Gynecologic and Obstetric Investigation

1   **When to consult a geneticist specialising in gestational trophoblastic disease**

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23

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26

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29

Abstract

Background:

Gestational trophoblastic disease comprises hydatidiform moles and a rare group of malignancies that derive from trophoblasts. Although there are typical morphological features that may distinguish hydatidiform moles from non-molar products of conception, such features are not always present, especially at early stages of pregnancy. Furthermore, mosaic/chimeric pregnancies and twin pregnancies make pathological diagnosis challenging while trophoblastic tumours can also pose diagnostic problems in terms of their gestational or non-gestational origin.

Objectives:

To show that ancillary genetic testing can be used to aid diagnosis and clinical management of GTD.

Methods:

Each author identified cases where genetic testing, including short tandem repeat (STR) genotyping, ploidy analysis, next generation sequencing and immunostaining for p57, the product of the imprinted gene *CDKN1C*, facilitated accurate diagnosis and improved patient management. Representative cases were chosen to illustrate the value of ancillary genetic testing in different scenarios.

Outcome:

Genetic analysis of placental tissue can aid in determining the risk of developing gestational trophoblastic neoplasia, facilitating discrimination between low risk triploid (partial) and high risk androgenetic (complete) moles, discriminating between a hydatidiform mole twinned with a normal conceptus and a triploid conception and identification of androgenetic/biparental diploid mosaicism. STR genotyping of placental tissue and targeted gene sequencing of patients can identify women with an inherited predisposition to recurrent molar pregnancies. Genotyping can distinguish gestational from non-gestational trophoblastic tumours using tissue or circulating tumour DNA, and can also identify the causative pregnancy which is the key prognostic factor for placental site and epithelioid trophoblastic tumours.

Conclusions and Outlook:

STR genotyping and P57 immunostaining have been invaluable to the management of gestational trophoblastic disease in many situations. The use of next generation sequencing and of liquid biopsies are opening up new pathways for GTD diagnostics. Development of these techniques has the potential to identify novel biomarkers of GTD and further refine diagnosis.

Introduction

Gestational trophoblastic disease (GTD) comprises a group of disorders associated with abnormal proliferation of trophoblastic cells including hydatidiform moles (HMs) and a rare group of malignant neoplasms collectively termed gestational trophoblastic neoplasia (GTN). GTN includes invasive mole, choriocarcinoma, as well as the very rare placental site and epithelioid trophoblastic tumours (PSTT and ETT). HMs carry a risk of development of GTN (Table 1), with the risk dependent on the type of HM. After evacuation of a HM, patients require monitoring of human chorionic gonadotropin (hCG) levels to identify persistent trophoblastic disease, for which the patients will require chemotherapy. A precise diagnosis ensures that patients at elevated risk of GTN are monitored appropriately and patients with low risk are able to attempt a new pregnancy with minimal delay.

The diagnosis of HM and the subdivision of these into complete hydatidiform moles (CHMs) and partial hydatidiform moles (PHMs) are in principle made from the morphological features. However, the distinction between CHM and PHM is not always easy, especially when the molar pregnancy is terminated early. Furthermore, some features of HMs can also be found in non-molar miscarriages such as hydropic chorionic villi (see [1,2] for further details). HMs are genetically different to non-molar pregnancies; PHMs typically contain an extra paternal genome and CHMs typically contain genomic DNA of paternal origin only [3] (Table 1). Morphological diagnosis can be supported by ancillary genetic techniques.

The majority of GTN are diagnosed clinically based on factors such as hCG levels, pregnancy history, and imaging. Except for PSTT and ETT, GTN are associated with survival rates of >98% with appropriate treatment and clinical management [5]. Genotyping can play an important role in diagnosis and management of GTN, being used to distinguish these from non-gestational tumours that also secrete hCG and to identify the causative pregnancy, the interval to which is the key prognostic factor for rare GTN subtypes.

In this article, we provide examples where consultation with a specialist GTD geneticist can aid diagnosis and how this can be informative/beneficial for patient management. The cases described in this article are inspired by true cases, but details have been amended to preserve anonymity of the patients. For readers interested in the technical aspects of genetic tests typically utilized, we recommend the following reviews [6–8]. Sections 1 to 6 demonstrate situations where testing helped guide the treatment by the gynecologists and/or the oncologists and sections 7 to 10 illustrate situations where testing was informative for pathologists.

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Section 1. When you wish to determine prognosis of a HM

Case 1.1: A woman has had a HM and wishes to start a new pregnancy quickly

Mary was 40 years old, G1P0. She and her husband had achieved a pregnancy after *in vitro* fertilisation. Three ova were fertilised, two of these appeared to be of a sufficient quality. One embryo was transferred to the uterus and led to a pregnancy, the other was cryopreserved. In week 11, ultrasonography (ULS) showed no heartbeat, and a HM was suspected. The level of hCG was 100,000 IU/L. Mary had a surgical termination of the pregnancy, and tissue was forwarded for histopathologic and genetic analyses. The histopathological examination showed findings compatible with a PHM. The karyotype of the evacuated tissue was 69,XXY (Figure 1) and genotyping showed that two genome sets originated from the father. According to the national guideline [9], after a triploid PHM, surveillance can be discontinued after 2 consecutive normal hCG measurements. Mary achieved this within 2 months, and therefore she and her husband could soon plan to start a new pregnancy.

Case 1.2: A patient becomes pregnant during surveillance after a HM

Sophie was 30 years old (G1P0). In week 10 of her first pregnancy, she started bleeding and had high hCG levels (200,000 IU/L). A HM was suspected by ULS so the uterus was evacuated. Genetic analyses, alongside histopathologic examination of the tissue, made the diagnosis of a triploid PHM. hCG surveillance was initiated and hCG levels decreased. However, two months post-evacuation hCG levels began to rise. ULS disclosed that Sophie was pregnant. Sophie and her husband were informed that the production of hCG in the new pregnancy would prevent efficient surveillance after the HM. However, the couple disliked the idea of terminating a normal pregnancy. As the PHM diagnosis was supported by genetic analysis confirming triploidy, the gynaecologist could inform Sophie and her husband with more certainty that the risk of GTN was very low. The couple decided to continue the pregnancy. Sophie was monitored by ULS examinations in week 12, 16 and 20. No sign of GTN developed during the pregnancy and a healthy child was born.

Case 1.3. When hCG following a HM fails to normalise

Diana was 35 years old, G2P1. In her second pregnancy she had a HM diagnosed in week 12 and the pregnancy was terminated. Histopathologic examination of the evacuated tissue showed morphology consistent with a PHM. All tissue had been formalin fixed and no genetic analyses were performed at this time. Initial hCG was 150,000 IU/L. For 3 weeks the hCG levels fell exponentially, however then the levels seemed to plateau as two measurements with one week interval showed values of approx.

130   2,600 IU/L. To obtain more information about the risk of GTN, genotyping was performed. Analysis of

131   STR (short tandem repeat) markers was performed on DNA isolated from 3 different parts of the

132   formalin fixed tissue containing villous tissue (representing the HM) and one sample containing

133   decidua (representing the mother, i.e. the patient). The three villous samples showed identical results,

134   consistent with a diandric triploid HM.

135

136   The genotyping result arrived at the same time as the third hCG value which showed plateauing. As

137   the gynaecologist now knew that the likelihood that Diana would develop GTN was very low, a

138   hysteroscopy was performed to check for possible retained molar tissue. An irregularity of yellow-

139   greyish tissue was removed from the right corner of the uterus. Histopathology showed a minimal

140   piece of degraded villous tissue with only faint signs of trophoblastic hyperplasia and no sign of

141   malignancy. Two months later, the patient was discharged after two normal hCG levels were obtained

142   (Figure 2).

143

144   **Comment.**

145   When using morphology alone, the risk of GTN after a PHM is 0.5-1%, which is significantly lower than

146   after a CHM [10]. GTN has been observed after triploid HMs: Seckl et al. reported 3 cases of

147   choriocarcinoma after triploid PHMs [11] and Cheung et al. reported 2 cases of metastatic GTN after

148   triploid PHMs [12]. However, in 4 cohort studies, a total of 265 patients were identified with a triploid

149   HM by karyotyping, or flow cytometry on fresh tissue where external controls were co-analysed. None

150   developed GTN, i.e. the estimated risk is 0% (95% CI: 0–1.4%) [13]. Accordingly, international

151   guidelines now recommend a less intensive surveillance after a triploid HM/PHM than after a CHM

152   [14].

153

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155   **Section 2. When you wish to distinguish a twin pregnancy from a PHM**

156   **Case 2.** Anna was 37 years old and (G2P1) pregnant in week 11, when a routine ULS disclosed a cystic

157   placenta, along with a living foetus, seemingly with a second normal placenta, suggesting a twin

158   pregnancy with a HM and a normal conceptus. No abnormalities were observed in the foetus.

159   However, as most conceptuses with a molar placenta and a foetus are PHMs, this diagnosis could not

160   be excluded. Anna and her partner wished to continue the pregnancy if the prognosis for the foetus

161   was good. The case was discussed in the foeto-medical multidisciplinary team, including

162   gynaecologists, clinical geneticists and pathologists, and genetic analyses were agreed on. A sample

163   was taken from the placenta that had a normal appearance on ULS. The placenta had a 46,XY

164   karyotype and genotyping of the sample along with parental blood samples revealed a diploid

165   biparental genome. This corroborated that Anna had a twin pregnancy with a normal foetus.

