includes their heterogeneity (402–405), and lack of level 1 evidence from clinical trials,(406) resulting in inadequate diagnosis and treatment.(407) Consequently, contemporaneous registry-based studies have documented poorer 5-year survival rates for rare cancers compared to common ones, and a failure for the former group to benefit from the gains in cancer survival that have occurred in recent decades. (408)

Recognition of the significance of rare cancers has led to several initiatives including; RARECARE – the project surveillance of rare cancers in Europe,(399) the establishment of European Reference Networks (ERNs),(409) in particular EUROCAN, the ERN for rare adult solid cancers, (410) and the International Rare Cancers Initiative.(411,412) These evolving international initiatives need to be integrated into care pathways for patients with rare cancers. One such rare tumour is Gestational Trophoblastic Disease (GTD) which arises from abnormal proliferation of trophoblastic cells in the placenta.(413) Hydatidiform mole (HM) or molar pregnancy is the most common pre-malignant type of GTD. HMs have two copies of the paternal genome and exist as partial or complete moles with the latter having a higher propensity for malignancy. (30,60) HM may be suspected on ultrasound and supported by elevated levels of human chorionic gonadotrophin (hCG), but diagnosis is made by histopathological examination of the evacuated products of conception (POC). At early HM presentation, diagnosis on morphology alone may be challenging due to GTD mimics and ancillary techniques (in-situ hybridisation, flow cytometry, molecular

genotyping) are often required to aid diagnosis. (56,289,294,414) The pathogenesis of GTD is unique in that the maternal tumour arises from gestational tissue rather than maternal tissue.(3) The World Health Organisation recognises four malignant GTD disorders; invasive mole, choriocarcinoma, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT).(8) In Europe, the incidence of choriocarcinoma is 1 per 40,000 pregnancies (22) whereas PSTT/ETT are even rarer accounting for 0.2% of all GTD.(187) These malignancies can occur months or years after a pregnancy and are often detected based on obstetric history and raised human chorionic gonadotrophin (hCG) levels.(16) However, choriocarcinoma is very responsive to chemotherapy and even patients presenting with advanced disease can achieve cure rates approaching 100%.(30,124,187)

In Ireland, the National Cancer Control Programme has established national multidisciplinary teams for sarcoma, neuroendocrine cancers and for GTD management to assist treatment in 8 National Cancer Centres.(232) The national GTD centre based at Cork University Maternity Hospital was established in 2017 by the National Cancer Control Programme (NCCP) as a monitoring and advisory centre. National clinical guidelines for the management of GTD developed by the NCCP with multidisciplinary involvement from members of the GTD centre in Cork recommend registration of all affected women with the GTD centre as a minimum standard of care.(108) The national GTD centre has a clinical lead and is supported by clinical nurse specialists who operate to best practice guidelines(415) and organise regular hCG follow-up testing at local hospitals and contact patients with their hCG results. Patient participation and involvement is integrated through advisory board membership and integration of patient survey results. The role of the expert GTD centre in Ireland has not yet been quantified. In the present study we assessed the impact of this initiative and reflect on how learning points from these cases could extend to other rare tumours nationally.

8.3 Material and methods

8.3.1 Study Design

Since its inception in 2017, the Irish national GTD centre has registered 748 GTD cases consisting of 10 women with gestational trophoblastic neoplasia (8 choriocarcinomas, 1 PSTT, 1 ETT). We performed a retrospective review of cases on the GTD registry and selected five illustrative cases treated at the GTD centre for review. In all cases presented, women provided informed consent in accordance with the Code of Ethics of the Declaration of Helsinki.

8.3.2 Context and inclusion criteria

For this case series, cases were selected for review based on their clinical significance, the diagnostic and treatment dilemma that they posed such as life-threatening presentations, disease types mimicking trophoblastic disease and cases where the clinical course was not as anticipated. A unifying theme in these cases was the need for multidisciplinary discussion often with international collaborators and integration with nationally developed clinical GTD guidelines.(108) The intent of the selected cases was to demonstrate the pivotal role of the national GTD centre in the management of a rare tumour.

8.3.3 Analytical Methodology

Biochemical analysis of hCG in Cork University Hospital was performed using the same hCG immunoassay throughout treatment. The laboratory is accredited to ISO15189 (2012) quality standards and participates in external quality assurance. The Abbott Architect™ total β-hCG assay was used to provide a rapid hCG result and the Roche Cobas® Elecsys total and free β-hCG assay was used to provide a confirmatory result. The Roche immunoassay is one of the few hCG assays approved for use in oncology, others are approved for use in early pregnancy only. Post-diagnosis hCG levels were monitored by the specialist GTD nurses and results were communicated to the patients in a timely manner with supportive counselling.

Histopathological assessment of trophoblastic tissue was performed by a perinatal pathologist and diagnosis was made using a combination of clinical history, morphology, and results of ancillary techniques, where indicated. Ploidy analysis was performed using

in-situ hybridisation and molecular genotyping was used to distinguish gestational from non-gestational choriocarcinoma.

Diagnostic imaging of women with GTD was performed in accordance with national clinical guidelines (108) using ultrasound, computed tomography (CT) of the thorax, abdomen and pelvis (CT-TAP) with contrast magnetic resonance imaging (MRI) of the brain where metastatic disease was suspected.

Clinical management was performed according to clinical practice guidelines of the European Organisation for the Treatment of Trophoblastic Diseases (EOTTD)(100). Following evacuation of the retained produces of conception (ERCP), hCG levels normalised in most women without the need for chemotherapy. When indicated, single agent chemotherapy was prescribed according to national regimens (i.e. methotrexate (MTX) 50mg intramuscular (IM) on days 1,3, 5, and 7 or Actinomycin-D (ACTD) 2mg intravenous (IV) bolus every 14 days)(416,417). Women who progressed to Gestational Trophoblastic Neoplasia (GTN) had FIGO staging and WHO modified prognostic scoring to aid selection of multi-agent chemotherapy. Challenging cases were discussed at MDT and escalated to external review by experts in international GTD networks where warranted.

8.4 Results

The characteristics of the five cases reviewed for this study are presented in Table 8.1. The impact of a national GTD centre in the clinical management of these cases is presented in Table 8.2. The salient lessons learnt from GTD to aid rare tumour management are presented in Table 8.4.

Table 8. 1: Characteristics of GTD and non-GTD cases.

Case	G, P	Age/ years	Disease classification	Subtype	FIGO score	Treatment	Treatment/ surveillance
1	G5P4	40	Molar Pregnancy	Ectopic	NA	MTX, ACTD, EMACO TAHBSO, TP/TE	4 years
2	G0P0	22	Germ Cell Tumour	Ovarian Dysgerminoma	NA	MTX, RPO, RSO	1 year
3	G3P1	32	Choriocarcinoma	Non-gestational	8	CE, DEM, TAH	>4 years
4	G1P1	35	Choriocarcinoma	Gestational	14	MTX, RML, BEP, EMACO	>4 years
5	G3P2	32	Choriocarcinoma	Gestational	16	DEM, CP/PE	>4 years

G = Gravidity; P = Parity. FIGO = The International Federation of Gynaecology and Obstetrics; FIGO score is used to stratify women into low/high risk categories of Gestational Trophoblastic Neoplasia; Low risk ≤6, High Risk ≥7, Ultra-high risk >12.

MTX= Methotrexate; ACTD = Actinomycin D; EMACO: Etoposide, Methotrexate, Actinomycin D, Cyclophosphamide and Vincristine; TAHBSO = Total Abdominal Hysterectomy and Bilateral Salpingo-Oophorectomy; RPO = Right Partial Oophorectomy; RSO = Right Salpingo-Oophorectomy; CE = Cisplatin and Etoposide**;** DEM = Dactinomycin, Etoposide, Methotrexate; TAH = Total Abdominal Hysterectomy TP/TE= Paclitaxel-Cisplatin and Paclitaxel-Etoposide; RML = Right Middle Lobectomy; BEP = Bleomycin, Etoposide and Cisplatin; . CP/PE = Carboplatin and Paclitaxel/Paclitaxel and Etoposide.

Table 8. 2: Impact of a GTD centre on clinical management

Case	Diagnosis	Impact of GTD service
1	Ectopic Molar Pregnancy	Persistent disease developed after surgical intervention. Lengthy hCG monitoring required. Adherence to GTD clinical practice guidelines.
2	Ovarian Dysgerminoma	Mimic of gestational trophoblastic disease. Alternative diagnosis suspected when unresponsive to methotrexate treatment. Diagnosis of germ cell tumour and surgical excision was curative.
3	Non-gestational Choriocarcinoma	Complexities of refractory disease and role of surgery. Avoidance of unnecessary toxic chemotherapy and long-term sequelae.
4	Gestational Choriocarcinoma	Genotyping required to classify choriocarcinoma. Sub-classification dictated prognosis and treatment pathway. Confirmation of gestational choriocarcinoma informed a switch to multiagent chemotherapy.
5	Gestational Choriocarcinoma	Life-saving therapy, initially induction based, in a new mother with extensive disease. Increased methotrexate dose to achieve central nervous system penetration. Tissue diagnosis not needed and potentially hazardous with risk of haemorrhage. Benefit of international networking.

8.4.1 Case One: Ectopic Molar Pregnancy

A 40-year-old female presented with irregular periods for 18 months postpartum. Despite a tubal ligation she had a positive pregnancy test 4 weeks after unprotected intercourse. On presentation, her serum hCG level was 6889 IU/L which fell to 3740 IU/L, 4 weeks later (Figure 8.1). There was no evidence of pregnancy found at dilation and curettage (D&C) and benign endometrial tissue was identified on histopathological investigation Two weeks later, serum hCG had risen to 4704 IU/L. Cross-sectional imaging using ultrasound and computerised tomography (CT) of the thorax, abdomen, and pelvis (CT-TAP) demonstrated a 2 cm follicle in the left ovary, consistent with an ectopic molar pregnancy. Following a multidisciplinary meeting (MDM), MTX therapy was initiated according to national clinical guidelines.(416) However, due to fluctuating hCG levels after 9 cycles of MTX, treatment was changed to ACTD as per national guidance.(417)

After 2 cycles of ACTD, hCG levels continued to rise and persistent disease in the left ovary was noted on imaging. A multidisciplinary decision was made to switch treatment to etoposide-MTX-ACTD- cyclophosphamide and vincristine (EMACO).(418) Pre-treatment hCG levels 240 days after initial presentation were 1174 IU/L declining to 10 IU/L after 3 cycles of chemotherapy, but hCG levels rose to 54 IU/L after an additional 2 cycles

MRI of the pelvis revealed an enhancement in myometrium and a 9 mm right adnexal cyst. Following additional MDM discussion at EURACAN, a total abdominal hysterectomy and bilateral salpingo-oophorectomy (TAHBSO) was performed (due to suspicion of an ovarian lesion), one year after initial presentation. The hCG level post-operatively was 196 IU/L but rose to 376 IU/L, 665 IU/L, 1488 IU/L and 3208 IU/L at 16, 17, 18 and 19 months respectively, after presentation. Fourth line chemotherapy with paclitaxel-cisplatin and paclitaxel-etoposide (TP/TE)(419) was initiated 19 months after initial presentation and hCG levels normalised 3 months later. Ultimately, chemotherapy was completed 2 years post-presentation, and the patient remains disease free on 2-year follow-up.

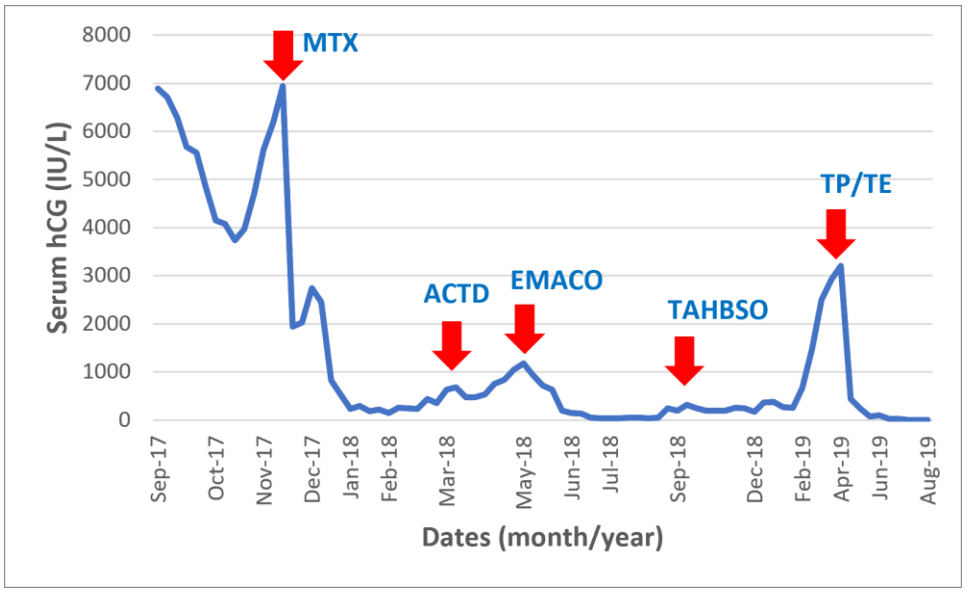

Figure 8. 1: Graph illustrating the change in hCG levels over time based on chemotherapy and surgical intervention.
MTX = Methotrexate; ACTD = Actinomycin-D; EMACO = Etoposide, Methotrexate, Actinomycin-D, Cyclophosphamide and Vincristine; TAHBSO = Total Abdominal Hysterectomy and Bilateral Salpingo-Oophorectomy; TP/TE = Paclitaxel-Cisplatin and Paclitaxel-Etoposide.

8.4.2 Case Two: Ovarian Dysgerminoma

A 22-year-old female with a background history of polycystic ovary syndrome (PCOS) presented with nausea and a positive pregnancy test, despite oral contraceptive use. Serial hCG measurements over four weeks were 6.1 IU/L, 8.5 IU/L, and 13 IU/L. A pelvic ultrasound demonstrated an empty uterus and cystic ovaries. Laboratory investigations were performed to investigate sources of false positive hCG results (urine pregnancy test, linear dilutions studies, PEG precipitation and use of alternative assays). In the subsequent 4 weeks, hCG levels declined to 11.2 IU/L and 7.3 IU/L. The patient was lost to follow up and re-presented 5 months after initial presentation with hCG levels of 284 IU/L (Figure 8.2). MTX treatment was commenced,(416) but hCG levels rose after a brief decline and concern was raised for a germ cell tumour rather than GTD. Cross-sectional imaging was normal. Following MDM discussion, combined oral contraceptive was initiated for 3 months without a treatment break.

Subsequent MRI of the pelvis revealed a 2.8 cm solid right adnexal mass suspicious of sex cord stromal tumour. Fifteen months after initial presentation, she had a right partial

oophorectomy performed with subsequent decline in hCG from 263 IU/L to 73 IU/L. Following surgery, hCG rose again to a peak of 131 IU/L and a completion right salpingo-oophorectomy was performed. Histopathological examination revealed an ovarian dysgerminoma and adjuvant therapy was not indicated. Two years post-oophorectomy, hCG levels remain normal (<1 IU/L). The rationale for inclusion of this case in the case series is that ovarian dysgerminoma is a great mimic of GTD, and this case provides valuable lessons in clinical management.

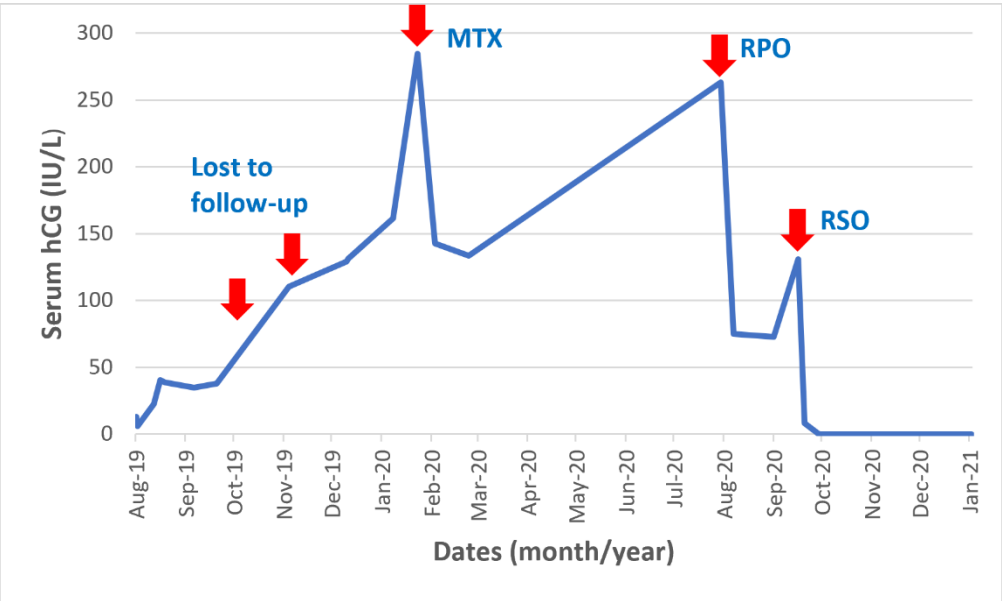

Figure 8. 2: Graph illustrating the change in hCG levels over time in response to chemotherapy and surgical interventions.
MTX = Methotrexate; RPO = Right Partial Oophorectomy; RSO = Right Salpingo-Oophorectomy.

8.4.3 Case Three: Non-gestational choriocarcinoma

A 32-year-old female with a past medical history of caesarean section presented with irregular vaginal bleeding for several months and a positive pregnancy test. A pelvic ultrasound showed no heartbeat or yolk sac. Eight weeks later, CT-TAP showed a 3.7 cm mass within the cervix. A follow-up MRI of the pelvis confirmed a heterogenous tumour centred in the posterior aspect of the cervix with elevated hCG at 110,340 IU/L (Figure 8.3).

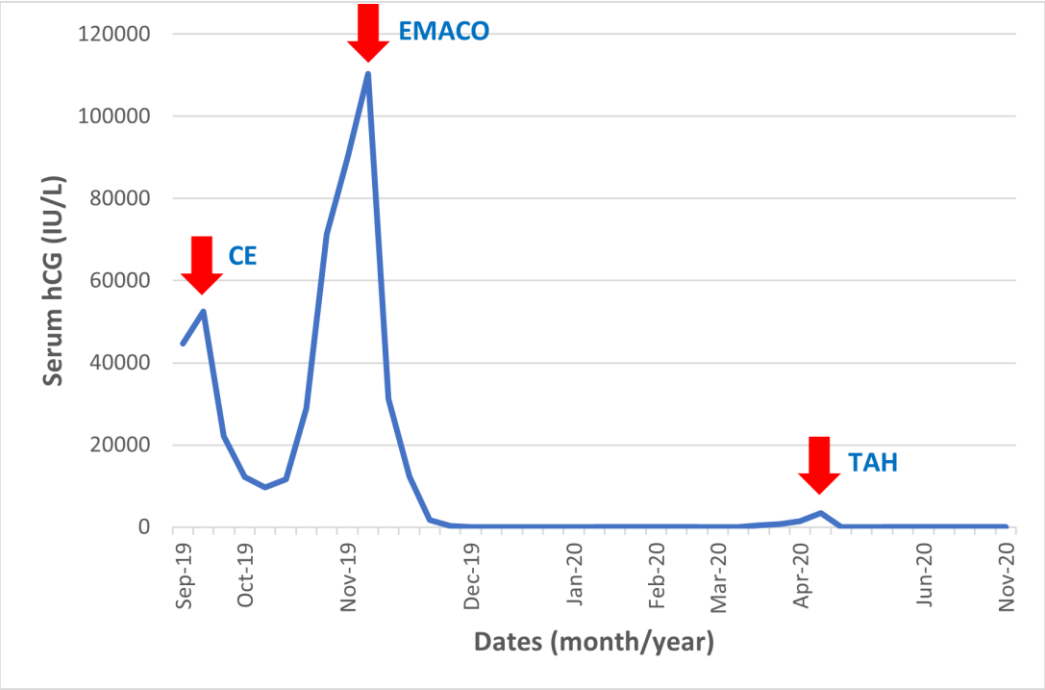

Figure 8. 3: Graph illustrating the change in hCG levels over time in response to chemotherapy and surgical interventions.
CE = Cisplatin and Etoposide; EMACO = Etoposide, Methotrexate, Actinomycin-D, Cyclophosphamide and Vincristine, TAH = Total Abdominal Hysterectomy.