166

167   Anna and her partner were informed about the risk of GTN and the likely outcome of normal live birth.

168   The couple decided to continue the pregnancy. The pregnancy was monitored closely, with imaging

169   (Figure 3) and measurements of hCG. The hCG levels dropped from 85,000 IU/L in week 11 to 20,000

170   in week 36. Apart from a few episodes of slight bleeding, the pregnancy was uneventful. In week 36

171   Anna went into labour spontaneously and delivered a healthy son. Genetic analyses of the molar

172   placenta disclosed that this was diploid and androgenetic, corroborating that it was a CHM. hCG

173   normalised within 6 weeks and stayed normal for the following 4 months, after which monitoring was

174   discontinued.

175

176

177   **Comment.**

178   Most multiple pregnancies including a HM, are "twins" including a diploid androgenetic HM and a

179   normal diploid biparental conceptus. Especially when diagnosed early, twinning between a diploid HM

180   and a normal diploid conceptus can be mistaken for a PHM. A detailed ULS can often identify two

181   separate placentas [15]. Due to the risk of bleeding, it is recommended to avoid taking a biopsy of a

182   HM. However, identifying a normal diploid biparental constitution of the normal placenta (or amniotic

183   fluid), can help discriminate between a triploid PHM, and twinning, and add to the information

184   available for the parents when they must decide whether to terminate or continue the pregnancy

185   [16,17].

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187

188   **Section 3. When a patient has recurrent molar pregnancies**

189

190   **Case 3.1**. Riona was 37 years old (G3P0) and presented with a second molar pregnancy following a

191   CHM, a year previously, and an intervening miscarriage. Pathological review of tissue from the two

192   molar pregnancies showed the second to have the morphology of a typical CHM while the first was

193   essentially a CHM but exhibited some atypical features. Due to the atypical features, the pathologist

194   requested genetic analysis. STR genotyping of maternal decidua and placental villi from fixed tissue

195   sections revealed that the genome of the placental villi in both cases was diploid and biparental rather

196   than having the two paternal contributions to the genome expected for a typical androgenetic CHM

197   (Figure 4). Diploid, biparental HMs suggest a diagnosis of familial recurrent HM (FRHM), an autosomal

198 recessive condition predisposing women to recurrent molar pregnancies. Sequencing of the gene
199 *NLRP7,* using DNA extracted from the patient's blood*,* showed the patient to be homozygous for
200 p.Pro716Ala, a pathogenic variant frequently found in patients with FRHM [18].

201

202 Women with FRHM are unlikely to achieve a normal pregnancy naturally but may do so by using a
203 donor ovum [19,20]. Subsequently the patient underwent *in vitro* fertilisation using a donor ovum and
204 was able to achieve a full-term pregnancy and a healthy female child two years later.

205

206 **Case 3.2**. Fatima was 37 years old (G7P2) and presented with a third CHM and a poor obstetric history.
207 A CHM ten years previously was followed by a miscarriage, second CHM, live birth, still birth, and a
208 further miscarriage before the third CHM, suggesting a possible diagnosis of FRHM, although live
209 births are extremely rare in women with FRHM. STR genotyping of maternal decidua and placental
210 villi from the third CHM revealed that the genome was androgenetic (Figure 4), demonstrating that
211 the patient had recurrent HMs of androgenetic origin and not FRHM.

212

213 Recurrent androgenetic CHMs (AnCHMs) are rare and the causes remain unclear. Subsequent
214 pregnancies may result in a normal term birth, although this is rare after three AnCHMs [21]. Having
215 already achieved one normal pregnancy, the patient wished to try and conceive naturally but
216 unfortunately experienced two further molar pregnancies, and a miscarriage. Both the fourth and fifth
217 HM were confirmed to be AnCHMs.

218

219 **Comment. Recurrent hydatidiform mole may have different aetiologies**
220 While the great majority of women with two HMs subsequently go on to have normal pregnancies, a
221 small number, particularly women with two consecutive CHMs and no normal pregnancies, will have
222 further molar pregnancies [21]. In these cases, STR genotyping of the molar tissue is important to
223 identify those women who have recurrent HM as a result of pathogenic variants in one of two genes,
224 *NLRP7* [22] or *KHDC3L* [23]. This condition, FRHM, is characterised by failure to establish correct
225 methylation of imprinted genes in the ovum [24,25], resulting in a molar phenotype in conceptuses
226 that are otherwise genetically normal with a contribution to the genome from both parents.

227

228 FRHM can be identified by showing that the molar tissue is diploid and biparental or, very occasionally,
229 triploid with two maternal contributions to the genome. Sequencing of the patient's DNA for
230 pathogenic variants in the genes *NLRP7* and/or *KHDC3L* can identify the variant(s) underlying the
231 condition, confirm the diagnosis and enable screening to determine whether other family members,

232 particularly nulliparous siblings, are likely to be affected. Since the genes associated with this condition

233 are important for normal functioning of the ovary, conventional *in vitro* fertilisation is not appropriate.

234 However, a normal term pregnancy can be achieved by using a donor ovum.

235

236 Women with recurrent AnCHMs, particularly those with three or more AnCHMs, generally have poor

237 obstetric history, including miscarriage, and stillbirth in addition to CHMs, but may occasionally have

238 one or more normal full-term pregnancies [21]. Little is known about the causes of recurrent AnCHMs,

239 which may differ in each case. Variants of three genes have been reported in patients with poor

240 reproductive history, including AnCHMs, but each of these only occur in a small number of cases [26].

241 Sequencing of our patient's DNA, (as a participant in a research project) did not reveal the presence

242 of any of these rare variants. Women with recurrent AnCHMs may conceive naturally and achieve a

243 normal term pregnancy. However, as in the case above, the more likely outcome of future

244 pregnancies, particularly after three or more AnCHM, is a CHM or miscarriage [21]. *In vitro* fertilisation

245 with preimplantation genotyping, to ensure embryos are non-molar, has been reported to result in

246 the successful live birth of twins for a woman with a history of a miscarriage and two AnCHM [27] and

247 may be an option to achieve a normal live birth. *In vitro* fertilisation using ovum donation may also be

248 successful but there is little experience of this to date. Further understanding of the underlying causes

249 of recurrent AnCHM is needed to improve advice available for these patients.

250

251

252 **Section 4. When you don't know if a choriocarcinoma is gestational**

253

254 **Case 4:** Nora was 67 years old (G1P1) and presented to the emergency department with abdominal

255 pain and diarrhoea. A colonoscopy revealed colitis and diverticular disease for which she was

256 treated. Nora is an ex-smoker, she has one daughter and her past medical history includes a

257 diagnosis of cervical cancer for which she received brachytherapy. An incidental chest X-ray revealed

258 a lung nodule and CT imaging confirmed a right upper lesion measuring 20 mm. Nora had a left

259 upper lobectomy with lymphadenectomy and histopathological examination revealed a lesion with

260 morphology and immunophenotype consistent with choriocarcinoma. Following her

261 histopathological diagnosis, serum hCG levels were measured and found to be elevated (400 IU/L).

262 Nora's diagnosis was discussed at a multidisciplinary team (MDT) meeting and the team decided to

263 adopt a watch and wait approach and commenced weekly hCG surveillance. Her hCG levels dropped

264 initially post-surgery but then rose steadily reaching a peak of 6,500 IU/L.

265

The differential diagnoses in this case included primary choriocarcinoma of the lung (which is extremely rare) and gestational choriocarcinoma. Nora's rising hCG level was discussed in the MDT and the oncologist requested genotyping to assist in the classification of her choriocarcinoma Nora's tumour tissue was sent to an expert centre for genotyping and DNA extracted from the tumour and normal lung tissue was analysed by STR analysis. The genotype of the tumour was the same as the genotype of normal tissue from Nora and no non-patient (paternal) alleles were observed in any of the STR loci (Figure 5A) which indicated a non-gestational choriocarcinoma. Following confirmation of her diagnosis, Nora's care was transferred to a medical oncologist team with the relevant expertise.

Comment

It is important to differentiate between gestational and non-gestational choriocarcinoma as they have different prognoses and clinical care pathways. Non-gestational choriocarcinomas have a poor prognosis and have a high propensity for metastasis [28]. More than half of all gestational choriocarcinomas are derived from a CHM, the remainder follow a pregnancy that has been terminated, a miscarriage, a full or preterm delivery or an ectopic pregnancy [29]. Genotyping plays a key role in the diagnosis by enabling the identification of non-patient alleles in gestational choriocarcinomas, compared to non-gestational choriocarcinomas in which the genotype reflects that of the patient.

Section 5. When the causative pregnancy is unknown, in PSTT or ETT

Case 5: Kirsty was 34 years old (G2P2) when she was diagnosed with a PSTT. It has been observed that the time period since the antecedent pregnancy is the most important prognostic factor for PSTTs, with an interval of 4 years or more associated with poor prognosis [30,31]. However, it has been shown that the antecedent pregnancy is not always the causative pregnancy [32,33]. In these cases, the interval since the causative pregnancy is likely to be more informative concerning the prognosis. Kirsty had two daughters, one three years old and the other seven years old. Therefore, genotyping of the daughters was requested to determine the causative pregnancy. The paternal alleles in the tumour matched those of the 7-year-old daughter, but only a subset matched those in the 3-year-old (Figure 5B). This indicated that the interval from the causative pregnancy was more than the 4 year cut-off, putting the patient in the high risk disease category [34]. Following a complete hCG response with EP-EMA chemotherapy, given the poor prognostic features, consolidation immunotherapy was offered and completed uneventfully.

300

Conclusion: An interval of four years or more from the antecedent pregnancy is associated with poor prognoses for both PSTT [30, 31] and ETT [35]. Although clinically the immediately antecedent pregnancy is typically perceived as the causative pregnancy, this is not always the case. In patients with PSTT or ETT who have had multiple previous pregnancies, STR genotyping is recommended to confirm the pregnancy of origin which can then be used to guide patient management [34].

Section 6. If you need a diagnosis and have no tissue

Case 6: Karen was 49 years old (G4P4) and presented to her local hospital with vaginal bleeding and elevated hCG. ULS showed no pregnancy and subsequent CT and MRI revealed a vaginal mass. Karen was admitted to a specialist GTD centre and underwent treatment for a suspected choriocarcinoma. Due to risk of profuse bleeding, it was not feasible to collect a tumour biopsy in order to determine if the tumour was gestational or non-gestational. Therefore, to facilitate genetic testing, a blood sample was collected before treatment began (hCG 26,000 IU/L): cell free DNA (cfDNA, from plasma) and genomic DNA (from buffy coat) were extracted. STR genotyping was performed, but no non-maternal alleles were detected in the cfDNA. As detection rates using STR genotyping of cfDNA are variable when serum hCG is less than 60,000 IU/L [36], it was not possible to conclude if the tumour was gestational or non-gestational in origin. A more sensitive technique, based on ultra-deep next generation sequencing of common single nucleotide polymorphisms (SNPs) [37], was employed and 43 of the 100 SNPs for which the patient was homozygous had non-patient alleles in the cfDNA (Figure 6), indicating that the tumour was gestational in origin. The patient continued single-agent treatment and she had a complete response.

Comment.

Although it is not possible to determine the tumour subtype using this method, cfDNA can be used to test the gestational or non-gestational origin of a tumour in cases where tumour tissue is not available and biopsy is contraindicated.

Section 7. When there is too little material for histological analysis

Case 7.1: Melanie was 42-year-old (G6P0) with 5 previous miscarriages and was admitted to the local early pregnancy assessment unit for medical management of suspected miscarriage. After evacuation,

334 products of conception (POC) were sent for routine pathological analysis where it was discovered that
335 only small and scanty chorionic villi were present in the sample. Whilst the morphological appearance
336 of the chorionic villi showed no striking features of hydropic change, the possibility of a HM could not
337 be excluded. Subsequent chromosome analysis of a fresh POC sample showed a 69,XXX karyotype. As
338 the morphological criteria for a molar phenotype were not fulfilled and chromosome analysis can not
339 define the parental origin of the genome, STR analysis was undertaken on DNA extracted from laser-
340 microdissected chorionic villi from fixed tissue. Genotyping of the maternal decidua and chorionic villi
341 showed inheritance of 1 maternal allele and 2 paternal alleles consistent with a diandric triploid
342 genotype. Melanie was enrolled at her GTD centre for hCG surveillance. As this pregnancy loss was
343 diagnosed as a triploid PHM, her hCG surveillance was completed after 2 normal hCG measurements,
344 2 months post-evacuation of uterus.
345
346 **Case 7.2**: Yvonne was 37 years old (G1P1), underwent an elective caesarean section after an
347 uneventful pregnancy, and gave birth to a healthy female infant. At the time of surgery, fatty cystic
348 material was identified underneath the placenta. This was removed from the uterine cavity and
349 submitted for pathological investigation. A disc of yellow/white tissue weighing 179 g and measuring
350 135x105x15 mm was identified. One aspect appeared pale and necrotic, and the other showed a
351 multicystic appearance, the largest cyst being 10 mm. Microscopy showed there was only a limited
352 number of enlarged, hydropic chorionic villi available for analysis. These showed circumferential
353 trophoblast proliferation and marked cellular atypia. Immunostaining for p57, a marker for the
354 presence of a maternal contribution to the genome [38,39], was negative across all the chorionic villi.
355
356 The morphological appearance favoured a diagnosis of a CHM, but the unusual clinical presentation
357 and limited number of chorionic villi did not allow a definitive diagnosis of CHM. STR analysis of DNA
358 extracted from laser-microdissected chorionic villi and maternal decidua within the same fixed tissue
359 block was performed. Results showed only the inheritance of paternal alleles in the chorionic villi,
360 indicative of a CHM.
361
362 Yvonne was thus diagnosed with a CHM in association with a normal pregnancy. She was enrolled into
363 an hCG surveillance program, in which her hCG normalized and monitoring was completed after 6
364 months.
365
366 **Comment**

367 These examples highlight cases where genetics was invaluable in aiding the definitive diagnosis of a
368 molar pregnancy where pathology alone did not fulfil the diagnostic criteria either due to the limited
369 or degenerate chorionic villi available for histological analysis (Case 7.1); or an unusual clinical
370 presentation combined with limited chorionic villi (Case 7.2). Whilst there was evidence of a triploid
371 karyotype in Case 7.1, it was challenging to diagnose as diandric or digynic without genotyping,
372 especially due to the lack of histological features. Furthermore, an unusual clinical presentation or a
373 sample with limited chorionic villi (Case 7.2) is challenging diagnostically to determine by histology
374 alone. In both scenarios, genotyping was able to confirm the presence of a molar component, so that
375 the patient could be offered the most appropriate management.

376

377

378 **Section 8: When the histology mimics a molar phenotype**

379

380 **Case 8:** Kathryn, 34 years old (G2P1) presented with an early foetal demise on ULS. Evacuation of the
381 uterus was performed. Local pathological investigations of the haemorrhagic membranous and
382 placental fragments showed the presence of decidua, membranes and chorionic villi. Some villi were
383 large and there was multifocal trophoblast budding. Preliminary pathological diagnosis was that of a
384 suspected PHM.

385

386 Histological analysis by a specialist in GTD identified obvious abnormal villous morphology associated
387 with a PHM phenotype, and immunostaining for p57 showed positivity in nuclei of cytotrophoblasts
388 and villous stromal cells. However, ploidy analysis by flow cytometry showed a diploid DNA content
389 (Figure 7). As this result was not consistent with a diagnosis of PHM, genotyping was initiated. Since
390 there was no access to fresh tissue, or maternal DNA, laser-microdissection and DNA extraction from
391 chorionic villi and maternal decidua from the same fixed tissue block was undertaken. STR analysis
392 showed results consistent with biparental diploidy, except in the locus on chromosome 16, where
393 three alleles were observed. This was subsequently confirmed by genotyping another four
394 chromosome 16 loci suggesting a trisomic 16 conceptus.

395

396 These results indicated that the patient required no hCG surveillance. She was informed of the trisomy
397 16 result and advised this most likely accounted for the pregnancy loss in this case. There was no
398 reason why she could now not attempt a new pregnancy.

399

Conclusion: In cases with atypical histology and normal p57 expression, genotyping by STR analysis is a useful tool to distinguish between PHM and non-molar pregnancy. In this case, atypical villous morphology, whilst mimicking the phenotype of a PHM, was attributed to an underlying chromosome abnormality (trisomy 16) which may explain the pregnancy loss. If genotyping was not performed, the patient may have been diagnosed as having a PHM and recommended to abstain from a new pregnancy until their hCG surveillance was completed satisfactorily.

Note: Discovery of a trisomy alone does not exclude the diagnosis of hydatidiform mole, as trisomy is occasionally seen in androgenetic HMs [40].

Section 9: When p57 immunostaining is discrepant or discordant

Case 9.1 Mandy was 27 years old (G1P0) and presented at the early pregnancy clinic at 10 weeks gestation with bleeding and inevitable miscarriage. POC were sent for routine pathological analysis, which showed enlarged and hydropic chorionic villi with lobulated outlines and widespread cistern formation. The appearance of scattered trophoblast pseudoinclusions were noted, alongside the absence of nucleated red blood cells. The differential diagnoses were hydropic pregnancy or PHM. Ploidy analysis of nuclei extracted from laser-microdissected fixed tissue (chorionic villi and maternal decidua) by flow cytometry showed a diploid DNA content. Immunostaining for p57 showed a discordant pattern with p57 negative stromal cells and p57 positive cytotrophoblast cells, as opposed to p57 staining where both stromal and cytotrophoblast cells are positive (as seen in both non-molar or diandric triploid pregnancies) or when p57 staining is completely absent in both stroma and cytotrophoblast (as seen in androgenetic moles).

To understand the equivocal p57 immunostaining, STR analysis of DNA extracted from the p57 positive and negative cells by laser-microdissection of fixed tissue was undertaken. The p57 positive cytotrophoblast cohort showed evidence of biparental diploidy, whereas the p57 negative stromal cells showed a pattern consistent with androgenetic diploidy.

Mandy was informed that she had experienced a mosaic/chimeric molar pregnancy (androgenetic and biparental components) and was entered into an hCG surveillance programme. In view of the limited knowledge regarding the risk of GTN, the hCG surveillance centre advised monitoring should follow

433   the same guidelines as for those women who experience a CHM. Mandy's hCG level normalised and

434   she completed her surveillance with no adverse events.

435

436   **Case 9.2:** Lucy was 34 years old (G3P2) and presented at 10 weeks gestation for ULS. The pregnancy

437   had the appearance of PHM with a demised foetus and a cystic placenta. Lucy's hCG level was 1.5

438   million IU/L. Following evacuation of the uterus, pathological analysis of the POC showed necrotic

439   decidua, implantation site and embryonic tissue with abundant chorionic villi across a range of sizes

440   which were enlarged, hydropic and irregular in outline. Scattered trophoblast inclusions and stromal

441   karyorrhexis, focal cistern formation and absence of nucleated red blood cells were noted. The

442   suspicion of a twin pregnancy was raised due to the presence of some villi appearing normal and some

443   with the morphological appearance of HM.