After multidisciplinary discussion, a laparoscopy and cervical mass biopsy confirmed choriocarcinoma involving the cervical mucosa with a FIGO score of 8 (Table 8.3). Genotyping was not performed as both the clinical and pathological evidence supported a diagnosis of non-gestational choriocarcinoma. Diagnosis was made eight months after initial presentation (Figure 8.3). The patient was commenced on chemotherapy with etoposide and cisplatin (CE) and hCG dropped to 61 IU/L after 3 cycles. Subsequently the patient received EMACO(418) and a further drop in hCG was observed (6 IU/L), 2 weeks after the first cycle of chemotherapy.

A second cycle of EMACO treatment was withheld to mitigate the complications of chemotherapy; neutropenia, infection, and arachnoiditis. At 15-months post-diagnosis, hCG levels were below 1.0 IU/L. Two months later, hCG started to rise again to 1382 IU/L. Repeat ultrasound of the pelvis was suggestive of a vascular cervical mass. Cross-sectional imaging showed no metastatic disease and hysterectomy was advised following MDM

discussion. Following a total abdominal hysterectomy, hCG levels dropped to 374 IU/L. One-month post-surgery, there was a further drop in hCG levels to 22 IU/L, with levels ultimately normalising (<1 IU/L) and remaining normal in follow-up surveillance monitoring for 2 years.

Table 8. 3: FIGO staging and WHO modified prognostic scoring system for Gestational Trophoblastic Neoplasia

FIGO stage	**Description**			
I	Gestational trophoblastic tumours strictly confined to the uterine corpus			
II	Gestational trophoblastic tumours extending to the adnexa or to the vagina, but limited to the genital structures			
III	Gestational trophoblastic tumours extending to the lungs with or without genital tract involvement			
IV	All other metastatic sties			
Risk scores	**0**	**1**	**2**	**4**
Age, years	<40	>40	-	-
Antecedent Pregnancy	Mole	Abortion	Term	
Interval from index pregnancy, months	<4	4-6	7-12	>12
Pre-treatment hCG mIU/ml	$<10^3$	$>10^3$-10^4	$>10^4$-10^5	$>10^5$
Largest tumour size including uterus[a], cm	-	3-4	≥5	-
Site of metastases including uterus	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

To stage and allot a risk factor score, a patient's diagnosis is allocated to a stage as represented by a Roman numeral I, II, III and IV. This is then separated by a colon from the sum of all the actual risk factor scores expressed in Arabic numerals, e.g., Stage I:4, Stage IV:9. This stage and score will be allotted for each patient. hCG = human chorionic

gonadotrophin [a]Size of the tumour in the uterus. Reproduced with permission by FIGO Committee on Gynecologic Oncology.(60)

Table 8. 4: Lessons learnt from Gestational Trophoblastic Disease to aid Rare Tumour Management

Centre Roles & Resource Benefits	Dedicated Nursing Service	Central Registration
National MDT Lead	Nurse liaison	Mandatory registration
Network establishment	Facilitates monitoring of care pathway	Quantify clinical cases nationally
Guideline Development	Psychological support for patients	Facilitate audit
Patient Participation & Involvement		Equity of access to care and advice
Clinical trial availability		
Clinical resource for management guidance		

8.4.4 Case Four: Gestational Choriocarcinoma

A 35-year-old female with a background history of inflammatory bowel disease presented with a history of abnormal vaginal bleeding.(420) Serial hCG measurements raised the possibility of an ectopic pregnancy. An ultrasound of the pelvis showed no visible intra- or extra-uterine pregnancy but the hCG level was elevated at 400 IU/L (Figure 8.4). A diagnosis of pregnancy of unknown location (PUL) was made and the lady received 2 doses of IM MTX(416) as per institutional protocol which failed to result in a consistent fall in hCG levels. hCG levels dropped from 496 IU/L to 150 IU/L following the second dose of MTX but then very quickly rose again to 1110 IU/L and reached 1685 IU/L after 7 weeks. Repeat ultrasound examination failed to locate a pregnancy.

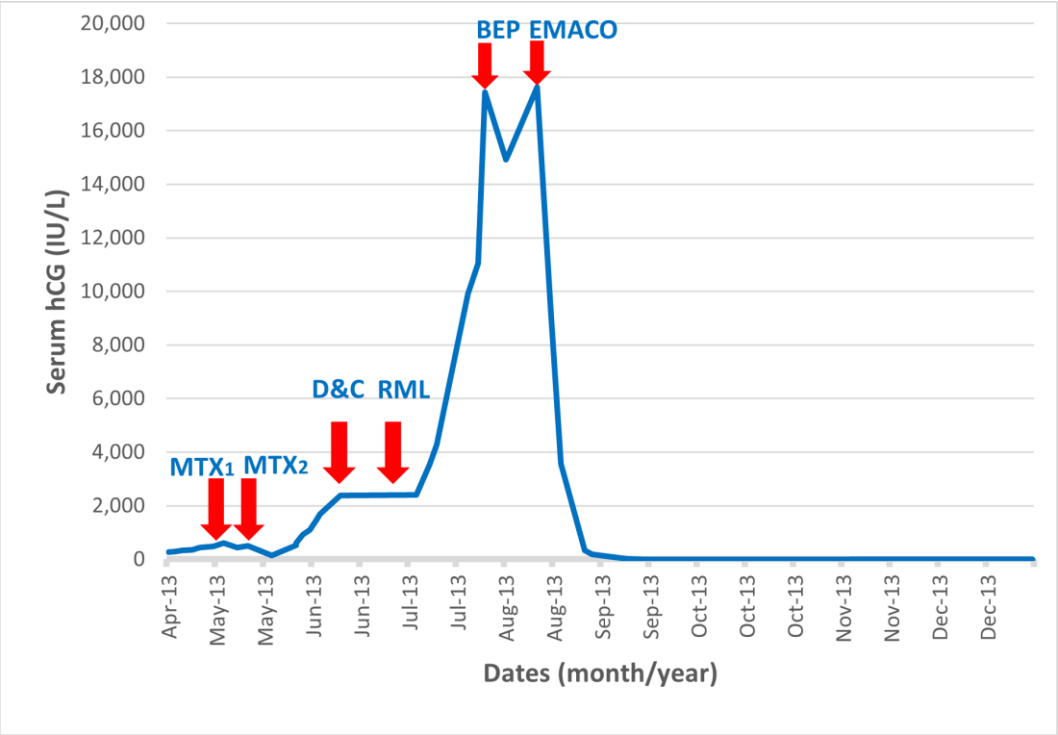

Figure 8. 4: Graph illustrating the change in hCG levels over time in response to surgical and oncological interventions.
MTX = Methotrexate; D&C = Dilation and Curettage; RML = Right Middle Lobectomy; BEP = Bleomycin, Etoposide and Cisplatin; EMACO = Etoposide, Methotrexate, Actinomycin-D, Cyclophosphamide and Vincristine.

Laparoscopy with concurrent dilation and endometrial curettage (D&C) showed no evidence of ectopic pregnancy and there was no significant abnormality detected. Histopathological examination of the D&C tissue demonstrated a proliferative endometrium with no evidence of pregnancy. An MRI of the pelvis confirmed this finding and showed multiple ovarian follicles. A CT of the abdomen and pelvis demonstrated a 1.6 cm enhancing lesion adjacent to the right atrium, and the case was discussed in a thoracic MDM.

A cardiac MRI revealed a lesion that appeared distinct from the pericardium, and a positron emission tomography (PET) scan revealed a low-level ^{18}F-fluorodeoxyglucose (FDG)-avid nodule adjacent to the right heart border with a maximum standardised uptake value (SUV max) of 2.4 reflecting low tumour aggressiveness. Ten weeks post-diagnosis, the lady underwent a lobectomy with partial excision of the right upper lobe, and lymphadenectomy. Histology demonstrated a necrotic lesion extending to the surface

of the pleura, and the tumour appeared to occur in the bronchus. The tumour had features of trophoblastic differentiation and was positive for hCG on immunohistochemistry favouring a choriocarcinoma.

A month post-surgery, hCG was still elevated at 2417 IU/L. The patient recovered from her cardiothoracic surgery and adjuvant chemotherapy of Bleomycin, Etoposide and Cisplatin (BEP) was administered in accordance with national guidelines(421) following MDM discussion. Chemotherapy was changed to EMACO(418) on receipt of the genotyping results which confirmed gestational choriocarcinoma as this was felt to be more GTD guideline concordant(27,91,100,108) and hCG levels normalised (<1.0 IU/L) and remains normal during follow-up surveillance. This unusual case highlights the need to closely monitor hCG levels following a PUL to detect disease persistence and diagnose GTN which is the most curable of all gynaecological malignancies.

8.4.5 Case Five: Gestational Choriocarcinoma

A 32-year-old female presented to her general practitioner (GP) 7-weeks postpartum. Following delivery of healthy twin boys at 35 weeks' gestation, she complained of right sided abdominal pain and redness in her left eye. Routine bloods were performed and revealed abnormal liver function tests (LFTs) and a haemoglobin of 8.0 g/dL, which were normal post-delivery. She was referred to the local hospital for medical assessment. On arrival, she had further investigations including a chest x-ray which revealed multiple bilateral pulmonary metastases and a hCG level of 507,150 IU/L. Her CT scan demonstrated diffuse necrotic pulmonary, liver, splenic, and nodal metastatic disease. MRI brain imaging demonstrated multiple intraparenchymal brain metastases. MRI pelvis confirmed the presence of 2 large necrotic masses within the myometrium, measuring 6 x 5.7cm and 5.4 x 5.5cm.

An initial liver biopsy was non-diagnostic and placental histology from her recent pregnancy was unremarkable, showing no evidence of intra-placental choriocarcinoma. These findings culminated in a diagnosis of an ultra-high risk choriocarcinoma with a FIGO score of 16 (Table 8.3). The patient was transferred to a specialist tertiary centre to begin intensive chemotherapy. She received 3 cycles of induction chemotherapy (Figure 8.5), with a 2-day regimen of etoposide (100mg/m2 IV D1-2) and cisplatin (20mg/m2 IV D1-

2).(422) She then proceeded to treatment with combination chemotherapy comprised of dactinomycin (0.5mg IV bolus D1), etoposide (100mg/m2 IV D1), methotrexate (300mg/m2 IV D1) (DEM), and folinic acid (15mg PO D2,3) alternating with etoposide (150mg/m2 IV D8) and cisplatin (75mg/m2 IV D8).(423)

After several cycles of chemotherapy, she developed cisplatin-induced ototoxicity with tinnitus and moderate-to-severe hearing loss in both ears. Six-months after her original presentation, her hCG plateaued at 9 IU/L and her case was discussed at the *EOTTD Annual Meeting* and treatment was changed to carboplatin (AUC4 IV D1) (rather than Cisplatin to abrogate further ototoxicity) and paclitaxel (135mg/m2 IV D1) alternating with paclitaxel (135mg/m2 IV D15) and etoposide (150mg/m2 IV D15). Her hCG normalized for the first time approximately 3 weeks after this change in chemotherapy and she remains in remission during follow-up surveillance

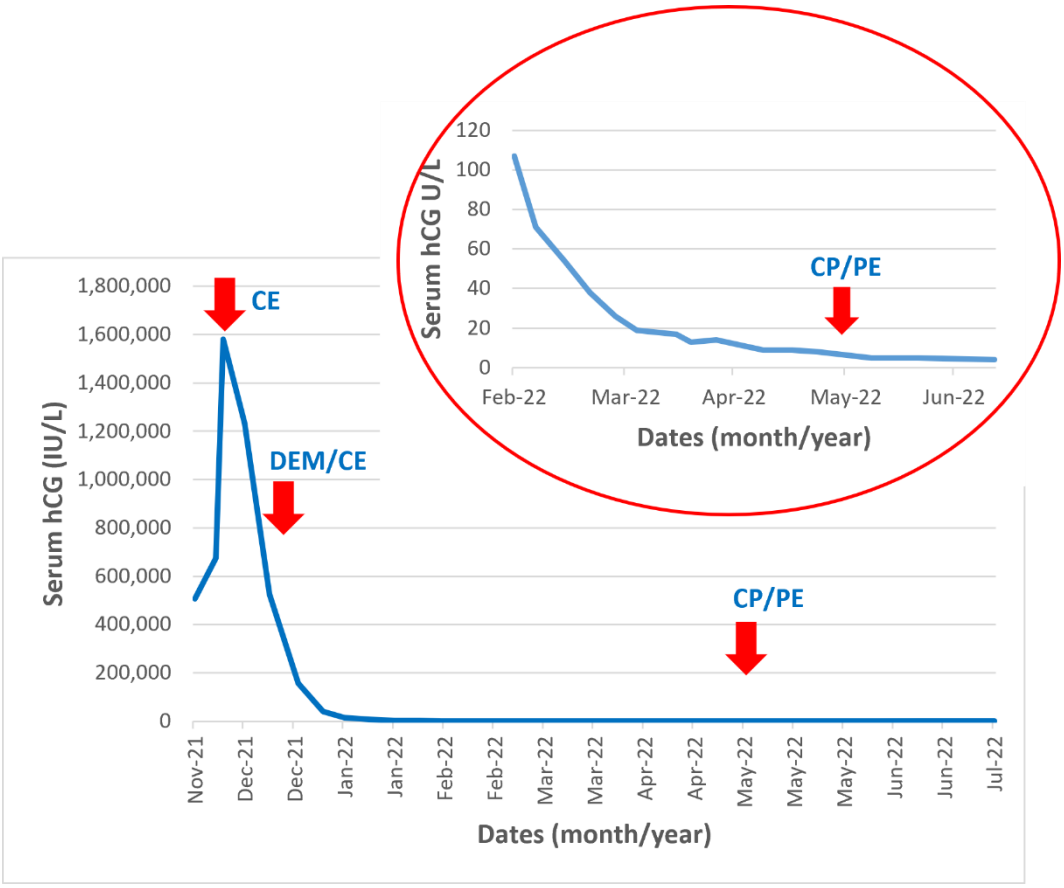

Figure 8. 5: Graph illustrating the change in hCG levels over time in response to chemotherapy.
CE = Cisplatin Etoposide; DEM = Dactinomycin, Etoposide, Methotrexate; CP/PE = Carboplatin and Paclitaxel/Paclitaxel and Etoposide. Inset figure shows plateauing of hCG levels in May 2022.

8.5 Discussion

The cases selected for this review reflect the complexity of managing women with GTD. It highlights the important role of an expert GTD centre and integrated MDT approach, further enhanced by experts from other GTD centres to managing women with this rare disorder and improving patient outcomes.(16,123,126) These range from challenges in differentiating the condition from other causes of hCG elevation to the treatment of critically ill patients where initial chemotherapy treatment can lead to fatal haemorrhage. The gravity of managing such cases is also reflected in the fact that many will have dependants or be hoping to initiate a family. The “Goldilocks Zone” (i.e. not too much treatment which will compromise quality of survival and not too little treatment which

will compromise cure) for such cases is narrow (424), and the transgenerational impact of successful management is substantial. The challenge is to achieve an early cure in these women with the least toxicity. (177)

Cancer is a lonely illness. During the consultative phase of the National Cancer Strategy 2017-2026 patients with rare cancers consistently commented on how this isolation was compounded by the lack of information on their cancer type. In the present study, patients had regular access to nurse specialists who recorded the progress of their illness, and to a multidisciplinary team with a global outreach to guide therapy.(266,425) This was particularly relevant to case 5 where international discussion guided management and reassured the patient about her treatment plan, and to case 1 whose treatment duration was 2 years.

As with other more common cancers, Irish, European and United States based consensus guidelines are established for patients with GTD.(30,39,40,49) These have evolved from the EOTTD Clinical Working Party, founded in Lyon, France in 2010.(100,427) This occurred 7 years before the European Rare Cancer Network was established.(428) As an exemplar in the area of rare tumour management, the EOTTD have standardised management plans through consensus, clinical trials and real-world data. Reviews of practices in specific countries, such as the UK (27), Belgium (304), Switzerland (429), and Ireland (232), have been published in recent years. These reviews generally concur, as intoned above, that national registration and treatment programmes and databases are quite effective in ensuring that there is effective information sharing among clinicians that are treating GTD despite the rareness of the disease complex.

In Ireland, a similar strategy for other rare tumours would include the assignment of a national clinical lead integrated with consensus working groups, with dedicated nurse specialist support conducted virtually who could also serve as nurse navigators to guide management and orchestrate care. Such a strategy would acknowledge the challenges patients face in dealing with multidisciplinary cancer management which requires a degree of self-reliance, and where adverse events can occur when self- reliance is missing.(430,431) Navigating care without a compass can also increase psychological distress, worsening cancer outcomes.(432) A specialist centre would also help address the concerns highlighted by 25% of GTD patients in our centre who reported deficits in the

understanding of their condition by medical professionals with whom they interacted outside the centre.(232)

In the context of extrapolating our current findings to the development of treatment strategies for other rare cancers, it is important to realize that GTD has a measurable biomarker (hCG) that is strongly correlated to tumour burden and causally related to the ultimate survival of patients. However, this is not true for several types of rare tumours such as cholangiocarcinoma (433,434) and may be the rule rather than the exception in rare cancers, for which the use of networking and cancer registries has been strongly suggested as approaches to improve patient survival.(435)

8.6 Conclusion

The cases presented here highlight the critical learning that is emerging for the treatment of rare cancers. The treatment pathways and clinical outcome of these cases demonstrate the importance of a national GTD centre with an expert clinical lead, dedicated nurse support and multi-disciplinary group adhering to national clinical guidelines to improve the outcome for women with this rare disorder. Importantly, within the ethos of the GTD centre patient participation and involvement is embedded. Moreover, the GTD centre fully integrates and networks with international colleagues, databases, and organisations for the management of rare cancers (e.g., EURACAN). Critical to the establishment of this national programme for rare disease is the need for registration to be mandatory, to allow equity of access for all patients to guideline-based care in an expert centre to ensure optimum patient management.

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Author Contributions:

Study design: SOR, MH, CMJ and HKC; Study data collection: MH, CMJ and CK.

Graphs: CMJ; Drafting manuscript; SOR, MH and CMJ.

Approval of final version of manuscript: SOR, MH, CJ, HKC, CK, SM, MKON and JC

Chapter 9 Proof-of-Concept study: remote capillary blood collection for hCG analysis in early pregnancy

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Chapter 9 Proof-of-Concept study: remote capillary blood collection for hCG analysis in early pregnancy

9.1 Abstract

Introduction

Serial measurement of serum human Chorionic Gonadotrophin (hCG) is frequently necessary for managing early pregnancy, including molar pregnancy, necessitating multiple visits to a maternity hospital for blood collection by venepuncture. This proof-of-concept study aimed to assess the clinical performance and user acceptability of capillary blood samples collected remotely, as an alternative to venous blood for hCG measurement.

Methods

Women attending the early pregnancy unit who required hCG measurement, were invited to participate. Following informed written consent, participants were shown how to collect capillary blood samples using the Mini-Collect® collection device. Matched venous and capillary blood samples were collected in clinic for comparison purposes. Participants were also supplied with a home collection kit in a prepaid envelope and asked to return capillary blood samples by post within 24 hours of collection, along with a completed user-satisfaction questionnaire. Statistical analysis was performed using Analyse-it® software.

Results

The study enrolled 71 participants and over a third of these women collected a capillary blood sample at home. The median age of participants was 33 years (range 29-36). Passing-Bablok linear regression (y=-0.037 + 1.04x) and Spearman correlation (r=0.999, p<0.0001), demonstrated good agreement and a positive correlation between venous and capillary samples, over a broad range of hCG values (1.2 to 224,0000 IU/L). The majority of capillary samples collected remotely (39%, 27/69) had sufficient blood volume for analysis (74%, 20/27). Respondents (77%, 18/25) found the collection device easy to use and expressed willingness to use a future service if available (80%, 20/25)

Conclusions

The study demonstrated excellent agreement between the hCG results obtained from both collection methods, suggesting that capillary blood can serve as a reliable alternative for venous hCG measurement. Responses from the study questionnaires demonstrated

women's eagerness to embrace home capillary blood testing, as a proactive means of participating in their own healthcare.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Home-based testing is becoming mainstream, driven by the surge in consumer demand for self-testing during the SARS-CoV-2 pandemic. Growth in this area has been seen in the use of capillary blood testing for glycaemic control in diabetes.

WHAT THIS STUDY ADDS

⇒ Demonstrates excellent agreement between capillary and venous blood for hCG.

⇒ Remote capillary blood collection is a viable alternative to venepuncture, particularly in clinical settings requiring frequent hCG monitoring.

⇒ Purchasing a home collection kit in a pharmacy would be acceptable and cost is not an impediment to this decision.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Encourages patient involvement in their healthcare through remote capillary blood hCG testing, promoting shared decision-making and collaboration.

⇒ Suggests the use of capillary blood collection could address challenges related to the centralisation of tumour marker testing due to analytical variation between immunoassays

9.2 Introduction

During early pregnancy, serial hCG measurement is frequently required for the clinical management of a pregnancy of unknown location (PUL), resulting in a significant volume of hCG tests conducted in Early Pregnancy Units (EPUs). These tests are crucial for triaging women and identifying those at high risk of complications, such as ectopic and molar pregnancies. However, visits to maternity hospital for phlebotomy following pregnancy loss can be distressing. Women with molar pregnancies, the most common form of Gestational Trophoblastic Disease (GTD), have reported that this adds to their distress during the hCG surveillance period, which can extend up to 6 months. Multiple factors such as arranging childcare and transport costs can impact a woman's ability to attend her nearest maternity hospital for phlebotomy to facilitate serial hCG monitoring.(232) Furthermore, general practitioners (GPs) are often uncomfortable monitoring blood tests for patients under tertiary care management.(436)

The search for alternative phlebotomy options for women experiencing pregnancy loss, including molar pregnancy, coupled with the need to centralise hCG testing for GTD management in Ireland, has necessitated a departure from standard laboratory testing.(108) Remote capillary blood collection (CBC) has emerged as a potential solution capable of addressing both challenges.

During the SARS-CoV-2 pandemic, remote CBC was employed to monitor glycosylated haemoglobin (HbA1c) levels as a measure of glycaemic control in patients with diabetes.(93) Capillary blood comprises a mixture of arterial and venous blood and interstitial fluid, and provides a viable alternative to venepuncture for diagnostic purposes, although some analyte concentrations may differ between sample types.(437) Recently, there has been a surge in consumer demand for at-home self-diagnostic testing with the market witnessing a proliferation of direct-to-consumer tests.(438,439)

Meeting the demand for centralised hCG testing, the possibility of home blood collection offers a practical solution to address the logistical challenges associated with establishing a central hCG testing laboratory. Centralised hCG testing ensures the use of an oncology-specific hCG assay and maintains consistency throughout treatment, aligning with National Clinical Practice Guidelines.(108) This approach streamlines access to hCG results

for specialist GTD nurses who play a pivotal role in monitoring hCG levels and providing support to women enrolled in the national GTD registry.

Remote blood collection also addresses many of the challenges related to patient access to phlebotomy services following virtual consultations.(93) It eliminates the need for women to visit maternity hospitals for phlebotomy following pregnancy loss and molar pregnancy, as previously mentioned**.**(232) This approach ensures clinicians have access to contemporaneous hCG results, allowing more effective consultations and facilitating shared decision-making, ultimately leading to improved patient satisfaction.(440) From the hospital's perspective, this solution saves on phlebotomy services and outpatient clinics.

This study sought to develop a prototype home testing solution by adapting capillary blood sampling for remote application and optimising the associated steps in this process. This proof-of-concept study had two primary objectives, (1) to assess the clinical utility and efficacy of capillary blood for hCG measurement in early pregnancy compared with standard venepuncture and (2) to evaluate the user acceptance of remote capillary blood sampling.

9.3 Methods

9.3.1 Study Design

This cross-sectional study enrolled pregnant women from the EPU at Cork University Maternity Hospital (CUMH), a large tertiary maternity facility, over a 6-month period (January-June 2023). Women attending the EPU who required hCG testing for scheduled care were invited to participate. After obtaining informed written consent, participants were provided with instructions on the CBC technique (Appendix IV), shown an online video tutorial on use of the MiniCollect® device, and received hands-on training during their clinic visit.(441) Participants were also provided with a home CBC kit containing a Mini-Collect® device (Figure 9.2), a questionnaire (Appendix V) and a pre-paid addressed envelope. Participants were instructed to collect approximately 250µl of capillary blood and to return this with the completed questionnaire within 24 hours of collection. The study was approved by the hospital research ethics committee (ECM4 (b) 01/11/2022 & ECM5 (8) 05/10/2022 & ECM3 (ss) 28/03/23).

The study objectives are divided into two groups: (A) prospective comparison of hCG results from matched venous and capillary blood samples collected in the EPU and (B) evaluation of the user acceptability of remote CBC.

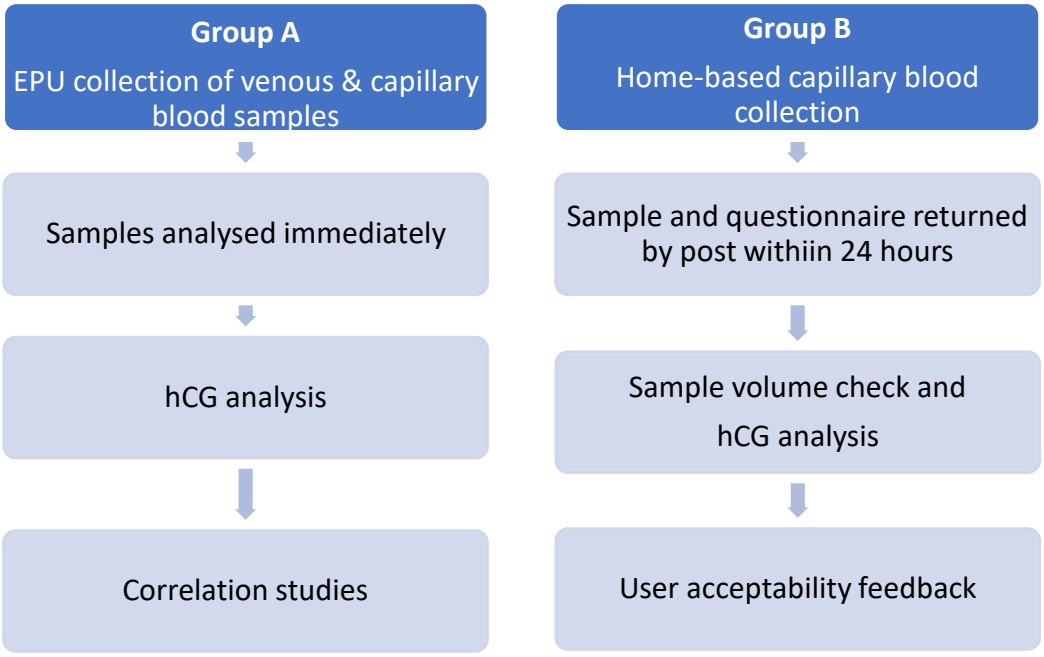

Figure 9. 1: Study Design

9.3.2 Questionnaire

A questionnaire comprising 14 Likert scale questions (Appendix IV) was designed to evaluate user acceptability of remote CBC. Six questions focused on the users' experiences of using the MiniCollect® device and home-based CBC. A further eight questions addressed the participant's experience of using the finger-prick lancet, their preference for this service compared to venepuncture and their willingness to pay for the service.

9.3.3 Capillary blood collection (CBC) in EPU

Prior to blood collection, participants washed their hands and prewarmed them using a rechargeable hot water bottle (DeVielle Ltd UK) for 10 minutes to enhance blood flow. They then gently massaged their fingers downward to stimulate blood circulation. The side of the middle or ring finger was chosen for fingerprick due to its adequate tissue

depth, preventing accidental bone injury.(442) The selected site was cleaned with disinfectant wipes (Clinell 2% Chlorhexidine in 70% Alcohol, GAMA healthcare Ltd UK) by the researcher and allowed to air-dry. Unistik® 3 sterile purple lancets (Owen Mumford Ltd, UK, reference AT1042), with safety Comfort Zone Technology™ and a 28-gauge needle were used to swiftly puncture the fingertip.(443) Blood was collected into a Mini-Collect® device (Greiner Bio-One, Austria, reference 450548) featuring an integrated scoop to facilitate easier blood droplet collection. These tubes without anticoagulant contain a separating gel and are preassembled with carrier tubes for laboratory centrifugation.

Home collection kits were pre-assembled into SpeciSafe® Multivial Biological Specimen UN3373 compliant mailing packs (Alpha laboratories ltd, reference SHO400), as depicted in Figure 9.2A. During home collection, the Mini-Collect® tube can be positioned upright in the transport container, as illustrated in Figure 9.2B. Once approximately 250µl blood is collected (halfway to the first graduation marking on the tube, Figure 9.2B), participants apply a sterile gauze pad to the puncture site to facilitate haemostasis.

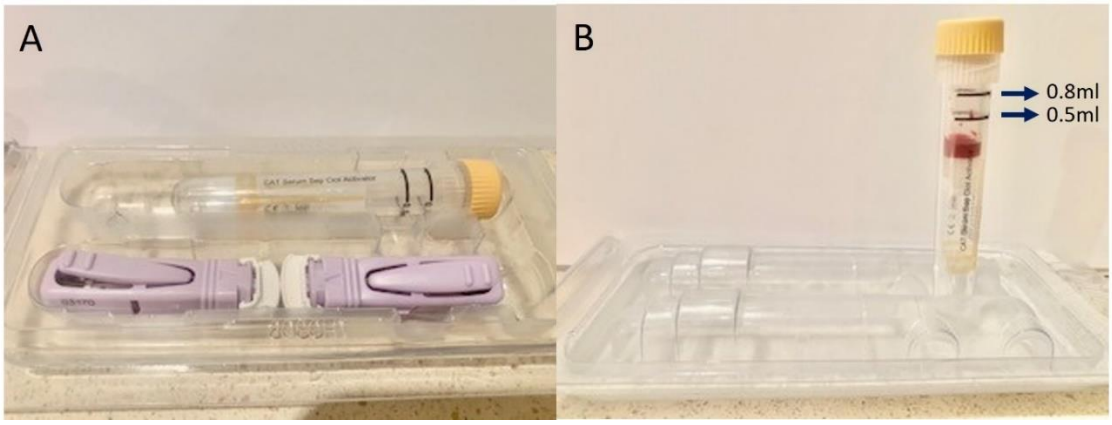

Figure 9. 2: (A) Home capillary blood testing kit. (B) SpeciSafe® transport container shown upright with graduation fill marks indicated.

9.3.4 hCG immunoassay

Analysis of hCG in venous and capillary blood samples was performed at Cork University Hospital's central laboratory using the Abbott Architect analyser (Abbott Diagnostics, Illinois, USA), adhering to manufacturer's instructions.(444) The Architect total beta-hCG (β-hCG) assay is accredited to ISO15189 (2012) international standards and the laboratory

participates in external quality assessment (EQA) for hCG through UK NEQAS. This assay uses a 2-step sandwich immunoassay with chemiluminescent microparticle technology and is linear from 1.2-15,000 IU/L). For the initial total β-hCG test, the architect analyser requires 75µl serum, with an additional 25µl needed for duplicate analysis.

Paired capillary and venous blood samples from the EPU were concurrently analysed in duplicate to minimise analytical bias and assay imprecision. Remote CBC samples received by post were visually assessed for volume adequacy using the tube graduations and processed immediately. After sample centrifugation (4,000g for 10 mins at 4^{o}C), serum was transferred into a micro-sample cup for Abbott® Architect® analysers (Abbott Diagnostics, reference 5106). Assay accuracy (mean) and precision (coefficient of variation, CV) was monitored by four levels of quality control (Technopath Multichem).

9.3.5 Statistical Analysis

Study data was recorded in a Microsoft Excel spreadsheet and statistical analysis was performed using Analyse-IT® software. The Shapiro-Wilk test assessed data normality, and descriptive statistics were applied to baseline study characteristics. Continuous parametric data was represented as mean and standard deviation (SD) and non-Gaussian data, as median and interquartile range. Spearman's rank coefficient assessed the correlation between venous and capillary blood hCG results, with a p value of <0.05 considered statistically significant. Passing-Bablok linear regression analysis and Bland-Altman difference plots were used to assess agreement and bias, following the Clinical Laboratory Standards Institute (CLSI) EP09-A3 guideline.(445)

9.4 Results

9.4.1 Study Characteristics

Seventy-four women initially consented to participate in the study, but three were excluded at the outset due to insufficient blood volume or lack of a matched venous sample. This led to a final study population of 71 women (Figure 9.3). The median age of participants was 33 years (interquartile range, 30-36) and the mean gestational age was 7 weeks (SD, 2.4). Clinical indications for hCG measurement in recruited women included ectopic pregnancy, PUL, molar pregnancy (n=3) and miscarriage.

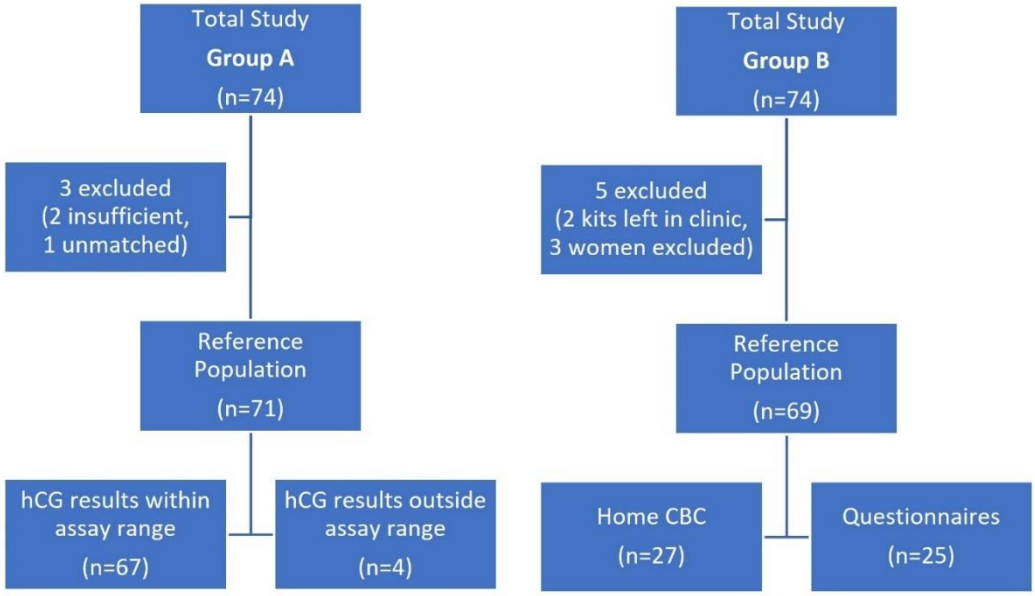

Figure 9. 3: Study recruitment schematic.
Group A: Paired venous and capillary blood samples collected in EPU. Group B: Home collected capillary blood samples. CBC, capillary bloods collected EPU, early pregnancy unit.

9.4.2 Statistical Analysis

Group A: Paired samples from EPU

The hCG results from venous blood were used as the reference comparator. The hCG assay demonstrated accuracy and precision, with a mean hCG concentration of 2.8, 21.7, 518, 15,185 IU/L and CV of 13%, 5%, 3% and 2.8%, respectively, reported for quality controls. The assay also achieved satisfactory EQA performance during the study period. The Shapiro-Wilk test showed the data was not normally distributed ($W=0.61$, $p<0.001$). Spearman's correlation coefficient revealed a significant positive correlation between hCG results from venous and capillary samples ($r=0.99$, $p<0.0001$). For robust statistical analysis, two approaches were employed for analysing group A samples (Figure 9.3): analysis of all hCG results (n=71) and analysis of a subset of hCG results within the linear range of the assay (n=67). This strategy was employed to mitigate potential errors resulting from specimen dilution samples with hCG concentrations exceeding the linear range of the assay, by 10 to 30 orders of magnitudes (Table 9.1). Despite this, both approaches yielded almost identical results, indicating excellent agreement between the collection methods across all hCG concentrations.

Passing-Bablok regression analysis (n=67) showed excellent agreement between venous and capillary blood samples with a slope of 1.03 (95% CI: 1.006 to 1.064), a minimal intercept of 0.084 (95% CI: -1.008 to 0.858) and a proportional bias of approximately 3% (Figure 9.4A). A Bland-Altman difference plot revealed a minimum mean difference of 26.6 IU/L, highlighting the consistency of results by both collection methods (Figure 9.4B).

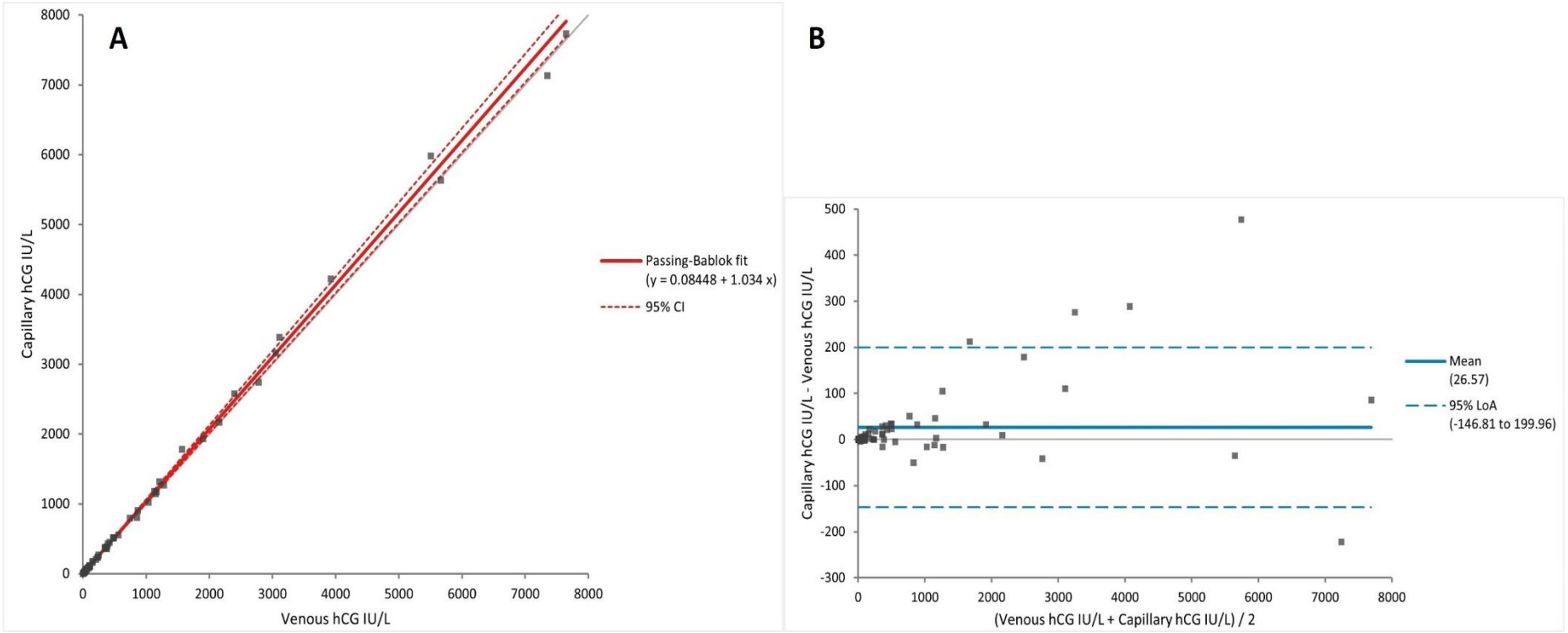

Figure 9. 4: (A) Passing-Bablok linear regression and (B) Bland-Altman difference plot (n=67) CI, confidence interval LOA, limits of agreement

Group B: Capillary Blood Collections in the home

Of the 74 women who consented to participate in the study, 3 were excluded at the outset due to insufficient or unmatched blood samples, 2 left the home collection kit behind in clinic, leaving 69 participants. Among these participants, 39% (27/69) returned a remote CBC, and most of the returned samples, 74% (20/27) had sufficient blood for hCG analysis. The volume of serum obtained from CBC ranged from a minimum of 20µl to a maximum of 250µl.