444

445   Laboratory investigations included ploidy analysis by flow cytometry and p57 immunostaining. Whilst

446   flow cytometry showed diploidy, the p57 immunostaining showed a variable pattern with large villi

447   entirely negative (Figure 8A), smaller villi showing a normal pattern (positive stroma and

448   cytotrophoblasts, Figure 8B) and a villous population with a discordant pattern (negative stroma,

449   positive cytotrophoblasts, Figure 8C).

450

451   To distinguish the genotype of these three distinct villous populations, STR analysis was performed on

452   laser-microdissected fixed tissue. Results showed the negative p57 chorionic villi had only paternal

453   alleles consistent with an androgenetic genome; the villi with normal p57 staining had both maternal

454   and paternal contribution (biparental diploidy); and the third population with discordant p57

455   expression (negative stroma and positive cytotrophoblast) contained both androgenetic genome

456   (stroma) and biparental diploidy (cytotrophoblast) components. The presence of a normal biparental

457   diploid component may explain the presence of embryonic tissue seen during pathological analysis.

458

459   Like Mandy, Lucy was informed of her mosaic/chimeric molar pregnancy and enrolled into an hCG

460   surveillance programme which was completed in an uneventful manner.

461

462   **Comment**

463   Cases of molar pregnancy with aberrant p57 immunostaining have been well-documented [42,43] and

464   whilst p57 immunostaining is often used as one of the first diagnostic tools to aid diagnosis of HM, it

465   can also expose more complex and equivocal cases. Advanced laboratory techniques such as laser-

466 microdissection and subsequent genotyping can help to unravel the complexities at a molecular and
467 cellular level, especially in cases with complex histological findings.

468

469 Many molecular studies have now shown that mosaicism or chimerism is not an uncommon finding in
470 HM [44–46]. Mosaicism/chimerism in relation to HM can be defined as the presence of an
471 androgenetic component in one population of villous cells, in addition to the presence of a normal
472 biparental population of cells where there is equal contribution of maternal and paternal genome.
473 The distribution of androgenetic and biparental cell lines may vary [42,47] and in some cases a viable
474 foetus may be present [43]. The risk of persistent gestational trophoblastic disease in these cases
475 remains to be established, partly because of the relatively small numbers identified at present, we
476 recommend that these mosaic/chimeric cases are followed-up as for a CHM.

477

478

479 **Section 10. When the p57 immunostaining pattern is not consistent with morphology**

480

481 **Case 10**. Emily was 33 years old (G3P2) and following evacuation of her suspected HM, pathology
482 review was performed on the POC. The chorionic villi were abnormal, many of which were hydropic
483 and showed irregular scalloped outlines and complex trophoblastic pseudo-inclusions, the majority
484 were avascular while others had collapsed vessels. Only focal excessive trophoblast was identified
485 and where present was arranged in an abnormal circumferential distribution. No extravillous
486 pleomorphic trophoblast was seen. p57 staining was unexpectedly negative. Therefore the case was
487 sent for review by a pathologist specializing in GTD. The morphological features were in keeping with
488 a PHM but could, more rarely, be seen in mosaic/chimeric pregnancies. Further p57 immunostaining
489 was requested on multiple blocks which showed absence of p57 expression in the cytotrophoblasts
490 and villous stromal nuclei. Due to the mismatch of the morphological and immunocytochemical
491 features of the case, genotyping was requested. STR genotyping of the maternal decidua and
492 placental villi revealed that the placental villi were diploid biparental, but no maternal allele was
493 detected at the *TH01* locus on chromosome 11p15.5 (Figure 9). In cytotrophoblasts and villous
494 stroma, *CDKN1C*, the gene which encodes p57, is normally expressed only from the maternal allele.
495 *CDKN1C* is located at chromosome 11p15.4, close to *TH01*. Therefore, loss of maternal *CDKN1C* is
496 also likely to have occurred with loss of the maternal *TH01*, explaining the p57-negative appearance
497 of the villi. GTN has been reported in some diploid biparental cases with absence of maternal
498 chromosome 11, therefore hCG monitoring was recommended [48].

499

Comment

Genotyping can be a useful tool to investigate POCs showing discrepancies between morphology and p57 immunostaining, the results of which can impact on patient management. CHMs expressing p57 due to retention of maternal chromosome 11 have also been reported [49].

Conclusion

GTD is nowadays a highly curable condition with an excellent overall survival rate [50]. Through the concerted and combined efforts and collaboration amongst the wider GTD community in recognising and diagnosing women earlier, alongside effective surveillance and treatment strategies, many women are successfully managed and treated accordingly. Guidelines on the management and treatment of GTD are well-documented [34,51] and international collaboration involving members of the EOTTD community have sought to establish recognised guidelines for diagnosis and treatment [14].

Whilst the guidelines provide a platform for scientists and clinicians working within the field of GTD and GTN, many parties may not work within specialised GTD units or teams, or have readily available access to all the necessary laboratory investigations and support required for GTD diagnosis. As such this paper aims to highlight scenarios where expert genetic investigation and interpretation may contribute to the overall care and management of women with possible GTD.

The first collection of cases (Section 1 to 6) is aimed at those scenarios where genetic investigation can aid the clinical management. For example, the correct diagnosis of PHM as demonstrated by STR analysis showing diandric triploidy, allows the patient to be managed conservatively on shorter-term hCG surveillance as opposed to longer-term surveillance associated with CHM (Cases 1.1, 1.2 and 1.3). Identification of FRHM is only possible after genetic investigation, and with this knowledge, couples may be offered the option of ovum donation to achieve pregnancy as opposed to an otherwise bleak obstetric outlook (Case 3.1). Similarly, identification of those women who experience recurrent androgenetic CHMs as opposed to FRHM is important in order to distinguish recurrence risk and to offer the appropriate genetic counselling and support (Case 3.2). The genetic identification of the gestational or non-gestational origin of a tumour helps guide the treatment strategies and decision making process for clinicians faced with hCG-producing tumours (Section 4). Genotyping to identify the causative pregnancy in cases of PSTT/ETT is an invaluable technique which can provide crucial

533 information regarding prognosis and treatment (Section 5). Additionally, recent advances in genetic

534 methodology can aid diagnosis even in the absence of a pathological specimen, and therefore direct

535 treatment strategies which otherwise may not have been considered (Section 6).

536

537 The second collection (Section 7 to 10) highlights the importance of genetic investigation to aid the

538 pathological diagnosis of GTD/GTN. In cases where the histology is equivocal and limited tissue is

539 available for a definitive diagnosis, genetic analysis of small amounts of DNA is invaluable to

540 distinguish between a diandric and digynic triploid (Cases 7.1 and 7.2). In addition, abnormal villous

541 morphology is often noted in pregnancy loss samples, which may be misleading or otherwise not

542 definitive for a HM. Underlying chromosomal abnormalities such as common trisomies often mimic

543 molar histology and without genetic analysis could be misinterpreted as an HM (Section 8). Routine

544 p57 immunostaining is often used as one of the front-line tests for HM but in cases where the p57

545 staining is discordant with the associated histological features, genetics can aid to decipher the

546 underlying chromosomal abnormalities causing such features and offer the most appropriate

547 diagnosis and management strategy (Section 10). Finally, genetics enables the identification of

548 mosaic/chimeric molar pregnancies which without analysis of the different villous population, may be

549 misinterpreted as an otherwise non-molar conception in less specialised centres (Cases 9.1 and 9.2).

550 Mosaicism/chimerism in HM can only truly be identified by genetic analysis and the prognostic

551 significance of mosaicism/chimerism in HM is still under review given the small number of cases

552 identified by genetics to date.

553

554 Overall, a wealth of knowledge is obtained when using genetic investigations in the clinical

555 management, or the differential diagnosis of a patient with suspected GTD/GTN. Whilst most cases

556 may be diagnosed through histological examination and p57 immunostaining alone, for those complex

557 and equivocal cases, knowing the underlying genetic constitution is invaluable. Nowadays, utilisation

558 of genetic testing represents the gold-standard within laboratory diagnosis, and is now much more

559 common practice, especially in those centres specialising in GTD diagnosis and surveillance [3,6,52].

560 Institutions which do not themselves have direct access to genetic testing for GTD/GTN management,

561 may therefore consider referral or consultation with a GTD specialist for further advice and support in

562 clinical management of routine, or most often, challenging or complex cases. Making the genetic

563 analyses is not often difficult as in many cases the same techniques as used in routine genetic

564 diagnostics, can be applied. However, choosing the optimal sample, handling of archival material, and

565 interpretation of the results can be difficult. Therefore we should aim to have a genetic referral

566 laboratory in each country.

567

568

569

570

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

573

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576
Author contributions
LM drafted and revised the manuscript and prepared figures.

GJM drafted and revised the manuscript and prepared figures.

CJ drafted and revised the manuscript.

IN revised the manuscript

RF drafted and revised the manuscript.

LS drafted and revised the manuscript and prepared figures.

584

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728
729

730 **Figure Legends**

731

732 **Figure 1. Q-banding of chromosomes showing a triploid karyotype (69,XXY) of a PHM.**

733

734 **Figure 2.** hCG normalisation after a triploid PHM without chemotherapy.

735

736 **Figure 3.** (A) MRI in week 16 showed that the myometrium was not invaded deeply. (B) ULS in week
737 18 revealed no abnormalities in the foetus.