Group B: Response to Study Questionnaire

Out of the 27 home testing kits returned for analysis, 25 included completed questionnaires. Most participants (72%, 18/25) found the Mini Collect® device easy to use. However, 28% (7/25) of participants reported soreness in the lanced finger post fingerprick procedure. Despite this discomfort, 80% (20/25) expressed willingness to take another capillary blood sample in the future. A significant proportion, 72% (18/25) preferred home CBC over the traditional method of visiting a hospital or GP for phlebotomy (Figure 9.5). Additionally, 52% (13/25) believed they had insufficient blood collected whereas only 26% (7/27) of samples were insufficient. All participants indicated their willingness to obtain a future home testing pack from a local pharmacy as necessary. When participants were asked if would pay for a CBC kit, there was an almost equal preference for both amounts given (less or greater than €10/$10), suggesting that cost may not be a major concern.

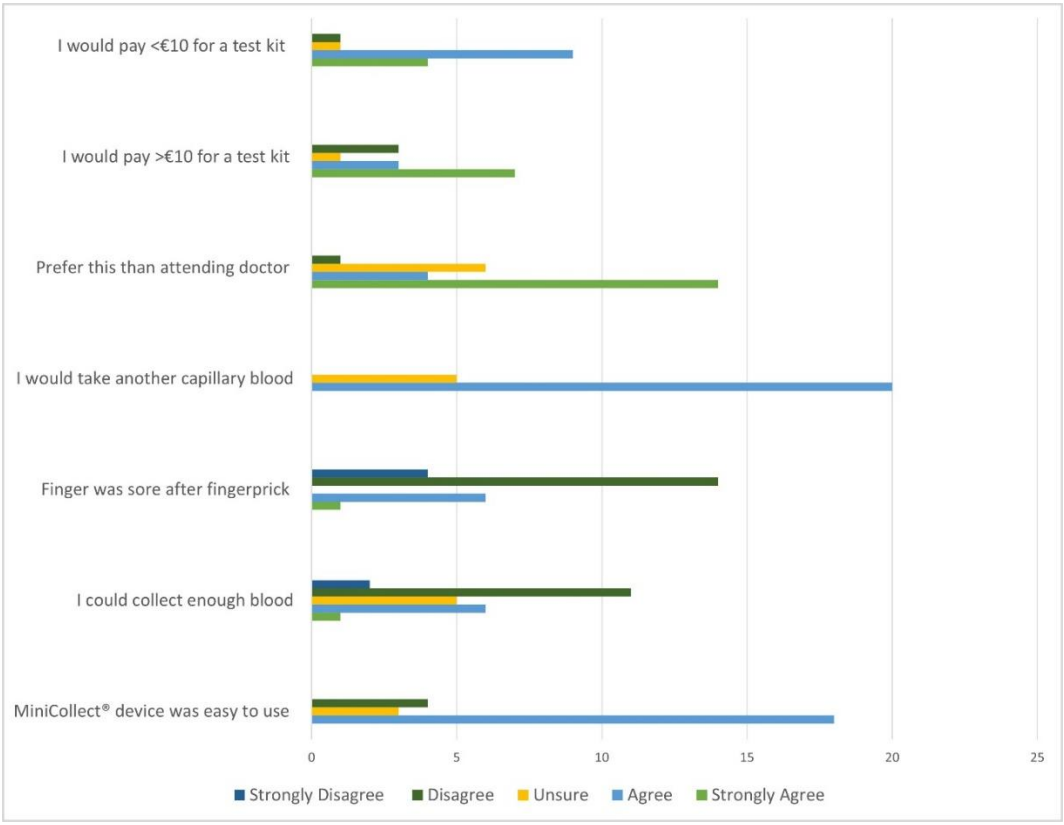

Figure 9. 5: Reponses to Questionnaire

9.5 Discussion

9.5.1 Summary of Main Results

This proof-of-concept study demonstrated that hCG results from venous and capillary blood sampling are equivalent making capillary blood hCG testing attractive for serial hCG monitoring in women with early pregnancy complications or GTD. There was positive feedback on the convenience of CBC, with women expressing a preference for CBC over traditional venepuncture.

9.5.2 Results in the Context of Published Literature

This study showed excellent agreement between venous and capillary serum hCG measurements, consistent with findings from other biochemistry studies that showed near-perfect agreement for certain analytes.(446) A small UK study comparing whole blood capillary and plasma venous samples for 15 biochemistry analytes found good correlation for HbA_1c and a positive bias for some lipid and liver function analytes.(447) Additionally, that study piloted a remote CBC kit to support virtual outpatient clinics during the SARS-CoV-2 pandemic, reporting excellent user-feedback, with 87% of participants finding CBC easy to perform.(447) Another Irish study found similar agreement between venous and capillary samples for HbA_1c.(93) A larger UK study validated remote CBC in an outpatient setting to enable shared decision making and found that CBC could accurately report specific tests for chronic disease management, facilitating telemedicine based outpatient care.(436) Another pilot study, evaluated remote CBC for tumour marker surveillance in colorectal cancer and reported a preference for finger-prick testing, particularly among younger participants (<65 years).(448)

9.5.3 Strengths and Weaknesses

While this proof-of-concept study had a relatively small size, it met the Clinical and Laboratory Standards Institute (CLSI) requirements for method comparisons and bias estimations.(445) Additionally, the range of hCG concentrations covered in patient samples included common clinical decision thresholds (1,500, 3,000 and 5,000 IU/L) for the management of ectopic pregnancy and miscarriage.(449) Another strength was the

availability of matched venous blood samples for each CBC in clinic, providing a control for analytical differences between matrices.(437)

However, there were limitations to the study design. The lack of a control arm to match remote CBC samples and enable immediate analysis, prevented the performance of stability studies. Secondly, the use of a MiniCollect® device with graduation fill lines (0.5/0.8ml) which did not reflect required blood volumes (0.1ml), may have deterred some participants. Moreover, patient information leaflets were only available in English, potentially discouraging non-English speakers from participating. In addition, while an online video was provided for guidance, a telephone helpline may have been more effective in supporting participants encountering difficulties using the CBC, possible affecting return rates. Nevertheless, the active involvement of the public in the study was a notable strength, while the questionnaire provided feedback on the efficacy and acceptability of remote CBC.

9.5.4 Implications for Practice and Future Research

This study highlighted a demand for and acceptance of remote CBC among pregnant women, suggesting a shift to a hybrid approach, involving both home and clinic-based blood sample collection. The biomarker hCG plays a pivotal role in investigating and managing GTD, suspected miscarriage, ectopic pregnancy, and PUL**.**(450,451) Larger prospective studies are necessary to validate these findings and to provide information on cost-benefit analysis.

Remote CBC shows promise for various applications beyond pregnancy monitoring. It could significantly contribute to infectious disease surveillance and facilitate the expansion of screening tests, as evidenced by recent Hepatitis C awareness campaigns.(452) Remote CBC could support chronic disease management by reducing hospital clinic visits and supporting telemedicine and video consultations**.**(436) It could simplify regular therapeutic drug monitoring for transplant recipients, offering a convenient alternative to hospitals for immunocompromised patients.(447,453) Furthermore, it may benefit people with disabilities who face challenges attending regular physician appointments or commuting long distances to access healthcare.

Future studies should include preanalytical validation to address concerns about the standardisation of capillary sampling procedures, which may compromise the quality and accuracy of test results(446,454) Additionally, the selection of collection tube, lancet and needle gauge may influence the blood volume collected and the discomfort experienced by users.(396,442,455) Integrating a QR code into the CBC kit could streamline the collection process by alerting the clinician and laboratory upon test dispatch.

Innovative blood collection systems, such as the Tasso-SST self-contained device for capillary blood samples and the Touch activated phlebotomy (TAP) II® device, using HALO™ technology have shown promise for the measurement of multiple analytes.(456–459) Additionally, Mitra microsampling devices using Volumetric Absorptive Microsampling (VAMs) technology absorb a fixed amount of capillary blood (10µl), which has been used for therapeutic monitoring of Prograf® in transplant patients.(460) These novel devices may be trialled in an updated CBC kit for a larger study proposed by our group. A Dutch study using a novel topper technology has successfully facilitated self-collection of blood for PSA testing in prostate cancer patients with favourable user feedback.(461)

The increasing consumer demand for home-based blood collection solutions empowers patients to actively participate in their healthcare.(440) Home-based CBC holds promise in centralising tumour marker testing for patients with rare tumours, such as hCG testing in trophoblastic disease. Previous studies have piloted tumour marker testing for CEA and PSA, for colorectal and prostate cancer, respectively.(448,461) This approach effectively addresses logistical challenges associated with intra-laboratory dispatch and analytical variation in commercial immunoassays for tumour markers. Feedback from our questionnaire indicates a preference for this type of follow-up among women experiencing pregnancy loss, indicating potential improvements in compliance for blood based diagnostic tests.

9.6 Conclusions

Capillary blood sampling, a minimally invasive technique traditionally used in diabetes, shows promise for numerous healthcare applications, including in pregnancy. This proof-of-concept study successfully demonstrated the equivalence of hCG capillary blood testing for hCG compared to standard venous blood sampling, achieving full clinical concordance. Validation of these findings by further larger prospective studies are necessary and should include a comprehensive cost-benefit analysis, before recommending the integration of capillary blood sampling into routine diagnostic practice.

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Author Contributions:

Study design: CMJ, PMOS, DHR and KOD**;** Study data collection: CMJ and RL; Statistics: CMJ and PMOS; Drafting manuscript; CMJ, PMOS, DHR and KOD. Approval of final version of manuscript: CMJ, POS, DHR, SC, JC, TMcC and KOD. KOD is the guarantor.

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Ethics approval: Written informed consent was obtained from all patients participating in this study in accordance with ethical approval from the hospital research ethics committee (ECM4 (b) 01/11/2022 & ECM5 (8) 05/10/2022 & ECM3 (ss) 28/03/23).

Chapter 10 Point of Care testing for hCG monitoring in early pregnancy

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Manuscript in preparation.

Chapter 10 – Point of Care testing for hCG monitoring in early pregnancy

10.1 Abstract

Objectives

Serial measurement of human Chorionic Gonadotrophin (hCG) helps guide patient management in early pregnancy. A point-of-care-testing (POCT) hCG solution has the potential to facilitate movement of an early pregnancy unit (EPU) to a remote location. This study evaluated use of the POCT Abbott i-STAT®1 to manage early pregnancy.

Methods

Women attending the EPU who required a hCG blood test were invited to participate. Following written consent, an additional lithium heparin blood sample was collected. Whole blood hCG was measured on the Abbott i-STAT®1 analyser in clinic and the remaining sample was sent to the hospital laboratory for hCG analysis on the designated comparator method, the Abbott Architect. Statistical analysis was performed using Analyse-IT software.

Results

Overall, 50 women were recruited to the study, representing intrauterine, molar, ectopic pregnancy, and miscarriage. hCG results outside the quantitative range of the POCT assay (n=14), although still broadly concordant with hCG results determined in the central laboratory, were excluded from statistical analysis. Comparative analysis of the remaining hCG results (n=47) from the i-STAT®1 and Abbott Architect assays demonstrated good agreement across the concentration range assessed (4-2072 IU/L). Spearman correlation was excellent at $r=0.99$, $p<0.0001$. Passing-Bablok linear regression showed good agreement, $y=1.18+0.96x$. Bland-Altman mean difference was -23.70 IU/L (3.5%).

Conclusions

The hCG results obtained from the iSTAT®1 analyser hCG were clinically concordant with those of the central laboratory method facilitating use of established clinical decision thresholds. However, a more extensive verification is required to investigate its clinical diagnostic utility in early pregnancy.

10.2 Introduction

Women often present to an Early Pregnancy Unit (EPU) due to vaginal bleeding and/or pelvic pain in the first trimester, prompting clinical examination and ultrasound assessment to determine pregnancy status. Serial measurement of serum human chorionic gonadotrophin (hCG) is instrumental in diagnosing early pregnancy complications.(462) The differential diagnosis may encompass pregnancy of unknown location (PUL), an ongoing or failing intrauterine pregnancy, ectopic pregnancy or molar pregnancy.(463)(464) PUL describes a situation where the pregnancy test is positive, but visualisation of the pregnancy via transvaginal ultrasound is not possible, which occurs in up to 42% of cases.(465)(466)

The National Institute for Health and Care Excellence (NICE) recommends a cut-off level of hCG of 1500 IU/L at which an intrauterine pregnancy should be visible on ultrasound.(449) Serial hCG monitoring is frequently requested as the change in hCG concentrations over a 48 hour period provides valuable insight into the viability of a pregnancy.(467) According to NICE guidance (NG126), a rise in hCG of greater than 63% within a 48-hour period is indicative of an ongoing pregnancy, while a decline of over 50% suggests a failing pregnancy. In cases where the change in serum hCG falls between these defined thresholds, clinical evaluation is necessary to determine the possibility of an ectopic pregnancy.(449)

Ectopic pregnancy is the leading international cause of death in the first trimester of pregnancy, predominantly occurring in the fallopian tube and leading to serious complications if left untreated.(468) Ectopic pregnancies occur in approximately 11 per 1,000 pregnancies, with a maternal mortality of 0.2 per 1,000 estimated ectopic pregnancies.(469)(470) There are 10,000 ectopic pregnancies reported annually in the UK.(471) In Ireland, the hospitalisation rate for ectopic pregnancy was 17.7 per 1,000 deliveries in 2016.(472) An audit conducted on tubal ectopic pregnancies at a major tertiary hospital in Ireland between 2020-2022, identified 212 cases. Management of these pregnancies included conservative measures (17.5%), medical intervention (34.9%), and surgical procedures (47.6%) (D. Hayes-Ryan, pers. Comm).

Blood hCG results play a crucial role in triaging women based on established clinical decision thresholds for ectopic pregnancy and PUL.(449)(471)(473)

Quantitative blood hCG measurement is preferred over qualitative urine tests due to the absence of degraded free β-hCG, termed hCG beta core fragment (hCGβcf) which often leads to false negative results in urines. (474)(475)(381) Serum hCG assays offer increased analytical sensitivity (2-5 IU/L) compared to qualitative urine POCT devices (20-25 IU/L).(475)(476) Use of POCT quantitative blood hCG assays provides a clinical decision threshold of 5 IU/L for early pregnancy detection and demonstrates better sensitivity in healthcare settings. POCT devices currently available to provide hCG in an outpatient setting include the Abbott iSTAT®1, Radiometer AQT90 FLEX® and VEDLAB Easy Reader (463,477–481)

This study aimed to assess the Abbott iSTAT®1 handheld analyser based on several key performance criteria, including ease of use, turn-around-time, assay specifications, analytical measuring range, inclusion in external quality assessment schemes, CE marking and compliance with the In-Vitro Diagnostic Regulation (IVDR).

10.3 Methods

10.3.1 Study Design

All women attending the EPU between January and June 2023, who required a hCG blood test for scheduled care, were invited to participate. Upon obtaining written consent, an additional lithium heparin blood sample was taken for POCT hCG analysis. Whole blood (WB) total β-hCG was measured on the Abbott i-STAT®1 analyser in the EPU and the remaining sample was sent to the central laboratory for analysis of plasma total β-hCG using the Abbott Architect (Abbott Diagnostics, Illinois, USA) analyser. Of note, hCG results measured on the i-STAT®1 were not used to inform patient management during the study. The study was approved by the CUMH research ethics committee (ECM4 (b) 01/11/2022 & ECM5 (8) 05/10/2022 & ECM3 (ss) 28/03/23).

10.3.2 Analytical Methods

The Abbott i-STAT®1 (Abbott Point of Care Inc, USA) is a handheld analyser which measures total β-hCG in a dry reagent cartridge (reference 05P58-25) (Table 10.1). It reports a quantitative result in the linear range of the assay (5-2,000 IU/L) and provides a qualitative result outside these thresholds. WB was loaded directly from the blood tube using the i-STAT®1 dispensing tip (reference 06F24-20) and performed according to manufacturer's instructions.(482) Controls with low (Abbott level 1, reference 05P59-01) and high (Abbott level 3, reference 05P59-03) hCG concentrations were analysed on alternate weeks during the study period. Levels were chosen to assess assay performance at the critical clinical decision thresholds. Accuracy of the i-STAT-1 analyser was further assessed using samples from the Welsh external quality assessment scheme (WEQAS).

Study plasma samples were analysed in the core laboratory on the Abbott Architect analyser using a total β-hCG assay, according to manufacturer instructions.(444) This assay is accredited to ISO15189 (2012) and was designated the comparator assay for accuracy assessment in this study. Internal liquid controls (Technopath Multichem 1A, reference O5P76-10) and External Quality Assessment (UK NEQAS) samples were analysed throughout the study period.

Table 10. 1: Comparison of point of care instruments to laboratory comparator

Parameters	Abbott i-STAT®1	Abbott Architect
Sample type	Plasma LH	Serum, Plasma LH
CE Marked	√	√
Minimum Volume	17µL	75µL
Linear Range (IU/L)	5-2,000	1.2-15,000
Hook effect (IU/L)	>300,000†	N/A
Assay Time (Minutes)	10	20
hCG isoforms	‡Total β-hCG	‡Total β-hCG
Test Principle/Detection	2-step ELISA/ECD	2-step ELISA/CMIA
WHO IS	5th (07/364)	4th (75/589)

LH: Lithium Heparin, CE: Conformité Européenne, WHO: World Health Organisation, IS: International standard, ‡Intact hCG and free β subunit, ECD: Electrochemical detection, CMIA; Chemiluminescent microparticle immunoassay, N/A: Not available, ELISA: Enzyme linked immunosorbent assay, †Sowder *et al*. reported a higher hook effect of 400,000 IU/L

10.3.3 Statistical analysis

Analysis was performed using Analyse-IT® software. Parametric testing of the data was performed using the Shapiro-Wilk test, and descriptive statistics were performed on the baseline study characteristics. Continuous parametric data were represented as mean and standard deviation (SD) and non-Gaussian data, as median and interquartile range. Whole blood hCG results from the i-STAT®1 were compared to those from the Abbott Architect analyser using Spearman's correlation coefficient with a p value of <0.05 considered statistically significant. Passing-Bablok linear regression analysis and Bland-Altman difference plots were used to assess method agreement and bias of the different assays respectively, in accordance with the Clinical Laboratory Standards Institute (CLSI) EP09-A3 guideline.(445).

10.4 Results

10.4.1 Study Characteristics

In total 61 women were recruited to the study, including women with intrauterine pregnancy (n= 11), PUL (n=12), ectopic pregnancy (n=6), miscarriage (n=25), molar pregnancy (n=3), intrauterine fetal demise (n=3) and retained products of conception (n=1). The median age of study participants was 33 years (interquartile range, 30-36) with a minimum to maximum maternal age of 20 to 40, repectively. The mean gestational age was 7 weeks (SD, ±2.4).

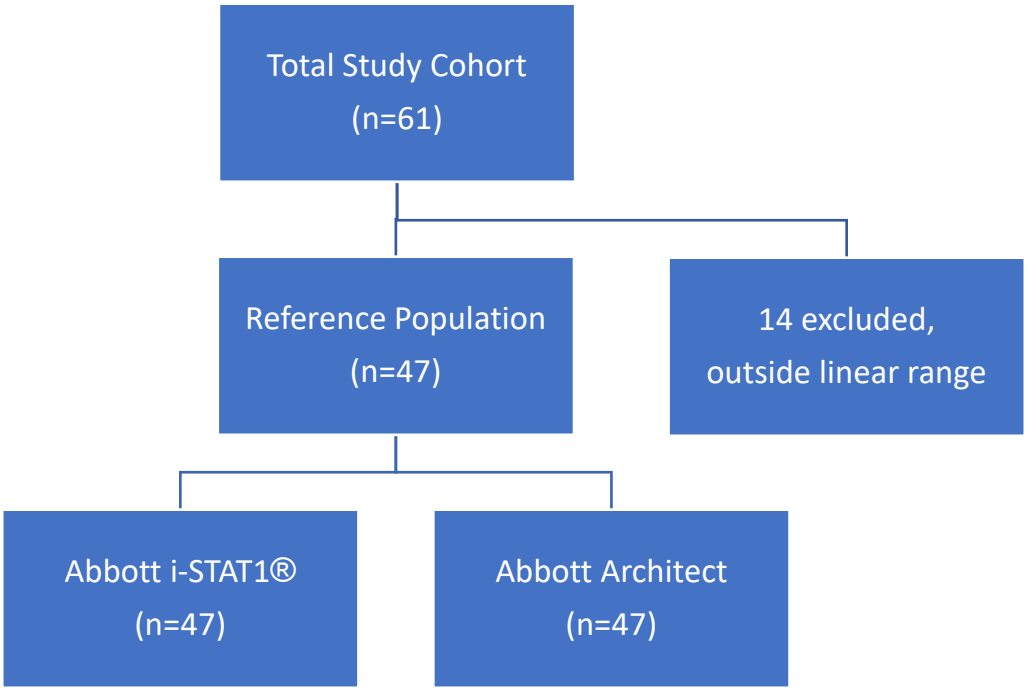

Figure 10. 1: Recruitment schematic

Table 10. 2: Specimens outside the linear range of the POCT hCG assays

Clinical Details	Abbott Architect	i-STAT®1
Ectopic	12,388	>2,000
IUFD	40,349	>2,000
IUP	101,664	>2,000
IUP	219,759	>2,000
IUP	2,600	>2,000
IUFD	3,041	>2,000
IUP	5,270	>2,000
IUP	6,897	>2,000
Miscarriage	<1.2	<5.0
Miscarriage	2,526	>2,000
Miscarriage	3,033	>2,000
Miscarriage	3,949	>2,000
Molar Pregnancy	<1.2	<5
RPOC	7,323	>2,000

IUFD: Intrauterine Fetal Demise, IUP: intrauterine pregnancy, RPOC: retained products of conception

10.4.2 Statistical Analysis

The Shapiro-Wilk test demonstrated that data from the i-STAT®1 was not normally distributed (W statistic: 0.78, P<0.0001). POCT results outside the linear range (n=14) of the I-STAT®1-1 although clinically in agreement with those of the central laboratory assay were excluded for statistical purposes only (see Figure 10.1 and Table 10.2). Good agreement was demonstrated between the β-hCG results from the Abbott i-STAT®1 and the Abbott Architect across the hCG concentration range assessed (4-2072 IU/L). Spearman correlation: r=0.995, p<0.0001, Passing-Bablok regression: Y=1.18+0.96x, Bland Altman difference plot: mean difference of -23.7 IU/L (-3.5%), see Table 10.3, Figures 10.2 & 10.3). Three of the six external quality assessment (EQA) samples were outside the measuring range of the assay (<5, >2,000 IU/L) but qualitative results were correct in all cases. The remaining EQA samples were acceptable with values within 2 standard

deviations (SD) of the WEQAS target mean value (see Table 10.4). Precision of the i-STAT®1 assay was acceptable with controls within their assigned range values and coefficients of variation (CV) within the manufacturer's CV targets (Level 1: 24.7, CV 15.8% and Level 2: 1571.5, CV 17%). Precision of the Abbott Architect assay was also acceptable for all 3 control levels (Level 1: 11.3%, Level 2: 4.2%, Level 3: 2.7%)

Table 10. 3: Method Comparison: Statistical assessment of agreement

Designated Comparator Method **Abbott Architect Total β-hCG**	***POCT Device*** **Abbott i-STAT®1 (n=47)**
Passing-Bablok Regression	Y=1.18+0.96x
Intercept (95% CI)	1.18 (-1.15 to 3.40)
Slope (95% CI)	0.96 (0.90 to 0.99)
Spearman Correlation	r=0.995, p<0.0001
Bland Altman Difference plot	**Mean Difference (IU/L):** -23.7
Mean % Difference (95% CI)	-23.7 (-43.7 to -3.70)
Lower LOA (95% CI)	-157.2 (-191.6 to -122.8)
Upper LOA (95% CI)	109.8 (75.4 to 144.2)
Bland Altman Difference plot	**Mean Difference (%)** -3.52
Mean Difference (95% CI)	-7.7 to 0.67
Lower LOA (95% CI)	-31.5 (-38.7 to -24.2)
Upper LOA (95% CI)	24.4 (17.2 to 31.6)

CI: Confidence Interval, LOA: Limits of Agreement

Table 10. 4: Accuracy verification for i-STAT®1

WEQAS Distribution, Sample number	i-STAT®1 Target Mean hCG IU/L	i-STAT®1 Method SD	i-STAT®1 EPU Mean hCG IU/L
WS0722, sample 1	46.08	5.83	44.1
WS0722, sample 2	>2000	NA	>2000
WS0722, sample 3	1433.5	36.37	1347.2
WS0922, sample 1	>2000	NA	>2000
WS0922, sample 2	<5	NA	<5
WS0922, sample 3	450.62	18.68	421.3

WEQAS: Welsh External Quality Assessment Scheme, NA: not available, EPU: early pregnancy unit

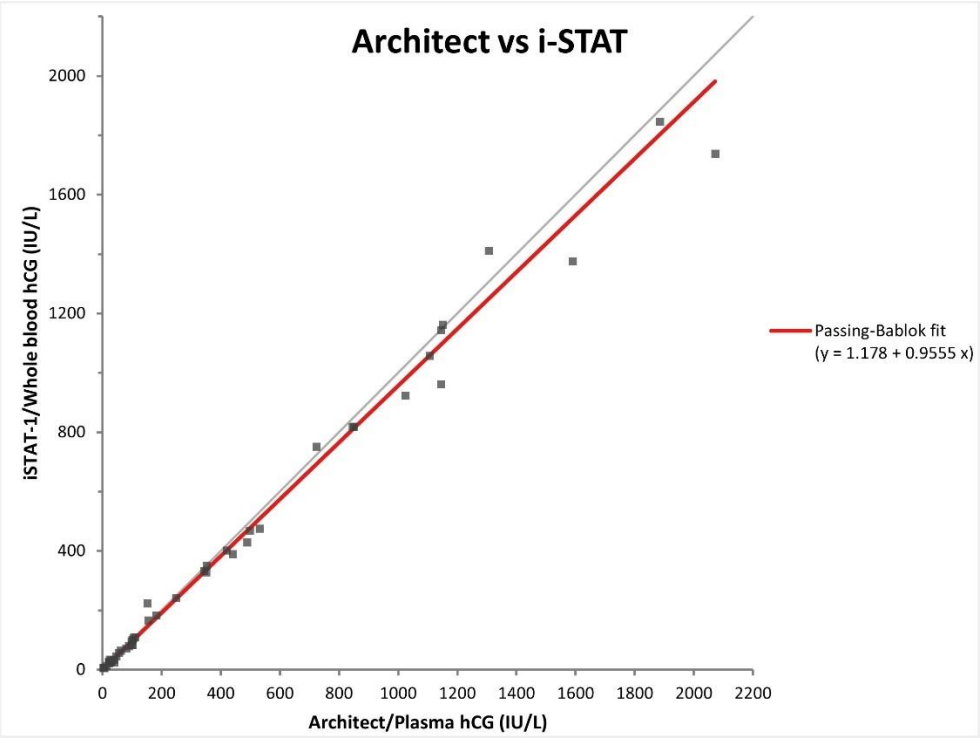

Figure 10. 2: Method comparison i-STAT®1 versus Architect: Passing-Bablok Regression

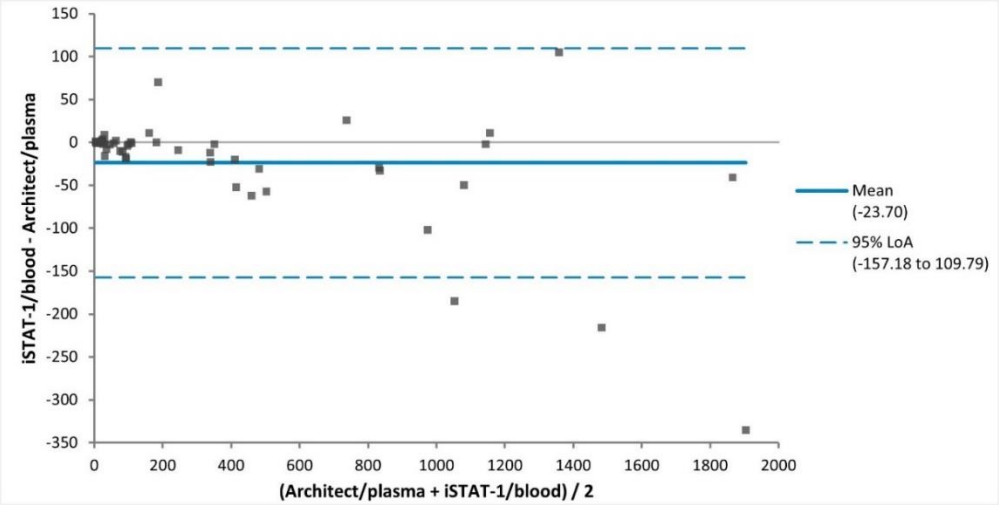

Figure 10. 3: Method Comparison (statistical assessment of agreement): i-STAT1 POCT device versus Abbott Architect (Designated comparator) method.

10.5 Discussion

This study demonstrated good agreement between the Abbott i-STAT®1 and the Abbott Architect with a minimal negative proportional bias of 4%. These findings provide confidence in hCG results at the clinical decision threshold (1500 IU/L) for PUL and suggest that the i-STAT®1 could be safely used for clinical management in the EPU.(449,466) The reported PUL rate in the local EPU varies between 8-12% of the total caseload (D. Hayes-Ryan, pers. Comm). During the study period, 49 of the 61 participants (80%) had hCG values less than 2,000 IU/L and these women had hCG measured for PUL or post-ectopic follow-up. This supports the view that most hCG results in PUL triage reside within the 1,000-2,000 IU/L range, which is within the quantitative range of the i-STAT®1 and supports its use to guide further management.(483) Most ectopic pregnancies following a PUL classification have an initial hCG <1000 IU/L.(484) However, it must be emphasised that hCG cannot be used alone to establish the diagnosis of ectopic pregnancy, and serial hCG results should always be interpreted in the context of the overall clinical picture.

There is limited data on quantitative hCG POCT devices, other than the Abbott i-STAT®1 and Radiometer AQT90 FLEX(477,478,480,481,485) The AQT90, a benchtop POCT device has a larger measuring range (2-5,000 IU/L) and could reduce the number of samples exceeding the 2,000 IU/L limit of the i-STAT®1. However, a minority of patients triaged for PUL exceeded this limit, as shown in this current study and a study by Kyriacou *et al*.(464) Despite this, the AQT90 holds promise as its expands the hCG measuring range increasing its usability in the EPU to triage PUL using the three established clinical thresholds for management of ectopic pregnancy (1,500: expectant, 3,000: medical and 5,000: surgical management.(449,470) Notably, use of hCG POCT for pregnancy management is not currently included in clinical management guidelines.(449,470,471) The gold standard for assessing early pregnancy remains the serial measurement of hCG using a standard laboratory blood test, despite some maternity hospitals lacking access to an on-site laboratory(449,463)

A limitation of our study was the lack of hCG results higher than that of the reported hook effect of the POCT assays. This is a phenomenon which occurs when hCG is present at such high concentrations that it saturates the anti-hCG antibody leading to false negative results and can lead to delayed or missed diagnosis with adverse outcome.(486,487) We

acknowledge that the highest hCG result assessed at 219,759 IU/L was less than manufacturer level claimed for the hook effect in the i-STAT®1 (300,000 IU/L). However, another study had placed the hook effect at 400,000 IU/L for this device.(477) The pregnancies most susceptible to the hook effect are molar pregnancy and when suspected, hCG results should be serially managed by a laboratory based total β-hCG assay.(487)

A notable strength of the Abbott I-STAT®1 POCT device is its portability as a handheld device, facilitating easy transfer between consultation rooms. Its user friendly interface and minimal maintenance requirement reduces the likelihood of errors, makes it more accessible for use by professionals outside the laboratory.(488) Additionally, it is the sole hCG POCT device included in the WEQAS POCT for quality assessment.

Therefore, POCT could potentially offer a rapid test βhCG result in the EPU, facilitating fast decision-making and patient triage within a shorter time frame. Rapid POCT hCG results are particularly beneficial in cases with a high suspicion of ectopic pregnancy, enabling management decisions based on the 48-hour hCG result.(449) Potential delays in blood hCG results could necessitate hospital admission and increase patient anxiety whereas immediate availability of results allows for prompt planning.(489) Such patient-centric approaches prioritise the patient needs, allowing decisions to be made during the clinic visit and possibly reducing unnecessary hospital admissions. POCT may also be used to provide a high sensitivity hCG test in the community for management of early medical abortion, where ectopic pregnancy is not a concern.(490)

This study adds to limited research on the possible clinical impact of POCT for hCG compared to existing laboratory-based testing. However, larger studies are necessary to evaluate the clinical impact of POCT hCG analysis on PUL triage protocols. A comprehensive cost-benefit evaluation is necessary to measure the impact of POCT hCG availability the EPU on hospital admissions and length of stay, similar to evaluations performed in emergency medicine.(485)

10.6 Conclusions

The i-STAT®1 demonstrated acceptable performance compared to central laboratory hCG results for quantifying β-hCG in early pregnancy. Use of POCT to measure blood hCG could lead to improved efficiencies in the EPU and safer clinical outcomes by facilitating a prompt diagnosis or exclusion of ectopic pregnancy. However, a more extensive prospective verification study, with a larger cohort of women is required before this POCT device could be recommended for routine clinical use.

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Guarantor: KOD

Author Contributions: Study design: CMJ, PMOS, DHR and KOD**;** Study data collection: CMJ and RL; Statistics: CMJ and PMOS; Drafting manuscript; CMJ, PMOS, DHR and KOD. Approval of final version of manuscript: CMJ, POS, DHR, SC, JC, TMcC and KOD.

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Conflict of interest: The author(s) declare no potential conflict of interest.

Patient consent for publication: not applicable.

Ethics approval: Written informed consent was obtained from all patients particpating in this study in accordance with ethical approval from the hospital research ethics committee (ECM4 (b) 01/11/2022 & ECM5 (8) 05/10/2022 & ECM3 (ss) 28/03/2023)

Data availability statement: In accordance with the journal's guidelines, we will provide our data for independent analysis by a selected team or by the editorial team for the purposes of additional data analysis or for the reproducibility of this study in other centres if such is requested.

Chapter 11 – Thesis Discussion

Chapter 11 – Thesis Discussion

Gestational trophoblastic disease (GTD) encompasses a spectrum of pregnancy-related disorders spanning benign and malignant conditions. Human chorionic gonadotrophin (hCG) accurately reflects the presence of trophoblastic cells and serves as a reliable biomarker for this disorder.(16) Molar pregnancy, a common benign form of GTD, typically carries an excellent prognosis while gestational trophoblastic neoplasia (GTN) is the most curative of all gynaecological malignancies. Timely diagnosis and appropriate treatment are essential for successful outcomes in both conditions, emphasising the pivotal role of hCG in management and detection of persistent disease.(229)

The establishment of the National Gestational Trophoblastic Disease (GTD) Registry, Monitoring and Advisory Centre in 2017 provided a unique opportunity to probe various aspects of GTD in Ireland, as explored in this thesis. The foundation of a national registry was further reinforced by the introduction of National Clinical Guidelines on the Diagnosis, Staging and Treatment of women with GTD, initially developed in 2015 and subsequently updated in 2022.(255)(108) Collectively, these two initiatives provided a structured framework to facilitate data collection and research to support evidence-based decision-making in the diagnosis and treatment of GTD.

This thesis contributes to the knowledge base of GTD through a series of nine research studies which address shortcomings in the diagnosis and monitoring of GTD. These studies cover various critical areas, including advancements in pathological diagnoses, the standardisation and harmonisation of hCG immunoassays and innovative approaches to hCG measurement remotely. In addition, the incorporation of patient perspectives in a service evaluation questionnaire, helped identify the main priorities for women post-GTD diagnosis, thereby offering a patient-centered approach to guide future research initiatives.

This chapter provides an interpretation of the research findings from the nine studies, detailed in chapters 2-10 and illustrated in Figure 11.1. These findings are categorised under the following headings:

1. Knowledge of GTD
2. Under-reporting of GTD
3. Biomarker evaluation
4. Novel methods of hCG analysis

This chapter also considers the implications of the study findings for clinical practice, as well as analysing potential areas for future research.

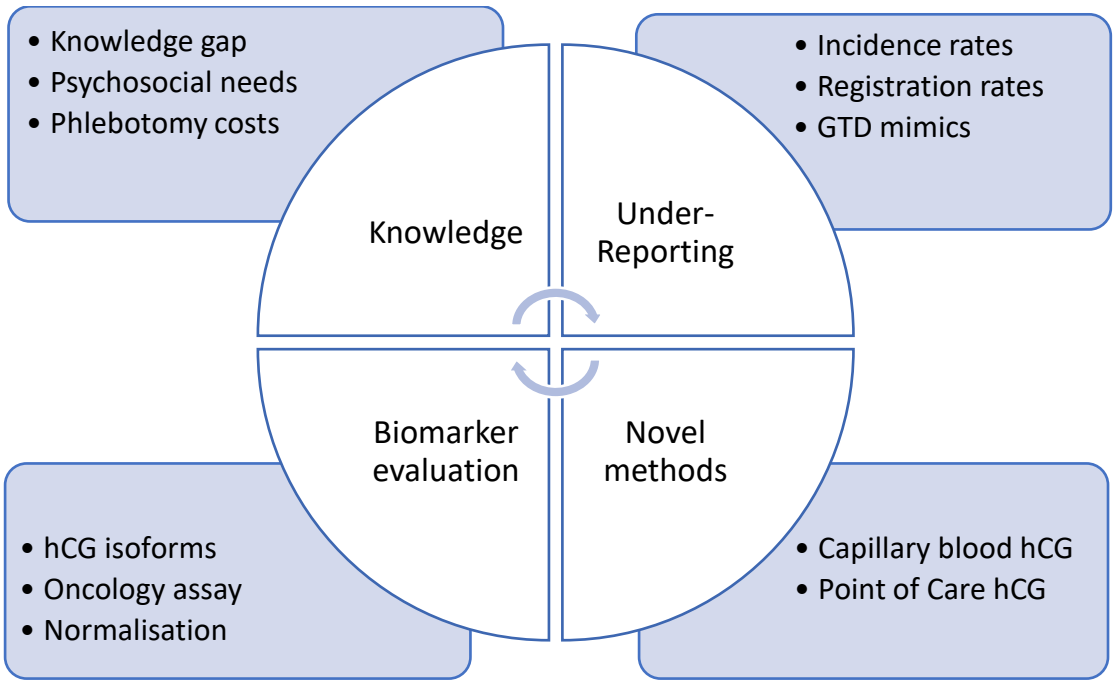

Figure 11. 1: Thesis study contributions to GTD field.