738

739 **Figure 4. STR genotyping traces from recurrent diploid biparental (A) and recurrent androgenetic (B)**
740 **moles.** A) In the patient's complete hydatidiform moles (CHMs) each locus has peaks of equivalent
741 height, one peak matching the patient (red arrowhead), while the other matches the partner (blue
742 arrowhead), indicating a diploid, biparental origin and a diagnosis of FRHM. B). In many cases the
743 partner's sample may not be available and the presence of alleles that do not match the patient are
744 assumed to be of paternal origin. In this patient's CHMs, a single allele is present at each locus,
745 indicating homozygosity, and the alleles do not match the maternal genotype, indicating that these
746 are alleles of paternal origin (blue arrowhead).

747

748 **Figure 5. Genotyping of trophoblastic tumours.** A) The alleles in the tumour are the same as those in
749 the patient, indicating that the tumour originated from the patient's own cells. B) Paternal alleles (blue
750 arrowhead) identified in the tumour indicating a gestational tumour. Comparing the genotypes of the
751 patient's children to the tumour, revealed a match to the 7-year-old daughter, but the 3-year-old
752 daughter did not have the same paternal alleles (e.g. at *CSF1PO*) and had paternal alleles (e.g. at
753 *D8S1179*) not present in the tumour (black arrowhead).

754

755 **Figure 6. Detecting circulating tumour DNA from a gestational tumour using deep sequencing of**
756 **common single nucleotide polymorphisms (SNPs).** At 43 of the 100 SNPs where the patient (red) is
757 homozygous (B allele frequencies of 0 or 1), non-patient alleles were detected in the cell free DNA
758 (black), indicating the presence of a gestational tumour.

759

760 **Figure 7. Ploidy analysis by flow cytometry**. DNA histogram of nuclei isolated from FFPE tissue
761 containing a mixture of a POC and decidua, stained with propidium iodide. X-axis shows fluorescence,
762 proportional to DNA content per nucleus. Y-axis shows number of nuclei. One G1 peak and one G2

763   peak are observed, indicating that the nuclei from the POC have the same ploidy as the nuclei from

764   maternal decidua, i.e. diploidy (41).

765

766   **Figure 8. Chorionic villi with p57 immunostaining.** Images show representative villi populations with

767   (A) negative p57 immunostaining in both stromal and cytotrophoblast cells; (B) positive "normal"

768   immunostaining in both stromal and cytotrophoblast cells; and (C) discordant immunostaining with

769   negative stromal and positive cytotrophoblast cells. All three types of chorionic villi were present

770   within the same POC.

771

772   **Figure 9. Absence of maternal *TH01* allele in placental villi with abnormal morphology.** Paternal

773   alleles in the placental villi are marked with a blue arrowhead and maternal alleles are marked with

774   a red arrowhead. At *D19S433* the origin of the alleles could not be determined.

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When to consult a geneticist specialising in gestational trophoblastic disease

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Running title

When to consult a GTD geneticist

Keywords

Genetics, genotyping, hydatidiform mole, trophoblastic tumour, p57 immunostaining

Word count

6337

Abstract

Background:

Gestational trophoblastic disease comprises hydatidiform moles and a rare group of malignancies that derive from trophoblasts. Although there are typical morphological features that may distinguish hydatidiform moles from non-molar products of conception, such features are not always present, especially at early stages of pregnancy. Furthermore, mosaic/chimeric pregnancies and twin pregnancies make pathological diagnosis challenging while trophoblastic tumours can also pose diagnostic problems in terms of their gestational or non-gestational origin.

Objectives:

To show that ancillary genetic testing can be used to aid diagnosis and clinical management of GTD.

Methods:

Each author identified cases where genetic testing, including short tandem repeat (STR) genotyping, ploidy analysis, next generation sequencing and immunostaining for p57, the product of the imprinted gene *CDKN1C*, facilitated accurate diagnosis and improved patient management. Representative cases were chosen to illustrate the value of ancillary genetic testing in different scenarios.

Outcome:

Genetic analysis of placental tissue can aid in determining the risk of developing gestational trophoblastic neoplasia, facilitating discrimination between low risk triploid (partial) and high risk androgenetic (complete) moles, discriminating between a hydatidiform mole twinned with a normal conceptus and a triploid conception and identification of androgenetic/biparental diploid mosaicism. STR genotyping of placental tissue and targeted gene sequencing of patients can identify women with an inherited predisposition to recurrent molar pregnancies. Genotyping can distinguish gestational from non-gestational trophoblastic tumours using tissue or circulating tumour DNA, and can also identify the causative pregnancy which is the key prognostic factor for placental site and epithelioid trophoblastic tumours.

Conclusions and Outlook:

STR genotyping and P57 immunostaining have been invaluable to the management of gestational trophoblastic disease in many situations. The use of next generation sequencing and of liquid biopsies are opening up new pathways for GTD diagnostics. Development of these techniques has the potential to identify novel biomarkers of GTD and further refine diagnosis.

Introduction

Gestational trophoblastic disease (GTD) comprises a group of disorders associated with abnormal proliferation of trophoblastic cells including hydatidiform moles (HMs) and a rare group of malignant neoplasms collectively termed gestational trophoblastic neoplasia (GTN). GTN includes invasive mole, choriocarcinoma, as well as the very rare placental site and epithelioid trophoblastic tumours (PSTT and ETT). HMs carry a risk of development of GTN (Table 1), with the risk dependent on the type of HM. After evacuation of a HM, patients require monitoring of human chorionic gonadotropin (hCG) levels to identify persistent trophoblastic disease, for which the patients will require chemotherapy. A precise diagnosis ensures that patients at elevated risk of GTN are monitored appropriately and patients with low risk are able to attempt a new pregnancy with minimal delay.

The diagnosis of HM and the subdivision of these into complete hydatidiform moles (CHMs) and partial hydatidiform moles (PHMs) are in principle made from the morphological features. However, the distinction between CHM and PHM is not always easy, especially when the molar pregnancy is terminated early. Furthermore, some features of HMs can also be found in non-molar miscarriages such as hydropic chorionic villi (see [1,2] for further details). HMs are genetically different to non-molar pregnancies; PHMs typically contain an extra paternal genome and CHMs typically contain genomic DNA of paternal origin only [3] (Table 1). Morphological diagnosis can be supported by ancillary genetic techniques.

The majority of GTN are diagnosed clinically based on factors such as hCG levels, pregnancy history, and imaging. Except for PSTT and ETT, GTN are associated with survival rates of >98% with appropriate treatment and clinical management [5]. Genotyping can play an important role in diagnosis and management of GTN, being used to distinguish these from non-gestational tumours that also secrete hCG and to identify the causative pregnancy, the interval to which is the key prognostic factor for rare GTN subtypes.

In this article, we provide examples where consultation with a specialist GTD geneticist can aid diagnosis and how this can be informative/beneficial for patient management. The cases described in this article are inspired by true cases, but details have been amended to preserve anonymity of the patients. For readers interested in the technical aspects of genetic tests typically utilized, we recommend the following reviews [6–8]. Sections 1 to 6 demonstrate situations where testing helped guide the treatment by the gynecologists and/or the oncologists and sections 7 to 10 illustrate situations where testing was informative for pathologists.

Section 1. When you wish to determine prognosis of a HM

Case 1.1: A woman has had a HM and wishes to start a new pregnancy quickly

Mary was 40 years old, G1P0. She and her husband had achieved a pregnancy after *in vitro* fertilisation. Three ova were fertilised, two of these appeared to be of a sufficient quality. One embryo was transferred to the uterus and led to a pregnancy, the other was cryopreserved. In week 11, ultrasonography (ULS) showed no heartbeat, and a HM was suspected. The level of hCG was 100,000 IU/L. Mary had a surgical termination of the pregnancy, and tissue was forwarded for histopathologic and genetic analyses. The histopathological examination showed findings compatible with a PHM. The karyotype of the evacuated tissue was 69,XXY (Figure 1) and genotyping showed that two genome sets originated from the father. According to the national guideline [9], after a triploid PHM, surveillance can be discontinued after 2 consecutive normal hCG measurements. Mary achieved this within 2 months, and therefore she and her husband could soon plan to start a new pregnancy.

Case 1.2: A patient becomes pregnant during surveillance after a HM

Sophie was 30 years old (G1P0). In week 10 of her first pregnancy, she started bleeding and had high hCG levels (200,000 IU/L). A HM was suspected by ULS so the uterus was evacuated. Genetic analyses, alongside histopathologic examination of the tissue, made the diagnosis of a triploid PHM. hCG surveillance was initiated and hCG levels decreased. However, two months post-evacuation hCG levels began to rise. ULS disclosed that Sophie was pregnant. Sophie and her husband were informed that the production of hCG in the new pregnancy would prevent efficient surveillance after the HM. However, the couple disliked the idea of terminating a normal pregnancy. As the PHM diagnosis was supported by genetic analysis confirming triploidy, the gynaecologist could inform Sophie and her husband with more certainty that the risk of GTN was very low. The couple decided to continue the pregnancy. Sophie was monitored by ULS examinations in week 12, 16 and 20. No sign of GTN developed during the pregnancy and a healthy child was born.

Case 1.3. When hCG following a HM fails to normalise

Diana was 35 years old, G2P1. In her second pregnancy she had a HM diagnosed in week 12 and the pregnancy was terminated. Histopathologic examination of the evacuated tissue showed morphology consistent with a PHM. All tissue had been formalin fixed and no genetic analyses were performed at this time. Initial hCG was 150,000 IU/L. For 3 weeks the hCG levels fell exponentially, however then the levels seemed to plateau as two measurements with one week interval showed values of approx.