11.1 Knowledge of GTD (chapters 2 and 3)

11.1.1 Main findings

In Chapter Two, a scoping review synthesised evidence to elucidate recent advancements in the diagnosis and management of GTD.(491) This review identified knowledge gaps and areas for future research, many of which are explored in this thesis.(16)(116) Collaborative efforts with European laboratories (Chapter 7) aimed to standardise hCG immunoassays and harmonise biomarker evaluation and reporting (Chapter 7).(487) The scoping review also underscored the importance of integrating ancillary techniques, such as molecular genotyping into pathological diagnostic pathways. Three studies in this thesis (Chapters 4, 5 and 6) evaluated the use of ploidy techniques to complement morphological assessment and enhance the accuracy of Hydatidiform Mole (HM) diagnosis to yield more reliable incidence rates.(297)(309)(275)(310)(328)

Chapter Three presented feedback from a patient experience questionnaire completed by women enrolled in the Irish national GTD registry. The feedback emphasised significant strengths in the coordinated care provided by GTD centres, while also revealing some gaps in service provision.(232) The study highlighted a lack of knowledge amongst healthcare professionals (HCP) outside GTD centres, emphasising the need for better education in this area. Rapid hCG result delivery and the role of the clinical nurse specialists in conveying critical results with context and reassurance was deemed a priority for women with GTD. Non-critical hCG results via text message were well-received, a finding confirmed in another questionnaire (Chapter Nine), suggesting a potential role for telemedicine in healthcare delivery.

This study also highlighted the need for psychosocial support and access to peer support groups, given the rarity of this condition. The psychological needs and bereavement counselling requirements of women following molar pregnancy had not been examined previously from a pregnancy loss perspective.(492) Additionally, the study emphasised the need for information booklets on GTD tailored for partners and family members, who play a vital role in supporting affected women.

11.1.2 Strengths and limitations

This study represents the first patient experience feedback of women enrolled in the national GTD registry. The study achieved a 43% response rate, representing excellent engagement, particularly given the sensitive nature of the subject matter. Anonymity of the postal survey allowed open expression, facilitating unbiased feedback, essential for the development and advancement of care services.(493) Quinlivan et al. attained a higher response rate (62%) through a postal survey targeting the partners of women with molar pregnancy. However, due to the lack of anonymity in their survey, they were able to send a reminder letter one month after the initial questionnaire.(114) Additionally, the anonymity of the current survey hindered the differentiation of feedback between women with GTN and those with molar pregnancy. This differentiation could have unveiled distinct priorities for each group. To overcome this limitation in future survey designs, incorporating data on the patient's diagnosis or removing anonymity could yield valuable insights.

The patient survey had certain limitations that merit consideration in future surveys. The study did not use validated questionnaires (e.g. Hospital Anxiety and Depression Scale, HADS) and did not incorporate the views of an expert in this area (e.g. psychologist). The retrospective design may have introduced recall bias, potentially affecting the study outcome. Other studies have addressed this by including a representative number of past patients to ensure a sufficient sample size for detecting changes in responses over time.(114) The Irish GTD Registry, now large enough, could adopt this approach for future surveys. In addition, the survey was only available in English, limiting participation from non-native English speakers. Using multilingual software to translate questionnaires and incorporating images for better comprehension could effectively cater to a multinational population. Furthermore, the perspectives of the women's partners were not included in the survey, but their viewpoints are equally valid. Limited research in this area has indicated that men are equally affected by pregnancy loss, often experiencing feelings of blame and heightened anxiety following a molar pregnancy.(113)(114)(494) However, open-ended questions in the survey did allow for some family related feedback.

11.1.3 Implications for practice and policy

The GTD knowledge gap among HCPs identified in the survey (Chapter Three) warrants a multifaceted approach for resolution. The HCPs identified included general practitioners (GPs), nurses/midwives and hospital registrars working in obstetrics and gynaecology. Integration of GTD education into medical and nursing/midwifery curricula would better inform future graduates.(232) Incorporating GTD education into postgraduate modules, dedicated study days, and conferences offers valuable opportunities for professionals to enhance their understanding of this rare disease. By involving patient representatives in these educational forums, healthcare providers can gain valuable insights and cultivate empathy towards patient perspectives, mirroring collaborative approaches observed in oncology services.(118)

Given the rarity of GTD, GPs may only encounter a woman with GTD infrequently throughout their career. Thus, integrating information into continuous professional development (CPD) programmes is crucial to ensure GPs are adequately equipped with the knowledge to provide care. Efforts aimed at raising public awareness of GTD in Ireland, such as featuring articles on molar pregnancy in mainstream media platforms like the RTE Brainstorm website (November 2023) can also play a significant role in enhancing understanding and recognition of this condition.(495)

The survey revealed several factors impacting women's ability to attend weekly phlebotomy clinics for hCG monitoring, including financial costs such as childcare, travel costs and GP fees.(232) In an effort to address phlebotomy costs, the National GTD Steering Committee has proposed extending the free GP care under the HSE Maternity and Infant Care Scheme, to women with molar pregnancies.(496) Additionally, women expressed a preference for a separate phlebotomy pathway to avoid sitting next to pregnant women in the early pregnancy unit. In response, local governance structures established a dedicated phlebotomy service for women with GTD at an offsite outpatient clinic.

National standards for bereavement care following pregnancy loss and perinatal death recommend follow-up care appointments for women experiencing pregnancy loss.(256) Follow-up calls with the GTD nurse specialist could serve as a means to connect women with bereavement counselling services. Although molar pregnancy is not included in most

European and International recurrent pregnancy loss guidelines, it is included in the Irish National Clinical Practice Guideline for recurrent miscarriage.(497)(498)(499) Therefore, bereavement counselling services should be available and offered to women with GTD irrespective of the stage of their pregnancy loss.

This survey emphasised the need for psychosocial support following molar pregnancy, addressing anxieties surrounding recurrence, infertility, and concerns about future pregnancies.(500) Partners of women with molar pregnancy may also experience heightened anxiety and depression, although, the presence of children in the relationship appears to have a protective role.(114) Research has indicated that GTD can impact various aspects of a couple's lives including sexual function, psychological well-being, and overall quality of life.(113)(245)(248)(501) These couples may benefit from a multidisciplinary approach to tackle these challenges, akin to the psycho-oncology service provided to cancer patients and their families.(119) In the UK, use of electronic Patient-Reported Outcome Measures (ePROMs) tailored to address the psychosocial needs of women with GTD has been explored. A GTD-specific ePROM (ePAQ-GTD) integrated into a virtual nurse-led rare cancer pathway demonstrated benefits such as improved communication, patient empowerment and enhanced decision-making.(116)(117) Similar e-health solutions could be considered to enhance psychosocial support for women with GTD in Ireland.

11.1.4 Implications for future work and research

Future scoping reviews on advances in GTD diagnosis should adopt the Population, Concept and Context (PCC) framework to precisely define the review's focus and develop a comprehensive search strategy. Additionally, inclusion and exclusion criteria for studies should be clearly documented, to provide transparency on the sources and types of evidence analysed. The PRISMA-ScR checklist (Preferred Reporting Items for Systematic Review and Meta-Analysis extension for Scoping Reviews), should be included as supplementary data. This will enable easier access to the sources of evidence and eligibility criteria, as recommended in the Joanna Briggs Institute Methological Guidelines.(502) Future surveys on pregnancy loss should include women with GTD as their emotional and psychological needs are similar to those of other women experiencing

miscarriage.(115) These surveys should employ validated questionnaires (e.g. HADS) and these should be available in multiple languages to cater to diverse multiethnic populations. For instance, the European Organisation for Research and Treatment of Cancer Quality of Life Group (EORTC-QLG) hve a 30-item questionanire designed to measure quality of life in cancer patients and a validated GTD questionnaire is currently under development by this group.(503) Additionally, it is imperative to evaluate the impact of molar pregnancy on the male partner. Data collection should encompass a range of question formats, including multiple-choice, Likert scale, categorical and open-ended questions.

Further exploration of the educational requirements of HCPs could involve structured interviews with midwives and hospital registrars and focus group discussions with GPs to inform educational strategies. The adoption of educational models like the EKC model (Empathy, Knowledge, and Care) in Sweeden, which aids in training HCPs caring for women with reproductive loss, holds significance.(261) In Ireland, the TEARDROP workshops developed by the Pregnancy Loss Research Group address the educational needs of HCPs managing pregnancy loss.(504) There is potential to adapt a similar workshop model for all types of early pregnancy loss, to address the perceived lack of knowledge in HCPs caring for women with molar pregnancy (Chapter Three).

Women with molar pregnancy face many challenges, often having to reconcile pregnancy loss with a cancer diagnosis.(113) The process of post-molar hCG monitoring can be distressing for them, as it may serve as a constant reminder of their loss.(115) Many women find solace in connecting with others who share similar experiences through social media posts, which aids them in coping with their own experiences of GTD.(505)(264) Some GTD centres host professional-led monthly support group meetings for newly diagnosed women. Irish women participate in these UK-based meetings hosted on virtual platforms and while this collaborative approach is welcome, it may not entirely meet the needs of Irish patients and warrants further evaluation. Recently, the Irish Society of Gynaecological Oncology initiated a podcast series featuring an episode on GTD, where medical specialists and patient representatives provided their perspectives. This podcast serves as valuable resource for women undergoing treatment.(506)

11.2 Under-reporting of GTD (chapters 4,5 and 6)

11.2.1 Main findings

Centralisation of services for gynaecological cancers to specialist centres in developed countries has resulted in improved outcomes and longer survival rates.(507) Several studies have emphasised the critical role of GTD centres in managing women with GTD, with research consistently showcasing improved outcomes for women treated at these specialist centres.(508)(123)(125) The European Organisation for Treatment of Trophoblastic Disease (EOTTD) has embarked on an ambitious initiative to establish national GTD registries in all European countries. While GTD registries have been established in many countries to gather clinical data for auditing incidence rates and disease management, their effectiveness has been hampered by documented under-reporting of GTD cases.(425)(120)(23)

Accurately calculating incidence rates is further complicated by inconsistencies in applying morphological criteria and intra-observer variability in the diagnosis of HM.(64) Hence, ancillary techniques such as p57 immunohistochemistry, ploidy analysis and molecular genotyping are often required to complement morphology and improve the accuracy of the diagnosis of HMs, particularly in early gestation. However, limited access to these additional techniques could hinder the collection of accurate incidence rates, providing an argument for centralised pathology review.

The scoping review (Chapter Two) identified the need for ancillary techniques such as molecular genotyping as an adjunct to morphological assessment of pregnancy tissue to improve the accuracy of HM diagnosis.(266) Responding to this need, Chapter Four introduced a novel scoring system for ploidy analysis aimed at enhancing the morphological diagnosis of partial hydatidiform moles (PHMs).(297) Adapted from the *HER2* in-situ hybridisation assay (D-DISH) used for breast cancer, this scoring system uses the internal chromosome 17 probe to assess ploidy status. Prior to integrating this technique into routine practice, its diagnostic utility underwent thorough evaluation, confirming that the clinical decision thresholds accurately categorised diploidy and triploidy in pregnancy tissue.

In Chapter Five, an evaluation audit using molecular genotyping validated use of the *HER2* D-DISH ploidy assay in cases of morphologically suspected PHMs.(275)(328) The audit demonstrated complete concordance with the initial diagnosis. Notably, this study emphasises the importance of initial morphological assessment by an experienced perinatal pathologist prior to *HER2* D-DISH ploidy determination to mitigate the risk of false positives due to digynic triploidy. It's worth noting that there is limited evidence indicating morphological overlap between digynic triploidy and diandric triploidy.(311)

Chapter Six presented the results of an audit of HM incidence rates within the local pathology service, following the introduction of ancillary techniques to enhance HM morphological assessment (p57 for Complete Hydatidiform Mole, CHM and HER2 D-DISH ploidy for PHM)(297). The audit revealed a higher incidence of HM, especially PHM, compared to previous reports.(28)(29) Additionally, the results of a national survey of pathology laboratories highlighted restricted accessibility to ancillary techniques, particularly ploidy techniques.(309)(310) The lower HM incidence rates observed nationally may reflect limited access or utilisation of ancillary techniques in some institutions.

Finally, an investigation into GTD registration rates in Ireland revealed concerning suboptimal registration rates with the national GTD registry, despite recommendations in the National Clinical Guidelines for GTD that all cases should be registered.(108) This recommendation has been reiterated by the department of pathology in the Royal College of Physicians of Ireland. Nearly half of the women with GTD in Ireland were unregistered, posing a significant clinical risk.

11.2.2 Strengths and limitations

The widespread availability of the *HER2* D-DISH technique in pathology laboratories, primarily to determine *HER2* amplification status in breast and gastric cancers, serves as a key enabler for the integration of this technique for ploidy analysis into routine diagnostic practice. Unlike molecular genotyping, no additional scientific expertise is required to interpret ploidy.

One potential limitation of the *HER2* D-DISH ploidy technique is weak staining, which could result in the under-counting of nuclear signals and misinterpretation of ploidy

status. However, in practice, cases with weak staining are restained and the majority are scored successfully, enabling accurate determination of ploidy. Since the introduction of this novel scoring technique, an audit has revealed a very low rate of equivocal scoring (1.02%).(297)

Another remote possibility is the misinterpretation of triploidy due to the presence of trisomy 17, given the use of chromosome 17 probes to determine ploidy. Given the rarity of trisomy 17 (1 in 1,000 miscarriages) and the lack of morphological overlap between trisomy 17 and PHM, this scenario is considered highly unlikely.(276)

11.2.3 Implications for practice and policy

It is important for countries to have complete GTD incidence data for a number of reasons. It avoids selection bias in patient registration, stores individualised patient information, informs clinical management and allows shared decision making based on reliable incidence data. Evidence suggests that clinical registries can enhance the quality of healthcare processes and improve patient outcomes.(509) They provide valuable repositories of epidemiological data, provide research opportunities, and facilitate clinical audit of the impact of treatment and service delivery. In addition, they reduce variation in clinical practice by offering centres of expertise, leading to subsequent healthcare cost savings.(126) However, in Ireland there is no national pathology register available to provide GTD incidence data and this information must be derived from national pathology audits.

Given the malignant potential of HMs, it is crucial to promptly and accurately identify them.(66) Implementation of the HER2 D-DISH ploidy assay using the “rule of 5” scoring system as an adjunct to morphological assessment has the potential to enhance the accuracy of diagnoses and improve the quality of epidemiological data. This approach could significantly reduce the incidence of uncertain diagnoses, as illustrated by the 27 atypical HMs documented in the national pathology survey. The innovative “rule of 5” scoring system can be seamlessly integrated into pathology laboratories with ready access to the *HER*-2 D-DISH assay, thereby enhancing the accuracy of HM diagnosis. National clinical management guidelines recommend registration of atypical cases with the national GTD centre, mandating clinical follow up and hCG monitoring as potential PHMs. By improving the accuracy of HM diagnosis, this approach could potentially alleviate the

anxiety associated with uncertain results, reduce the need for prolonged hCG surveillance monitoring, and enable the earlier initiation of a new pregnancy.

The disparity in HM incidence rates uncovered in the National Pathology Survey may reflect inconsistent use of ancillary techniques to support morphological diagnosis of HMs, emphasising the need for centralised pathology review. However, more widespread implementation of the *HER2* D-DISH ploidy analysis, to support morphology, could improve the accuracy of HM diagnosis at units where primary diagnoses are made, thereby reducing the reliance on centralised HM secondary pathology review. This is particularly important as centres providing centralised review can become overloaded with inappropriate referrals if confidence in local units is lacking. Another concern with reliance on centralised review is that there is nothing for them to review if a local unit has missed the diagnosis. Therefore, best approach may be to improve confidence in diagnostics in local units by providing education and supporting the implementation of simple techniques like p57 immunohistochemistry and the *HER2* D-DISH ploidy assay. This approach would ensure timely access for all women to an accurate diagnosis.

The low adherence to voluntary registration of GTD cases by clinicians with the national GTD registry identified in this study highlights the need for improved reporting practices and presents a compelling case for mandatory registration to ensure equitable access for all women to expert care for this highly treatable condition.(332) The lack of a national pathology register to identify unregistered GTD cases necessitates use of pathology audits to obtain this information. The Health Service Executive GTD steering committee in collaboration with the Faculty of Pathology at the Royal College of Physicians of Ireland have already advocated for registration with the GTD centre, but this advocacy should be reinforced or even mandated. The Faculty of Pathology could also help standardise reporting practices in pathology by issuing guidance to ensure the provision of more accurate HM incidence rates.

11.2.4 Implications for future work and research

In advance of widespread adoption of the “rule of 5” ploidy scoring system, larger prospective studies are needed nationally and internationally to validate its reproducibility. This step is crucial as indiscriminate adoption of published clinical decision thresholds may lead to incorrect diagnostic classification.(153) Considering the

widespread availability of the *HER2* D-DISH assay in pathology laboratories, replicating this study to validate the findings should be readily achievable.

Conducting large-scale prospective studies by pathologists across various institutions could offer valuable insights into resolving the ongoing debate on whether digynic triploidy causes morphological confusion with diandric triploidy.(311) The dearth of evidence on this subject poses a significant challenge in determining whether this potential risk is purely theoretical or practical in clinical diagnostic practice.(290)(274)(291) Unlike PHM, digynic triploids do not necessitate hCG monitoring, making accurate diagnosis a priority for both the clinician and the patient who may be able to avoid follow-up hCG surveillance monitoring.(275)

The disparity in local and national incidence rates and the number of atypical HMs recorded in this study (Chapter Six), highlights the need for standardised diagnostic reporting pathways to enhance quality assurance.(309) Future pathology surveys should collect data on the utilisation patterns of ancillary techniques (e.g. percentage of total POCs tested for ploidy), as these practices could impact diagnostic rates. Such data may also contribute to discussions around the harmonisation of diagnostic pathways for HM. Additionally, these surveys should incorporate information on patient ethnicity and maternal age, factors which could potentially impact national HM incidence rates.

11.3 Biomarker evaluation (chapters 7 and 8)

11.3.1 Main findings

hCG is an excellent tumour biomarker, as its concentration in blood accurately reflects tumour burden.(510) As part of an initiative by the EOTTD to harmonise and standardise hCG reporting, Chapter Seven presents the responses from an electronic survey conducted among EOTTD affiliated laboratories that measure hCG for GTD monitoring.(90) Reponses were obtained from 13 countries and five of these countries have established GTD referral centres.

The study questionnaire highlighted significant variability in operational and reporting practices between laboratories, even among those using the same hCG immunoassay. The study also addressed analytical and biological interferences in hCG measurement, through practical examples, further highlighting the importance of scientists and clinicians knowing the limitations of individual hCG immunoassays. In addition, the survey revealed

the use of different decision thresholds for normality and use of hCG immunoassays not approved for oncology, posing a potential clinical risk. The consequences of using different cut-off thresholds could include increased patient anxiety, extra courses of MTX or delayed detection of disease recurrence. This study also evaluated various laboratory service provision scenarios to assess their effectiveness in hCG monitoring for GTD. Only two laboratories (UK and Netherlands) reported centralised hCG testing for GTD and these centres benefit from a wealth of data, scientific knowledge, and clinical expertise built up over many years managing this rare disease.

The Roche Elecsys® (Total + free β-hCG) oncology approved immunoassay, emerged as the most frequently used commercial immunoassay in the survey. However, even among laboratories using the same Roche assay, there were differences in the sensitivity (limit of quantification, LOQ) quoted (0.1-0.5 IU/L), confidence intervals used to determine normality (95%, <5 IU/L and 99%, <1 IU/L) and awareness of hCG isoforms detected.

Chapter Eight demonstrated the pivotal role of serum hCG and molecular genotyping in the management of five challenging cases.(332) These cases demonstrated the importance of investigating persistent low level HCG elevations to identify disease persistence and guide chemotherapeutic or surgical intervention. This is best exemplified in case one (ectopic molar pregnancy) where hCG levels initially dropped on third line chemotherapy to 10 IU/L and then rose to 54 IU/L necessitating surgical intervention and fourth line chemotherapy. Collaboration with a reference laboratory was required to confirm the low-level hCG elevation and treatment took two years requiring multidisciplinary team management to achieve a cure. Notably, an ectopic pregnancy was not confirmed on histopathological examination of tissue following TAHBSO. A benign paratubal cyst was observed in the right ovary with focal decidua noted on the surface of the left overy but there was no trophoblastic lesion identified. Therefore, this case could represent a high-risk choriocarcinoma after term pregnancy rather than an ectopic molar pregnancy. Case two (ovarian dysgerminoma) also highlighted the need for close collaboration between clinical and scientific teams where low level hCG elevations may mimic GTD.

Molecular genotyping is become integral to GTD management, as demonstrated in case four (gestational choriocarcinoma) where confirmation of the gestational origin of the

choriocarcinoma led to a change in the chemotherapy regimen.(268) This case highlights the importance of meticulous monitoring of hCG in pregnancies of unknown location to promptly diagnose GTN. Non-gestational has a worse prognosis than gestational choriocarcinoma, as highlighted in case three, where hCG levels normalised following chemotherapy and started to rise again necessitating a total abdominal hysterectomy.

11.3.2 Strengths and limitations

The survey conducted among European laboratories (Chapter Seven) represents the first comprehensive effort to gather insights from laboratories offering hCG monitoring to women with GTD. Despite a low response rate, the 21 responses provided viewpoints from 13 countries, involving scientists directly engaged in hCG measurement for GTD management across Europe. Although, the survey's findings are valuable, they also offer a foundation for refining future survey iterations. This includes considering national policies regarding hCG test provision for GTD management, the use of a national shared laboratory information system, specialist scientists' interpretation of reports, participation of clinicians and scientists in MDTs, and the physical proximity of laboratories to GTD referral centres. Such local details offer context for the questionnaire responses.

11.3.3 Implications for practice and policy

The findings from the survey conducted among EOTTD-affiliated laboratories (Chapter Seven), reinforces the need for standardisation of serum hCG immunoassays to reduce inter-assay variability.(487) Use of a total β-hCG assay rather than an assay measuring only intact hCG is essential for appropriate GTD management.(511)(90) The EOTTD hCG working group is actively pursuing the harmonisation of assay detection limits (LOQ), quality parameters and clinical decision thresholds for commercial immunoassays, aligning with their ambition to enhance the consistency and accuracy of hCG measurement for GTD management.

The study also presented two options for hCG test provision: centralised hCG testing reference laboratories using a bespoke hCG radioimmunoassay (RIA) or decentralised hCG testing using commercial two-site sandwich immunoassays. The first option ensures the provision of a standardised assay by experience scientists who can leverage cumulative years of data on one assay to establish clinical decision thresholds and construct hCG

regression curves or hCG ratios to predict GTN progression and chemotherapy resistance.(133)(162) In contrast, decentralised testing allows routine diagnostic laboratories use commercial immunoassays approved solely for early pregnancy detection, potentially posing significant clinical risks. Decentralised testing is further complicated by managed service contracts in large diagnostic laboratories, where financial considerations often dictate the choice of laboratory platforms which can result in a change of service providers at short notice.(487)

The continued reliance on laboratory developed tests for hCG measurement by RIA in reference centres is facing growing challenges, ranging from safety concerns regarding isotope usage to operational issues in a tightly regulated laboratory setting. This environment is governed by European Union (EU) Directives overseeing in-vitro diagnostics (IVD) tests and requires strict adherence to international accreditation standards, ISO15189 2012.(373) Such regulatory frameworks may significantly influence national decisions regarding future hCG services for GTD.

11.3.4 Implications for future work and research

The lack of standardised immunoassays for GTD-derived hCG poses a challenge to data comparability and hampers efforts to harmonise data across institutions. Future endeavours should prioritise the development of practice guidelines for hCG measurement, addressing the pre-analytical, analytical, and post-analytical concerns highlighted in the survey. Moreover, the EOTTD hCG working group is also collaborating with UK NEQAS, an external quality assurance (EQA) provider to organise a pilot scheme for GTD laboratories.(512)

Considering the lack of centralised hCG testing for women with GTD in Ireland, laboratory services endeavour to ensure timely provision of serial hCG monitoring, using appropriate hCG assays, conducted in accredited laboratories by experienced scientists, as recommended in the National Clinical Practice Guidelines.(108) Inter-laboratory connectivity to the national GTD centres will be facilitated as part of a national laboratory connectivity project (MEDLIS).

Laboratory scientists and clinicians must remain vigilant regarding unexpected findings and correlate laboratory hCG results with clinical presentation to identify false low or

positive hCG results. Specialised education and training opportunities are crucial for scientists and clinicians engaged in GTD management, to address deficiencies highlighted in the laboratory survey. Participation in programmes such as the EOTTD trainee mentorship programme or the ERASMUS staff mobility training (which I had the opportunity to participate in to visit Charing Cross GTD centre) offers professionals the chance to visit GTD reference centres for collaboration on projects or to facilitate knowledge exchange.(513)

Chapter Eight presented cases that highlighted the benefits of a national GTD centre with an expert clinical lead, dedicated nursing support, access to a specialist MDT group, patient participation, working to national clinical guidelines and integrating with international experts to effectively manage this rare disease.

In contrast to other cancers, GTN has a high cure rate, an excellent biomarker, widely accepted practical clinical guidelines and a well-established community of experts dedicated to management of this rare malignancy.(100,127)

11.4 Novel methods of hCG analysis (chapter 9 and 10)

11.4.1 Main findings

Given the absence of a centralised hCG testing service for women with GTD in Ireland, innovative approaches aimed at achieving national harmonisation of hCG testing were explored. Chapter Nine evaluated the clinical efficacy and user acceptance of capillary blood samples collected remotely.(514) This study showed the equivalence of hCG capillary blood testing to standard venous blood hCG testing demonstrating complete clinical concordance. Feedback from the user satisfaction questionnaire demonstrated the acceptance of this collection technique, with participants expressing a clear preference for capillary blood sampling over traditional visits (hospital/clinic/GP practice) for blood tests.

Chapter Ten assessed the use of point-of-care testing (POCT) for hCG measurement in the early pregnancy unit (EPU) following a planned relocation of the unit away from the maternity hospital.(515) A mini-verification of a POC analyser, the Abbott i-STAT®1 was performed and showed good agreement with the main laboratory Abbott Architect for quantifying β-hCG in early pregnancy. The Abbott i-STAT®1 POCT device could be used by

clinicians to triage patients based on a hCG clinical decision threshold of 1500 IU/L, to guide patient management for a suspected PUL or ectopic pregnancy.

11.4.2 Strengths and limitations

Consumer demand, one of the main drivers for home testing, surged followed the interest in self-testing during the recent SARS-CoV-2 pandemic.(438) Concurrently, capillary blood collection was introduced to facilitate monitoring of glycosylated haemoglobin (HbA_1C) in diabetes.(93) A notable strength of the capillary blood study (Chapter Nine) was that it aligned with this growing trend, with participants expressing a willingness to readily adopt this sample collection method if it were routinely available to them. However, a limitation of the capillary blood study was that the associated study literature was only produced in English. This may have deterred some participants, despite the presence of self-explanatory photographs to guide capillary blood collection. This issue can be rectified in future larger prospective studies arising from this proof-of-concept study, by incorporating multilingual production formats.

11.4.3 Implications for practice and policy

The application of remote capillary blood collection extends far beyond pregnancy monitoring and holds promise for centralising tumour marker testing for patients with rare tumours.(448) While hCG serves as an excellent tumour marker for trophoblastic disease, serum hCG immunoassays exhibit considerable inter-assay variability and necessitate harmonisation.(487) Leveraging capillary blood samples collected remotely facilitates centralised laboratory testing with an oncology-approved immunoassay throughout a patient's treatment and surveillance journey. Furthermore, feedback from the study questionnaire indicated a preference for this approach to follow-up serial testing, suggesting potential improvements in adherence to follow-up blood-based diagnostic tests if this sample collection modality were routinely available. This favourable response suggests that a future roll out would enhance the likelihood of patient compliance with follow-up monitoring, a critical factor for women undergoing treatment for GTD.

Implementation of hCG POCT in the EPU could help triage women at risk of pregnancy complications by using well-established clinical decision thresholds for ectopic pregnancy and PUL and may reduce the number of women who need to be admitted to hospital for

care. However, the use of hCG POCT in the management of early pregnancy is not currently included in national or international clinical guidelines for ectopic pregnancy, recurrent miscarriage or molar pregnancy.(498)(108)(21) Rather, guidelines recommend use of laboratory-based serial hCG measurements, as advised in the National Institute for Health and Clinical Care (NICE) on the diagnosis and initial management of ectopic pregnancy and miscarriage.(469) Additional research is warranted to assess existing hCG clinical decision thresholds in POCT instruments to identify how they compare to central laboratory analysers.

11.4.4 Implications for future work and research

The proof-of-concept capillary blood study requires further evaluation in a larger cohort and replication in other institutions to validate its reproducibility and reliability. Future studies should expand their scope to include a more diverse range of nationalities to obtain a broader perspective view on user acceptability. Some of the perceived barriers to home collection included training needs, collection time, anxiety related to finger-pricking, concerns about adequate blood volume. A large-scale rollout should involve meeting patient representative groups to advocate for home testing and the offer of training directly in patients' homes. Additionally, an alongside comprehensive cost-benefit analysis is required to justify the incorporation of capillary blood testing into clinical practice.

Further research is also needed to standardise the capillary blood collection procedure and optimise the volume of blood collected.(454) Careful consideration should also be given to pre-analytical factors such as the type of lancet used, needle gauges and plastic used in collection containers, as well as sample stability issues. Additional considerations for home testing include alternative sampling methods such as urine, saliva and dried blood spots. Use of qualitative rapid urine pregnancy tests (one-step immunoassay) for hCG monitoring in GTD is not recommeneded as these lateral flow devices are prone to false negative and false positive results.(381,516) The lack of a quantitative commercial immunoassay for urine hCG measurement limits the widespread use of urine for hCG monitoring.(517) In addition, current LDTs for urine hCG use RIA and most diagnostic laboratories are no longer licenced to use radioactivity. However, there are some non-isotopic competitive hCG immunoassays in development which may replace RIA. These

include use of dissociation-enhanced lanthanide fluroescence and chemiluminescence.(394) Dried blood spots (DBS) offer a viable alternative, where there is a large patient catchment area and women reside in geographical areas remote from GTD centres.(94) The Capitainer® DBS card facilitates quantitative measurement, overcoming many of the challenges associated with DBS use.(94)

11.5 Future Research Studies

Several overarching themes for future research are identified in this thesis, which span across multiple studies from the thesis. Suggested future studies are briefly outlined here.

11.5.1 Educational Deficit (Chapter 2)

1. Incorporate information on GTD into undergraduate/postgraduate modules, CPD and conference programmes through collaborate with the National Universities of Ireland and the Irish College of General Practitioners to address knowledge gaps
2. Conduct public awareness campaigns to raise the profile of GTD as a rare disease, as illustrated in dissemination efforts in this thesis; RTE Brainstorm articles and presentations to the All-Ireland Rare Disease Inter-Disciplinary Network (RAiN).
3. Use trainee mentoring (EOTTD) and staff mobility programmes (Erasmus+) to facilitate education and training for clinicians and scientists in reference GTD centres.

11.5.2 Extending the scope of questionnaires (Chapters 3, 6,7)

1. Use of validated questionnaires (e.g. HADS) or use of a validated GTD questionnaire, currently under development by the EORTC-QLG.
2. Develop a multi-lingual patient questionnaire, tailored to different nationalities, and including the partner's experience of molar pregnancy.
3. Design a national pathology questionnaire to collect data on GTD diagnostic rates, use of ancillary techniques to support morphological assessment.
4. Combine a European laboratory questionnaire with a sample exchange programme to collect data on local GTD centres, IT connectivity and workforce qualifications.

11.5.3 Standardisation of diagnosis (Chapters 4,5,6,7)

1. Implement practical guidelines for HM reporting in pathology nationally, to ensure adherence to best practice, enhance diagnostic accuracy, and obtain more precise incidence rates.(66)
2. Organise collaborative studies among pathologists to investigate whether digynic triploidy causes morphological confusion with PHM, as clinical management pathways will differ.
3. Develop of a core outcome set for GTD to facilitate international consensus on a universal denominator for incidence studies and allow more meaningful international comparisons.
4. Standardise quality parameters across each commercially available hCG immunoassay and incorporate key metrics such as limits of quantitation, high dose hook effect, normalisation levels.
5. Integrate molecular genetics into the GTD diagnostic pathway to better inform clinical management, considering the following (1) CHM zygosity testing (47), (2) Gene fusion testing for APSN (LPCAT::TERT) (11), STR genotyping for choriocarcinoma (14)

11.5.4 GTD Centre Resources (Chapters 3, 6)

1. Organise professional-led monthly virtual meetings for newly diagnosed women to disseminate evidence-based information.
2. Provide supportive information booklets for partners/families of women with GTD.
3. Increase awareness amongst medical professional bodies regarding low registration rates for GTD.

11.5.5 Innovative Solutions (Chapters 3,9)

1. Organise prospective large-scale capillary blood studies for women with GTD and incorporate cost-benefit analysis, to assess the feasibility of implementing this approach for hCG monitoring in women with GTD, which may address some of the costs associated with weekly phlebotomy for hCG measurement.

2. Consider alternative sample types (DBS, saliva, urine) and/or alternative laboratory techniques for hCG measurement, such as liquid chromatography mass spectrophotometry warrant further study.

11.6 Summary

At the outset of this work, a knowledge gap concerning GTD among healthcare professionals was identified. This lack of understanding combined with the psychological, emotional, and financial burden highlights the many challenges faced by women with GTD. These findings also emphasise the need for greater education about this rare condition and equivalent bereavement counselling needs for all women regardless of the reason for pregnancy loss.

Two separate studies in this thesis noted under-registration of women with GTD. The national pathology questionnaire revealed under-registration of almost half of the women with GTD and a third of these had CHM, posing a significant clinical risk. Feedback in the patient experience survey also reported women having to instigate registration themselves.

One of the outputs of this thesis was the validation of a ploidy assay as an adjunct to morphological examination to improve the accuracy of PHM diagnosis. Implementation of this technique has led to more accurate local HM incidence rates and suggests an under-reporting of HMs nationally and possibly internationally. Under-reporting of cases nationally may be linked to limited access to ancillary techniques which highlights the need to implement practical guidelines to standardise HM diagnostic pathways.

A key finding from the survey of EOTTD-affiliated laboratories on hCG operational and reporting practices revealed substantial variability both between and within laboratories, even among those using the same commercial immunoassay. This underscores the imperative to standardise hCG assays and harmonise reporting practices across laboratories.

The presentation of challenging GTD cases in five case reports emphasised the crucial role of both hCG and molecular genotyping in determining risk stratification, disease recurrence and chemotherapy resistance. These cases also showcased the value of a collaborative, multidisciplinary multinational approach in managing this rare disease.

A proof-of-concept study using capillary blood collection for hCG measurement was performed to address some of the challenges of hCG centralisation. Analysis of paired capillary and venous samples showed excellent agreement, indicating that capillary blood could serve as a viable alternative for hCG monitoring. Feedback on the user acceptability of capillary blood collection was overwhelmingly positive, leading to plans for a larger prospective study to evaluate its impact on clinical pathways and conduct a health economic assessment in women with GTD.

Furthermore, in exploring the accessibility of hCG testing in the community, POCT analyser was evaluated for their its suitability in triaging women in a remote early pregnancy unit for scheduled care. The POCT device showed clinically concordant with laboratory hCG results facilitating the use of clinical decision thresholds for pregnancies of unknown location. While there is a lack of information on the use of POCT for GTD management, further studies are warranted in this area.

11.7 Conclusion

In conclusion, the research presented in this thesis provides valuable clinical insights and has broadened the knowledge base on GTD in Ireland. It highlights a lack of standardisation in the pathological diagnosis of GTD, and in the measurement of the biomarker, hCG. In addition, it identified under-reporting of incidence rates, poor compliance with voluntary registration of women with GTD and a knowledge gap regarding molar pregnancy amongst healthcare professionals. This work offers an opportunity to positively impact diagnostic practices and introduce innovative methods to improve the accuracy of GTD diagnosis and enhance the accessibility of hCG monitoring to all women with GTD.

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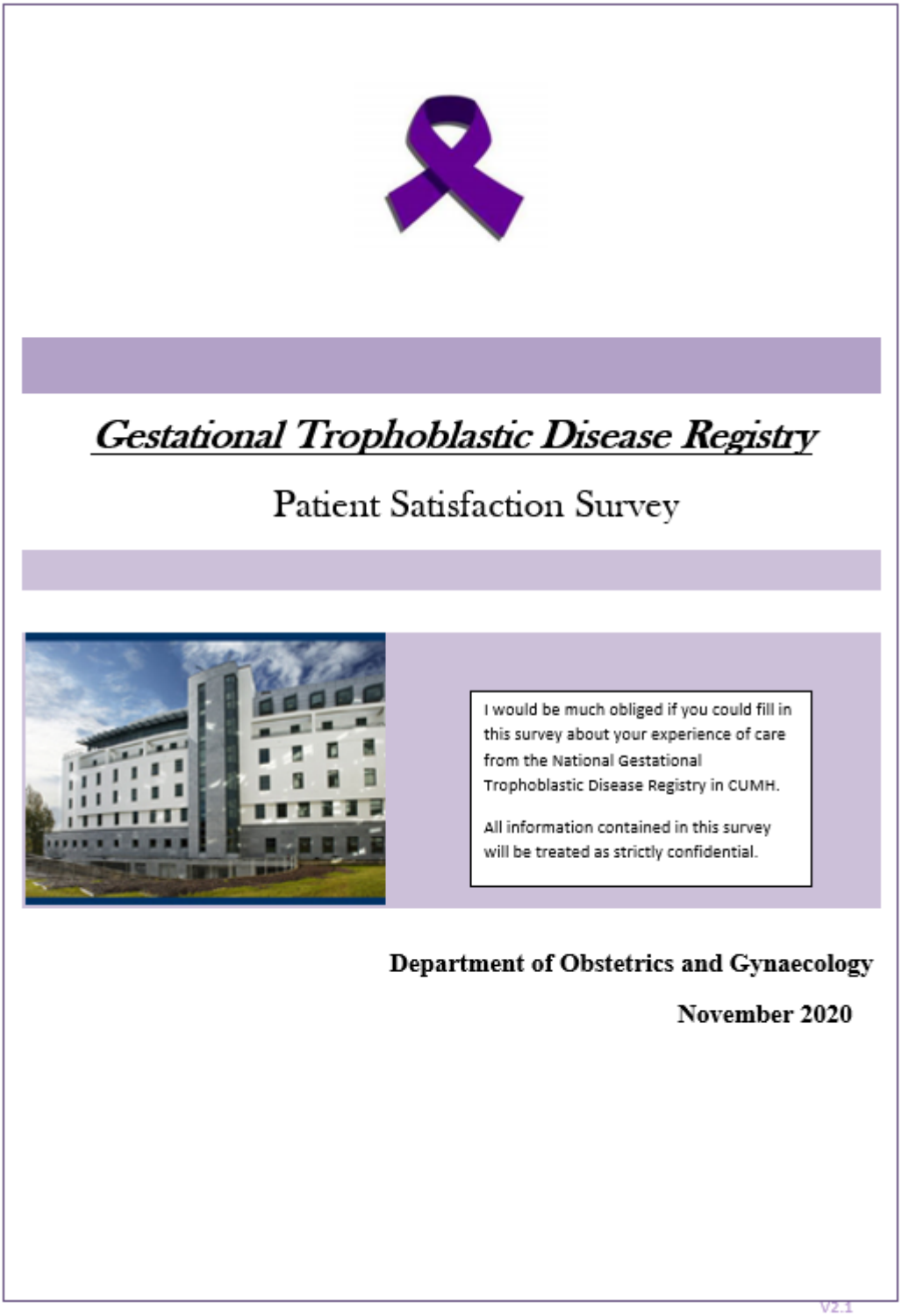
Supporting materials

Appendix I - Gestational Trophoblastic Disease Registry. Patient Satisfaction Survey



Patient Information Leaflet

Study Title: Survey of women on the National GTD Registry on their experience of services offered.