2,600 IU/L. To obtain more information about the risk of GTN, genotyping was performed. Analysis of STR (short tandem repeat) markers was performed on DNA isolated from 3 different parts of the formalin fixed tissue containing villous tissue (representing the HM) and one sample containing decidua (representing the mother, i.e. the patient). The three villous samples showed identical results, consistent with a diandric triploid HM.

The genotyping result arrived at the same time as the third hCG value which showed plateauing. As the gynaecologist now knew that the likelihood that Diana would develop GTN was very low, a hysteroscopy was performed to check for possible retained molar tissue. An irregularity of yellow-greyish tissue was removed from the right corner of the uterus. Histopathology showed a minimal piece of degraded villous tissue with only faint signs of trophoblastic hyperplasia and no sign of malignancy. Two months later, the patient was discharged after two normal hCG levels were obtained (Figure 2).

Comment.

When using morphology alone, the risk of GTN after a PHM is 0.5-1%, which is significantly lower than after a CHM [10]. GTN has been observed after triploid HMs: Seckl et al. reported 3 cases of choriocarcinoma after triploid PHMs [11] and Cheung et al. reported 2 cases of metastatic GTN after triploid PHMs [12]. However, in 4 cohort studies, a total of 265 patients were identified with a triploid HM by karyotyping, or flow cytometry on fresh tissue where external controls were co-analysed. None developed GTN, i.e. the estimated risk is 0% (95% CI: 0–1.4%) [13]. Accordingly, international guidelines now recommend a less intensive surveillance after a triploid HM/PHM than after a CHM [14].

Section 2. When you wish to distinguish a twin pregnancy from a PHM

Case 2. Anna was 37 years old and (G2P1) pregnant in week 11, when a routine ULS disclosed a cystic placenta, along with a living foetus, seemingly with a second normal placenta, suggesting a twin pregnancy with a HM and a normal conceptus. No abnormalities were observed in the foetus. However, as most conceptuses with a molar placenta and a foetus are PHMs, this diagnosis could not be excluded. Anna and her partner wished to continue the pregnancy if the prognosis for the foetus was good. The case was discussed in the foeto-medical multidisciplinary team, including gynaecologists, clinical geneticists and pathologists, and genetic analyses were agreed on. A sample was taken from the placenta that had a normal appearance on ULS. The placenta had a 46,XY

karyotype and genotyping of the sample along with parental blood samples revealed a diploid biparental genome. This corroborated that Anna had a twin pregnancy with a normal foetus.

Anna and her partner were informed about the risk of GTN and the likely outcome of normal live birth. The couple decided to continue the pregnancy. The pregnancy was monitored closely, with imaging (Figure 3) and measurements of hCG. The hCG levels dropped from 85,000 IU/L in week 11 to 20,000 in week 36. Apart from a few episodes of slight bleeding, the pregnancy was uneventful. In week 36 Anna went into labour spontaneously and delivered a healthy son. Genetic analyses of the molar placenta disclosed that this was diploid and androgenetic, corroborating that it was a CHM. hCG normalised within 6 weeks and stayed normal for the following 4 months, after which monitoring was discontinued.

Comment.

Most multiple pregnancies including a HM, are "twins" including a diploid androgenetic HM and a normal diploid biparental conceptus. Especially when diagnosed early, twinning between a diploid HM and a normal diploid conceptus can be mistaken for a PHM. A detailed ULS can often identify two separate placentas [15]. Due to the risk of bleeding, it is recommended to avoid taking a biopsy of a HM. However, identifying a normal diploid biparental constitution of the normal placenta (or amniotic fluid), can help discriminate between a triploid PHM, and twinning, and add to the information available for the parents when they must decide whether to terminate or continue the pregnancy [16,17].

Section 3. When a patient has recurrent molar pregnancies

Case 3.1. Riona was 37 years old (G3P0) and presented with a second molar pregnancy following a CHM, a year previously, and an intervening miscarriage. Pathological review of tissue from the two molar pregnancies showed the second to have the morphology of a typical CHM while the first was essentially a CHM but exhibited some atypical features. Due to the atypical features, the pathologist requested genetic analysis. STR genotyping of maternal decidua and placental villi from fixed tissue sections revealed that the genome of the placental villi in both cases was diploid and biparental rather than having the two paternal contributions to the genome expected for a typical androgenetic CHM (Figure 4). Diploid, biparental HMs suggest a diagnosis of familial recurrent HM (FRHM), an autosomal

recessive condition predisposing women to recurrent molar pregnancies. Sequencing of the gene *NLRP7,* using DNA extracted from the patient's blood*,* showed the patient to be homozygous for p.Pro716Ala, a pathogenic variant frequently found in patients with FRHM [18].

Women with FRHM are unlikely to achieve a normal pregnancy naturally but may do so by using a donor ovum [19,20]. Subsequently the patient underwent *in vitro* fertilisation using a donor ovum and was able to achieve a full-term pregnancy and a healthy female child two years later.

Case 3.2. Fatima was 37 years old (G7P2) and presented with a third CHM and a poor obstetric history. A CHM ten years previously was followed by a miscarriage, second CHM, live birth, still birth, and a further miscarriage before the third CHM, suggesting a possible diagnosis of FRHM, although live births are extremely rare in women with FRHM. STR genotyping of maternal decidua and placental villi from the third CHM revealed that the genome was androgenetic (Figure 4), demonstrating that the patient had recurrent HMs of androgenetic origin and not FRHM.

Recurrent androgenetic CHMs (AnCHMs) are rare and the causes remain unclear. Subsequent pregnancies may result in a normal term birth, although this is rare after three AnCHMs [21]. Having already achieved one normal pregnancy, the patient wished to try and conceive naturally but unfortunately experienced two further molar pregnancies, and a miscarriage. Both the fourth and fifth HM were confirmed to be AnCHMs.

Comment. Recurrent hydatidiform mole may have different aetiologies

While the great majority of women with two HMs subsequently go on to have normal pregnancies, a small number, particularly women with two consecutive CHMs and no normal pregnancies, will have further molar pregnancies [21]. In these cases, STR genotyping of the molar tissue is important to identify those women who have recurrent HM as a result of pathogenic variants in one of two genes, *NLRP7* [22] or *KHDC3L* [23]. This condition, FRHM, is characterised by failure to establish correct methylation of imprinted genes in the ovum [24,25], resulting in a molar phenotype in conceptuses that are otherwise genetically normal with a contribution to the genome from both parents.

FRHM can be identified by showing that the molar tissue is diploid and biparental or, very occasionally, triploid with two maternal contributions to the genome. Sequencing of the patient's DNA for pathogenic variants in the genes *NLRP7* and/or *KHDC3L* can identify the variant(s) underlying the condition, confirm the diagnosis and enable screening to determine whether other family members,

particularly nulliparous siblings, are likely to be affected. Since the genes associated with this condition are important for normal functioning of the ovary, conventional *in vitro* fertilisation is not appropriate. However, a normal term pregnancy can be achieved by using a donor ovum.

Women with recurrent AnCHMs, particularly those with three or more AnCHMs, generally have poor obstetric history, including miscarriage, and stillbirth in addition to CHMs, but may occasionally have one or more normal full-term pregnancies [21]. Little is known about the causes of recurrent AnCHMs, which may differ in each case. Variants of three genes have been reported in patients with poor reproductive history, including AnCHMs, but each of these only occur in a small number of cases [26]. Sequencing of our patient's DNA, (as a participant in a research project) did not reveal the presence of any of these rare variants. Women with recurrent AnCHMs may conceive naturally and achieve a normal term pregnancy. However, as in the case above, the more likely outcome of future pregnancies, particularly after three or more AnCHM, is a CHM or miscarriage [21]. *In vitro* fertilisation with preimplantation genotyping, to ensure embryos are non-molar, has been reported to result in the successful live birth of twins for a woman with a history of a miscarriage and two AnCHM [27] and may be an option to achieve a normal live birth. *In vitro* fertilisation using ovum donation may also be successful but there is little experience of this to date. Further understanding of the underlying causes of recurrent AnCHM is needed to improve advice available for these patients.

Section 4. When you don't know if a choriocarcinoma is gestational

Case 4: Nora was 67 years old (G1P1) and presented to the emergency department with abdominal pain and diarrhoea. A colonoscopy revealed colitis and diverticular disease for which she was treated. Nora is an ex-smoker, she has one daughter and her past medical history includes a diagnosis of cervical cancer for which she received brachytherapy. An incidental chest X-ray revealed a lung nodule and CT imaging confirmed a right upper lesion measuring 20 mm. Nora had a left upper lobectomy with lymphadenectomy and histopathological examination revealed a lesion with morphology and immunophenotype consistent with choriocarcinoma. Following her histopathological diagnosis, serum hCG levels were measured and found to be elevated (400 IU/L). Nora's diagnosis was discussed at a multidisciplinary team (MDT) meeting and the team decided to adopt a watch and wait approach and commenced weekly hCG surveillance. Her hCG levels dropped initially post-surgery but then rose steadily reaching a peak of 6,500 IU/L.

The differential diagnoses in this case included primary choriocarcinoma of the lung (which is extremely rare) and gestational choriocarcinoma. Nora's rising hCG level was discussed in the MDT and the oncologist requested genotyping to assist in the classification of her choriocarcinoma Nora's tumour tissue was sent to an expert centre for genotyping and DNA extracted from the tumour and normal lung tissue was analysed by STR analysis. The genotype of the tumour was the same as the genotype of normal tissue from Nora and no non-patient (paternal) alleles were observed in any of the STR loci (Figure 5A) which indicated a non-gestational choriocarcinoma. Following confirmation of her diagnosis, Nora's care was transferred to a medical oncologist team with the relevant expertise.

Comment

It is important to differentiate between gestational and non-gestational choriocarcinoma as they have different prognoses and clinical care pathways. Non-gestational choriocarcinomas have a poor prognosis and have a high propensity for metastasis [28]. More than half of all gestational choriocarcinomas are derived from a CHM, the remainder follow a pregnancy that has been terminated, a miscarriage, a full or preterm delivery or an ectopic pregnancy [29]. Genotyping plays a key role in the diagnosis by enabling the identification of non-patient alleles in gestational choriocarcinomas, compared to non-gestational choriocarcinomas in which the genotype reflects that of the patient.

Section 5. When the causative pregnancy is unknown, in PSTT or ETT

Case 5: Kirsty was 34 years old (G2P2) when she was diagnosed with a PSTT. It has been observed that the time period since the antecedent pregnancy is the most important prognostic factor for PSTTs, with an interval of 4 years or more associated with poor prognosis [30,31]. However, it has been shown that the antecedent pregnancy is not always the causative pregnancy [32,33]. In these cases, the interval since the causative pregnancy is likely to be more informative concerning the prognosis. Kirsty had two daughters, one three years old and the other seven years old. Therefore, genotyping of the daughters was requested to determine the causative pregnancy. The paternal alleles in the tumour matched those of the 7-year-old daughter, but only a subset matched those in the 3-year-old (Figure 5B). This indicated that the interval from the causative pregnancy was more than the 4 year cut-off, putting the patient in the high risk disease category [34]. Following a complete hCG response with EP-EMA chemotherapy, given the poor prognostic features, consolidation immunotherapy was offered and completed uneventfully.

Conclusion: An interval of four years or more from the antecedent pregnancy is associated with poor prognoses for both PSTT [30, 31] and ETT [35]. Although clinically the immediately antecedent pregnancy is typically perceived as the causative pregnancy, this is not always the case. In patients with PSTT or ETT who have had multiple previous pregnancies, STR genotyping is recommended to confirm the pregnancy of origin which can then be used to guide patient management [34].

Section 6. If you need a diagnosis and have no tissue

Case 6: Karen was 49 years old (G4P4) and presented to her local hospital with vaginal bleeding and elevated hCG. ULS showed no pregnancy and subsequent CT and MRI revealed a vaginal mass. Karen was admitted to a specialist GTD centre and underwent treatment for a suspected choriocarcinoma. Due to risk of profuse bleeding, it was not feasible to collect a tumour biopsy in order to determine if the tumour was gestational or non-gestational. Therefore, to facilitate genetic testing, a blood sample was collected before treatment began (hCG 26,000 IU/L): cell free DNA (cfDNA, from plasma) and genomic DNA (from buffy coat) were extracted. STR genotyping was performed, but no non-maternal alleles were detected in the cfDNA. As detection rates using STR genotyping of cfDNA are variable when serum hCG is less than 60,000 IU/L [36], it was not possible to conclude if the tumour was gestational or non-gestational in origin. A more sensitive technique, based on ultra-deep next generation sequencing of common single nucleotide polymorphisms (SNPs) [37], was employed and 43 of the 100 SNPs for which the patient was homozygous had non-patient alleles in the cfDNA (Figure 6), indicating that the tumour was gestational in origin. The patient continued single-agent treatment and she had a complete response.

Comment.

Although it is not possible to determine the tumour subtype using this method, cfDNA can be used to test the gestational or non-gestational origin of a tumour in cases where tumour tissue is not available and biopsy is contraindicated.

Section 7. When there is too little material for histological analysis

Case 7.1: Melanie was 42-year-old (G6P0) with 5 previous miscarriages and was admitted to the local early pregnancy assessment unit for medical management of suspected miscarriage. After evacuation,

products of conception (POC) were sent for routine pathological analysis where it was discovered that only small and scanty chorionic villi were present in the sample. Whilst the morphological appearance of the chorionic villi showed no striking features of hydropic change, the possibility of a HM could not be excluded. Subsequent chromosome analysis of a fresh POC sample showed a 69,XXX karyotype. As the morphological criteria for a molar phenotype were not fulfilled and chromosome analysis can not define the parental origin of the genome, STR analysis was undertaken on DNA extracted from laser-microdissected chorionic villi from fixed tissue. Genotyping of the maternal decidua and chorionic villi showed inheritance of 1 maternal allele and 2 paternal alleles consistent with a diandric triploid genotype. Melanie was enrolled at her GTD centre for hCG surveillance. As this pregnancy loss was diagnosed as a triploid PHM, her hCG surveillance was completed after 2 normal hCG measurements, 2 months post-evacuation of uterus.

Case 7.2: Yvonne was 37 years old (G1P1), underwent an elective caesarean section after an uneventful pregnancy, and gave birth to a healthy female infant. At the time of surgery, fatty cystic material was identified underneath the placenta. This was removed from the uterine cavity and submitted for pathological investigation. A disc of yellow/white tissue weighing 179 g and measuring 135x105x15 mm was identified. One aspect appeared pale and necrotic, and the other showed a multicystic appearance, the largest cyst being 10 mm. Microscopy showed there was only a limited number of enlarged, hydropic chorionic villi available for analysis. These showed circumferential trophoblast proliferation and marked cellular atypia. Immunostaining for p57, a marker for the presence of a maternal contribution to the genome [38,39], was negative across all the chorionic villi.

The morphological appearance favoured a diagnosis of a CHM, but the unusual clinical presentation and limited number of chorionic villi did not allow a definitive diagnosis of CHM. STR analysis of DNA extracted from laser-microdissected chorionic villi and maternal decidua within the same fixed tissue block was performed. Results showed only the inheritance of paternal alleles in the chorionic villi, indicative of a CHM.

Yvonne was thus diagnosed with a CHM in association with a normal pregnancy. She was enrolled into an hCG surveillance program, in which her hCG normalized and monitoring was completed after 6 months.

Comment

These examples highlight cases where genetics was invaluable in aiding the definitive diagnosis of a molar pregnancy where pathology alone did not fulfil the diagnostic criteria either due to the limited or degenerate chorionic villi available for histological analysis (Case 7.1); or an unusual clinical presentation combined with limited chorionic villi (Case 7.2). Whilst there was evidence of a triploid karyotype in Case 7.1, it was challenging to diagnose as diandric or digynic without genotyping, especially due to the lack of histological features. Furthermore, an unusual clinical presentation or a sample with limited chorionic villi (Case 7.2) is challenging diagnostically to determine by histology alone. In both scenarios, genotyping was able to confirm the presence of a molar component, so that the patient could be offered the most appropriate management.

Section 8: When the histology mimics a molar phenotype

Case 8: Kathryn, 34 years old (G2P1) presented with an early foetal demise on ULS. Evacuation of the uterus was performed. Local pathological investigations of the haemorrhagic membranous and placental fragments showed the presence of decidua, membranes and chorionic villi. Some villi were large and there was multifocal trophoblast budding. Preliminary pathological diagnosis was that of a suspected PHM.

Histological analysis by a specialist in GTD identified obvious abnormal villous morphology associated with a PHM phenotype, and immunostaining for p57 showed positivity in nuclei of cytotrophoblasts and villous stromal cells. However, ploidy analysis by flow cytometry showed a diploid DNA content (Figure 7). As this result was not consistent with a diagnosis of PHM, genotyping was initiated. Since there was no access to fresh tissue, or maternal DNA, laser-microdissection and DNA extraction from chorionic villi and maternal decidua from the same fixed tissue block was undertaken. STR analysis showed results consistent with biparental diploidy, except in the locus on chromosome 16, where three alleles were observed. This was subsequently confirmed by genotyping another four chromosome 16 loci suggesting a trisomic 16 conceptus.

These results indicated that the patient required no hCG surveillance. She was informed of the trisomy 16 result and advised this most likely accounted for the pregnancy loss in this case. There was no reason why she could now not attempt a new pregnancy.

Conclusion: In cases with atypical histology and normal p57 expression, genotyping by STR analysis is a useful tool to distinguish between PHM and non-molar pregnancy. In this case, atypical villous morphology, whilst mimicking the phenotype of a PHM, was attributed to an underlying chromosome abnormality (trisomy 16) which may explain the pregnancy loss. If genotyping was not performed, the patient may have been diagnosed as having a PHM and recommended to abstain from a new pregnancy until their hCG surveillance was completed satisfactorily.

Note: Discovery of a trisomy alone does not exclude the diagnosis of hydatidiform mole, as trisomy is occasionally seen in androgenetic HMs [40].

Section 9: When p57 immunostaining is discrepant or discordant

Case 9.1 Mandy was 27 years old (G1P0) and presented at the early pregnancy clinic at 10 weeks gestation with bleeding and inevitable miscarriage. POC were sent for routine pathological analysis, which showed enlarged and hydropic chorionic villi with lobulated outlines and widespread cistern formation. The appearance of scattered trophoblast pseudoinclusions were noted, alongside the absence of nucleated red blood cells. The differential diagnoses were hydropic pregnancy or PHM. Ploidy analysis of nuclei extracted from laser-microdissected fixed tissue (chorionic villi and maternal decidua) by flow cytometry showed a diploid DNA content. Immunostaining for p57 showed a discordant pattern with p57 negative stromal cells and p57 positive cytotrophoblast cells, as opposed to p57 staining where both stromal and cytotrophoblast cells are positive (as seen in both non-molar or diandric triploid pregnancies) or when p57 staining is completely absent in both stroma and cytotrophoblast (as seen in androgenetic moles).