Principal Investigators: Caroline Joyce, *Principal Clinical Biochemist;* Dr Keelin O'Donoghue, *Consultant Obstetrician & Gynaecologist* and Dr John Coulter, *Clinical Lead for Gestational Trophoblastic Disease*

Sites of Investigation: Cork University Maternity Hospital (CUMH)

Thank you for taking the time to read this patient information leaflet. It gives detailed information about the research study which you should read carefully so that you understand it fully and can make an informed decision. You will be asked to complete a questionnaire if you wish to participate.

Why is this study needed?

In Ireland, all women with a molar pregnancy are registered with the National GTD Registry, Monitoring and Advisory Centre in CUMH where patient care is coordinated by the Clinical Lead, Dr John Coulter. This study will help us understand the needs and experience of women diagnosed with GTD. We believe your opinions will help inform the services and resources available to future patients on this Registry.

What is my role?

As part of this study you are asked to complete one questionnaire on your experience as a patient registered with the National GTD Registry. The questionnaire should take 10-15 minutes to complete.

Important things you need to know:

If you take part in the study, withdraw from the study, or do not participate in the study there will be no change to your care from the National GTD Registry.

How we will use your personal data?

During this study, no identifiable information (name, date of birth, address, etc) will be collected. All questionnaires will be anonymous.

Will this be confidential?

All the information collected on the questionnaire will be completely anonymous as no identifiable information will be collected.

Data processing and storage:

The study is being conducted by a UCC postgraduate student; Caroline Joyce based in CUMH. This means that UCC is the Data Controller for this research project and that UCC owns the data collected as part of the study. All data will be stored on password protected devices only accessible to the research team without any identifiable information in accordance with both UCC and CUMH data protection policies.

Is this study approved by an Ethics Committee?

Yes, ethical approval for the study was granted from the Cork Research Ethics Committee of the Cork teaching hospitals and if you have any concerns about this study, you may contact them by phone at 021-4901901 or by email: crec@ucc.ie

Do I have to sign anything?

No, if you agree to participate, we ask you to please complete the study questionnaire

Contact Information:

Should you need help filling out the questionnaire or would like further information, please contact Caroline Joyce by email at caroline.joyce@hse.ie. Alternatively, if you wish to discuss GTD related issues, please contact the GTD specialist nurses at GTD@hse.ie or by phone at 086 0082561. ***If you have any complaints in connection with our processing of your personal data, you may contact UCC's Data Protection Officer (DPO): DPO, Information Compliance Section, Office of Corporate & Legal Affairs, University College Cork, Western Road, Cork E: foi@ucc.ie Tel: +353 21 4903949***

V2.1

Thank you for taking the time to share your views on the National Gestational Trophoblastic Disease (GTD) Registry. All responses to this questionnaire will be treated in confidence and will be used to improve quality of care we offer at the GTD Registry. Please ✓ one box in each question.

A. Registration

1. How did you hear about the National GTD Registry?

- My Clinician ☐
- HSE website ☐
- Social Media ☐
- Other_____ ☐

2. Who suggested registration with the National GTD Registry?

- My clinician ☐
- Myself ☐

3. How long since your diagnosis of a molar pregnancy/trophoblastic disease?

- Less than 12 months ☐
- 1-3 years ☐
- Over 3 years ☐

4. How long after your diagnosis were you registered with the National GTD Registry?

- Less than 1 year ☐
- 1-3 years ☐
- Over 3 years ☐

5. Was your diagnosis of molar pregnancy/trophoblastic disease clearly explained?

Yes ☐ No ☐

6. Was your treatment regimen clearly explained to you?

Yes ☐ No ☐

7. Were you given contact details for GTD nurses?

Yes ☐ No ☐

8. Do you think all GTD patients should be registered with the National GTD Registry?

Yes ☐ No ☐

Please include any further comments on registration here:

_____

V2.1

B. Patient Knowledge

9. Had you heard of a molar pregnancy before your diagnosis?

Yes ☐ No ☐

10. Did you visit the link on the CUH website to GTD/Molar Pregnancy?

http://www.cuh.hse.ie/Cork-University-Maternity-Hospital/Gynaecology/GTD-Centre/What-Is-GTD/

Yes ☐ No ☐

11. Have you seen the HSE patient information leaflet on molar pregnancy?

Yes ☐ No ☐

If yes, how did you receive this information? Please ✓ one box

My clinician ☐
HSE website ☐
GTD Nurses ☐

12. Do/Did you know the name of the clinician responsible for your care?

Yes ☐ No ☐

13. Do you have concerns about the management of future pregnancies?

Yes ☐ No ☐ N/A ☐

If YES, were these concerns addressed by staff at the GTD registry?

Yes ☐ No ☐

C. Diagnosis of GTD at local hospital

Please ✓ the appropriate box to indicate the extent to which you agree/disagree with the following statements:

	Strongly disagree	Disagree	Unsure	Agree	Strongly agree
14. My clinician clearly explained my diagnosis	☐	☐	☐	☐	☐
15. I was given sufficient time to ask questions about my diagnosis.	☐	☐	☐	☐	☐
16. Other medical professionals with whom I interacted throughout my treatment understood my condition	☐	☐	☐	☐	☐

V2.1

D. Monitoring hCG levels

17. Were hCG results communicated to you in a timely manner by the GTD nurses?

Yes ☐ No ☐

18. Where did you provide blood samples for hCG measurement?

Please ✓ one box

- Early Pregnancy Clinic ☐
- Phlebotomy unit in local hospital ☐
- Primary care/GP surgery ☐
- Other _____ ☐

19. When providing blood, were you impacted by any of the following?

Please ✓ all boxes that apply

- GP charged for bloods ☐
- GP appointment required ☐
- Had to explain why I needed bloods ☐
- I have a fear of needles ☐
- I needed childcare to attend phlebotomy ☐
- Other _____ ☐

20. Would you prefer to submit a urine sample for hCG monitoring?

Yes ☐ No ☐

21. How would you like to receive your hCG results from the GTD Registry?

Please ✓ one box

- Text Message ☐
- Email ☐
- Telephone call from GTD nurse ☐
- By letter ☐
- Other _____ ☐

22. Do you think hCG levels should be provided by experienced scientists in a central laboratory with quality assurance?

Yes ☐ No ☐

E. Patient experience

23. How would you describe your satisfaction with care provided by the GTD Registry?

Please ✓ one box

- Highly satisfied ☐
- Satisfied ☐
- Dissatisfied ☐
- Very dissatisfied ☐

V2.1

24. Please list your priorities based on your experience as a patient on the GTD Registry?
Please rate from 1 to 5; (1 = high priority; 5= low priority)

Early registration	
Rapid hCG results	
Literature explaining GTD	
Psychological support	
Availability of GTD nurse	

25. How would you describe your experience of dealing with the GTD specialist nurses?
Please ✓ one box

Highly professional	
Adequate time for discussion	
Sufficient information provided	
Other _____	

26. Please suggest any improvements you believe would benefit the GTD Registry?

_____

_____

_____

27. Please suggest any additional resources required by patients on the GTD Registry?

_____

_____

_____

Thank you for taking the time to complete this survey.

V2.1

Appendix II – National Pathology Survey 2019

Gestational Trophoblastic Disease Laboratory Survey

For the year 2019 could you please complete the questions below with numbers from your laboratory?		
Laboratory Name:		
How many Products of Conception did your laboratory examine in 2019?		
How many Complete Hydatidiform Moles did you diagnose in 2019?		
How many Partial Hydatidiform Moles did you diagnose in 2019?		
How many cases of 'Atypical' products of conception were reported? [These would include cases where a “hydatidiform mole could not be excluded” or where a case is “suspicious for hydatidiform mole” but a firm diagnosis of partial mole or complete mole could not be made].		
How many cases of “P57-discordant villi” did you diagnose in 2019?		
How many Gestational Choriocarcinomas did you diagnose in 2019?		
How many Atypical Placental Site Nodules did you diagnose in 2019?		
How many Placental Site Trophoblastic Tumours/ Epithelioid Trophoblastic Tumours did you diagnose in 2019?		
Do you have P57 immunohistochemistry within your laboratory?		Yes ( ) No ( )
	If yes, is the stain accredited?	Yes ( ) No ( )
	If you don’t have P57, are you aware that the Faculty of Pathology recommends that you have access to P57 if you report products of conception?	Yes ( ) No ( )
	Do you plan to introduce P57 to your laboratory?	Yes ( ) No ( )
	If so when?	
Do you have in house Ploidy Analysis for triploidy detection?		Yes ( ) No ( )
	If yes, what type?	
	If yes, is the test accredited?	
Do you have in house Molecular Genotyping for trophoblast disease?		Yes ( ) No ( )
	If yes, is the test accredited?	Yes ( ) No ( )
If a second opinion or additional testing is needed what laboratory or laboratories do you refer your cases to?		
	How many external referrals did you send in 2019 for this purpose?	

When reporting molar pregnancies or cases suspicious for molar pregnancy do you add the following recommendation, that was endorsed by the Faculty of Pathology, to your laboratory report " Patient registration with the National Gestational Trophoblast Disease Centre is recommended?	Yes ( ) No ( )
General Comments	

Thank you for your co-operation in completing this survey.

Appendix III - EOTTD hCG Survey 2021

Introduction

Lack of comparability between immunoassays used for the quantitation of human chorionic gonadotrophin (hCG) in patients with gestation trophoblastic disease (GTD) impairs the commutability of research data and is a barrier to the standardisation of clinical practice.

The EOTTD hCG working group has acknowledged that understanding the variation in hCG assays currently in use at centres responsible for the management and follow-up oof GTD patients is critical in developing guidance and setting standards. As such, we invite you to complete this short survey, the results of which will be reviewed by the EOTTD hCG working group.

On behalf of the EOTTD hCG working group, thank you for your participation.

Section 1: hCG methods

Please detail the following characteristics for the methods currently in your centre for the measurement of hCG for **molar pregnancy/GTD** patients. Additional methods can be added in subsequent pages.

Q1 Method principle (e.g. ELISA, RIA)

Q2 Manufacturer (e.g. Siemens)

Q3 Instument/platform (e.g. Immulite)

Q4 Sample Matrix

Q5 Standardisation/calibration

Q6 Units of measurement

Q7 Sensitivity (limit of quantitation)

Q8 Specificity for hCG isoforms

Q9 Antibody/antiserum used and species

Q10 Licenced for use in oncology patients (Yes/No)

Q11 If you answered NO to Q10, has the assay been validated in-house on GTD samples (Yes/No)

Q12 Do you have another method, if yes repeat questions Q1-Q11 for this method

Section 2: Quality aspects

Please detail the quality control measures currently used in your laboratory

Q24 Which internal quality controls (IQC) do you use (eg. Biorad, in-house)

Q25 What levels of QC do you use (eg. Level 1 = 10 IU/L, Level 2 = 25 IU/L, Level 3 = 200 IU/L)

Q26 Do you use any other quality control parameters

Q27 Do you participate in External Quality Assurance (EQA/proficiency testing)

Q28 If you answered YES to Q29,, please state

Provider of laboratory accreditation (eg. UKAS)

Accreditation standard (eg. ISO 15189)

Section 3: Reporting

Q31 If you use an in-house/home-made hCG assay, please state how raw data is processed (eg. 4-parameter logistic regression) and which software package is used to generate results.

Q32 Which reference ranges/clinical cut-offs do you quote for hCG

- Serum/plasma
- Urine
- CSF

Q33 If you report urine hCG results, do you correct for urine creatinine

Q34 If you answered YES to Q33, please state any cur-offs used for the reported ratio

Q35 What is the source of your reference ranges/cut-offs (published literature, derived in-house etc.)

Q36 Do you apply hCG regression curves

Q37 If you answered YES to Q36, please state with regard to curves used (post-evacuation/MTX resistance) and source data (molar evacuation dates etc.)

Q38 Do you display hCG results graphically on your reports

Q39 Who is responsible for reporting hCG results in your centre

Q40 Do you report hCG results with comments offering clinical advice

Q41 Are hCG results discussed at multi-disciplinary team meetings

Q42 Do you report hCG results directly to patients

Q43 Do you use more than one method

Q44 If you answered YES to Q43, which method was used to report the final result and why

Q45 Do you include any technical comments/limitations of the assay on your report?

Section 4: Operational aspects

Q46 How often do you perform hCG assays

Q47 What turnaround time do you offer for hCG testing

Q48 Do you offer urgent hCG testing outside of working hours (evenings/weekends)

Q49 IF you answered YES to Q48, please specify which hCG method is used

Q50 How many hCG samples do you process annually for GTD purposes

Q51 If known, how many GTD patient do you provide hCG testing for annually

Q52 Do you use the same hCG assay for assessment of non-GTD patients (testicular/ovarian germ cell tumours etc.)

Q53 If you answered YES to Q52, please specify which other conditions are tested for using the same method

Section 5: Non-GTD assays

Please provide information on any hCG assays used for **non-GTD** patients. Please leave blank if not appropriate.

Q53 Please specify which other assay/s you use in your centre for non-GTD referrals

Q55 Please specify which other non-GTD conditions are tested for by these methods?

Appendix IV – Capillary Blood Collection Instructions

Study Title: Human Chorionic Gonadotrophin (hCG) determination from capillary blood collection devices: comparison of home prepared capillary blood with standard venous blood analysis

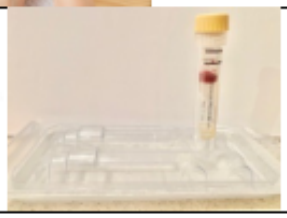
Capillary Blood Collection Instructions

Home kit contents:

- Purple lancet
- Yellow MiniCollect® blood tube
- Biochemistry Request Form
- Plastic postage pack and envelope

Checklist: before returning sample include

✓Completed Request Form with **date/time**
✓Labelled capillary blood tube
✓Completed questionnaire

	Instruction	Image for Guidance
1	Before you start, you may wish to re-watch the video on capillary blood collection. Note: Try to avoid taking the sample on a Friday or at weekends, as it may take too long to reach the laboratory.	https://www.youtube.com/watch?v=YeL-S3g80Kc&t=67S MiniCollect® Capillary Blood Collection System handling clip
2	Wash your hands with warm water and soap and towel dry. Note: warming your hands with a hot water bottle will increase blood flow to the fingers.	
3	You may find it easier to take blood from your non-dominant hand (ie, if you are right-handed, take the blood from the **middle or ring finger** on your left hand). Massage your hand from the palm down to your finger to increase the blood flow in your finger.	
4	Remove the yellow cap from the MiniCollect® tube by pressing on the ribbed area of the tube to push the cap upwards. Place the tube in an upright position in the plastic pack provided as shown for blood collection.	 
6	Wipe the finger with an alcohol wipe and air dry. Remove the white cap from the purple lancet by twisting the cap anticlockwise until it comes lose. Position the lancet on your finger so you cut across the friction ridges and puncture the skin in one quick continuous stroke.	 
7	A small drop of blood will form - wipe away the first drop of blood with a tissue. Keep your capillary blood flowing by using the fingers on your opposite hand to massage from the palm of the hand down to the finger.	

Ethical approval for this study was provided by the Clinical Research Ethics Committee of the Cork Teaching Hospitals

Capillary Blood Collection Instruction v1.0

8	Collect the blood drops into the collection tube using the scoop as shown. Tap the tube on a hard surface to push the blood into the tube. Fill the MiniCollect® tube halfway to the upper fill line (0.5) as shown in the image in note 4. **Note:** Please return the blood tube even if blood is not filled midway as there may be sufficient blood for analysis.	
9	Once you have finished collecting blood, replace the yellow cap on the MiniCollect® tube and push downwards until you hear a **click**, to ensure the tube is **securely closed**. Apply firm pressure to the puncture site with a tissue or gauze pad to stop the bleeding and apply a plaster	
10	Gently invert the MiniCollect® tube 5-10 times to mix. Attach the completed **sample identity label** with your details included.	 Mix tube 5-10 times MRN: _____ Name: _____ DOB _____ Study #
11	Place the filled MiniCollect® tube and the used/unused lancets in the **plastic pack** as shown. Place the plastic pack in the padded **pre-addressed stamped envelope** **Please note:** The **PURPLE** lancet is a single-use lancet, the needle retracts into the device immediately after use to avoid needlestick injury.	 
12	Complete the study questionnaire and place it in the padded envelope with all items on your checklist. **Note:** please complete the questionnaire even if you are not providing a blood sample, as this feedback is very valuable.	
13	Store the sample at room temperature until posted. Post your sample to the Biochemistry laboratory as soon as possible (ideally on the same day or within 24 hours of sample collection)	Post within 24 hrs
14	Dispose of all the waste kit contents safely in your household waste.	

Thank you for participating in this research study!

Appendix V – Study Questionnaire: hCG determination from capillary blood collection devices

Pregnancy Loss Research Group,
Department of Obstetrics and Gynaecology,
University College Cork
Cork University Maternity Hospital

Study Questionnaire for the Study: hCG determination from capillary blood collection devices

Thank you for taking the time to share your views on your experience of using the capillary blood collection devices as part of our study. Responses to this questionnaire may be used to improve the quality of care to pregnant women.
Please return this questionnaire with the capillary blood sample you collect at home.

Please ✓ one box in each question.

		Strongly Agree	Agree	Unsure	Disagree	Strongly Disagree
The video on YouTube showing how to collect capillary blood was helpful						
The written instructions on collecting capillary blood were easy to follow						
The MiniCollect® collection device was easy to use						
I was able to collect enough blood from the fingerprick to fill the tube						
It was easy to seal the cap on the collecting device						
Posting samples to the laboratory within 24h of collection was easy						
My finger was sore after the fingerprick						
I would take another capillary sample in the future if required						
I would prefer to use this service than attend the doctor for bloods						
I would be more likely to have my bloods checked if this service was available						
I would be happy to pay for a test collection pack	> €10					
	< €10					
I would collect the testing pack in a pharmacy.						
I would like to receive a testing pack in the post from my doctor.						
I would like to receive a text message with my blood results						