To understand the equivocal p57 immunostaining, STR analysis of DNA extracted from the p57 positive and negative cells by laser-microdissection of fixed tissue was undertaken. The p57 positive cytotrophoblast cohort showed evidence of biparental diploidy, whereas the p57 negative stromal cells showed a pattern consistent with androgenetic diploidy.

Mandy was informed that she had experienced a mosaic/chimeric molar pregnancy (androgenetic and biparental components) and was entered into an hCG surveillance programme. In view of the limited knowledge regarding the risk of GTN, the hCG surveillance centre advised monitoring should follow

the same guidelines as for those women who experience a CHM. Mandy's hCG level normalised and she completed her surveillance with no adverse events.

Case 9.2: Lucy was 34 years old (G3P2) and presented at 10 weeks gestation for ULS. The pregnancy had the appearance of PHM with a demised foetus and a cystic placenta. Lucy's hCG level was 1.5 million IU/L. Following evacuation of the uterus, pathological analysis of the POC showed necrotic decidua, implantation site and embryonic tissue with abundant chorionic villi across a range of sizes which were enlarged, hydropic and irregular in outline. Scattered trophoblast inclusions and stromal karyorrhexis, focal cistern formation and absence of nucleated red blood cells were noted. The suspicion of a twin pregnancy was raised due to the presence of some villi appearing normal and some with the morphological appearance of HM.

Laboratory investigations included ploidy analysis by flow cytometry and p57 immunostaining. Whilst flow cytometry showed diploidy, the p57 immunostaining showed a variable pattern with large villi entirely negative (Figure 8A), smaller villi showing a normal pattern (positive stroma and cytotrophoblasts, Figure 8B) and a villous population with a discordant pattern (negative stroma, positive cytotrophoblasts, Figure 8C).

To distinguish the genotype of these three distinct villous populations, STR analysis was performed on laser-microdissected fixed tissue. Results showed the negative p57 chorionic villi had only paternal alleles consistent with an androgenetic genome; the villi with normal p57 staining had both maternal and paternal contribution (biparental diploidy); and the third population with discordant p57 expression (negative stroma and positive cytotrophoblast) contained both androgenetic genome (stroma) and biparental diploidy (cytotrophoblast) components. The presence of a normal biparental diploid component may explain the presence of embryonic tissue seen during pathological analysis.

Like Mandy, Lucy was informed of her mosaic/chimeric molar pregnancy and enrolled into an hCG surveillance programme which was completed in an uneventful manner.

Comment

Cases of molar pregnancy with aberrant p57 immunostaining have been well-documented [42,43] and whilst p57 immunostaining is often used as one of the first diagnostic tools to aid diagnosis of HM, it can also expose more complex and equivocal cases. Advanced laboratory techniques such as laser-

microdissection and subsequent genotyping can help to unravel the complexities at a molecular and cellular level, especially in cases with complex histological findings.

Many molecular studies have now shown that mosaicism or chimerism is not an uncommon finding in HM [44–46]. Mosaicism/chimerism in relation to HM can be defined as the presence of an androgenetic component in one population of villous cells, in addition to the presence of a normal biparental population of cells where there is equal contribution of maternal and paternal genome. The distribution of androgenetic and biparental cell lines may vary [42,47] and in some cases a viable foetus may be present [43]. The risk of persistent gestational trophoblastic disease in these cases remains to be established, partly because of the relatively small numbers identified at present, we recommend that these mosaic/chimeric cases are followed-up as for a CHM.

Section 10. When the p57 immunostaining pattern is not consistent with morphology

Case 10. Emily was 33 years old (G3P2) and following evacuation of her suspected HM, pathology review was performed on the POC. The chorionic villi were abnormal, many of which were hydropic and showed irregular scalloped outlines and complex trophoblastic pseudo-inclusions, the majority were avascular while others had collapsed vessels. Only focal excessive trophoblast was identified and where present was arranged in an abnormal circumferential distribution. No extravillous pleomorphic trophoblast was seen. p57 staining was unexpectedly negative. Therefore the case was sent for review by a pathologist specializing in GTD. The morphological features were in keeping with a PHM but could, more rarely, be seen in mosaic/chimeric pregnancies. Further p57 immunostaining was requested on multiple blocks which showed absence of p57 expression in the cytotrophoblasts and villous stromal nuclei. Due to the mismatch of the morphological and immunocytochemical features of the case, genotyping was requested. STR genotyping of the maternal decidua and placental villi revealed that the placental villi were diploid biparental, but no maternal allele was detected at the *TH01* locus on chromosome 11p15.5 (Figure 9). In cytotrophoblasts and villous stroma, *CDKN1C*, the gene which encodes p57, is normally expressed only from the maternal allele. *CDKN1C* is located at chromosome 11p15.4, close to *TH01*. Therefore, loss of maternal *CDKN1C* is also likely to have occurred with loss of the maternal *TH01*, explaining the p57-negative appearance of the villi. GTN has been reported in some diploid biparental cases with absence of maternal chromosome 11, therefore hCG monitoring was recommended [48].

Comment

Genotyping can be a useful tool to investigate POCs showing discrepancies between morphology and p57 immunostaining, the results of which can impact on patient management. CHMs expressing p57 due to retention of maternal chromosome 11 have also been reported [49].

Conclusion

GTD is nowadays a highly curable condition with an excellent overall survival rate [50]. Through the concerted and combined efforts and collaboration amongst the wider GTD community in recognising and diagnosing women earlier, alongside effective surveillance and treatment strategies, many women are successfully managed and treated accordingly. Guidelines on the management and treatment of GTD are well-documented [34,51] and international collaboration involving members of the EOTTD community have sought to establish recognised guidelines for diagnosis and treatment [14].

Whilst the guidelines provide a platform for scientists and clinicians working within the field of GTD and GTN, many parties may not work within specialised GTD units or teams, or have readily available access to all the necessary laboratory investigations and support required for GTD diagnosis. As such this paper aims to highlight scenarios where expert genetic investigation and interpretation may contribute to the overall care and management of women with possible GTD.

The first collection of cases (Section 1 to 6) is aimed at those scenarios where genetic investigation can aid the clinical management. For example, the correct diagnosis of PHM as demonstrated by STR analysis showing diandric triploidy, allows the patient to be managed conservatively on shorter-term hCG surveillance as opposed to longer-term surveillance associated with CHM (Cases 1.1, 1.2 and 1.3). Identification of FRHM is only possible after genetic investigation, and with this knowledge, couples may be offered the option of ovum donation to achieve pregnancy as opposed to an otherwise bleak obstetric outlook (Case 3.1). Similarly, identification of those women who experience recurrent androgenetic CHMs as opposed to FRHM is important in order to distinguish recurrence risk and to offer the appropriate genetic counselling and support (Case 3.2). The genetic identification of the gestational or non-gestational origin of a tumour helps guide the treatment strategies and decision making process for clinicians faced with hCG-producing tumours (Section 4). Genotyping to identify the causative pregnancy in cases of PSTT/ETT is an invaluable technique which can provide crucial

information regarding prognosis and treatment (Section 5). Additionally, recent advances in genetic methodology can aid diagnosis even in the absence of a pathological specimen, and therefore direct treatment strategies which otherwise may not have been considered (Section 6).

The second collection (Section 7 to 10) highlights the importance of genetic investigation to aid the pathological diagnosis of GTD/GTN. In cases where the histology is equivocal and limited tissue is available for a definitive diagnosis, genetic analysis of small amounts of DNA is invaluable to distinguish between a diandric and digynic triploid (Cases 7.1 and 7.2). In addition, abnormal villous morphology is often noted in pregnancy loss samples, which may be misleading or otherwise not definitive for a HM. Underlying chromosomal abnormalities such as common trisomies often mimic molar histology and without genetic analysis could be misinterpreted as an HM (Section 8). Routine p57 immunostaining is often used as one of the front-line tests for HM but in cases where the p57 staining is discordant with the associated histological features, genetics can aid to decipher the underlying chromosomal abnormalities causing such features and offer the most appropriate diagnosis and management strategy (Section 10). Finally, genetics enables the identification of mosaic/chimeric molar pregnancies which without analysis of the different villous population, may be misinterpreted as an otherwise non-molar conception in less specialised centres (Cases 9.1 and 9.2). Mosaicism/chimerism in HM can only truly be identified by genetic analysis and the prognostic significance of mosaicism/chimerism in HM is still under review given the small number of cases identified by genetics to date.

Overall, a wealth of knowledge is obtained when using genetic investigations in the clinical management, or the differential diagnosis of a patient with suspected GTD/GTN. Whilst most cases may be diagnosed through histological examination and p57 immunostaining alone, for those complex and equivocal cases, knowing the underlying genetic constitution is invaluable. Nowadays, utilisation of genetic testing represents the gold-standard within laboratory diagnosis, and is now much more common practice, especially in those centres specialising in GTD diagnosis and surveillance [3,6,52]. Institutions which do not themselves have direct access to genetic testing for GTD/GTN management, may therefore consider referral or consultation with a GTD specialist for further advice and support in clinical management of routine, or most often, challenging or complex cases. Making the genetic analyses is not often difficult as in many cases the same techniques as used in routine genetic diagnostics, can be applied. However, choosing the optimal sample, handling of archival material, and interpretation of the results can be difficult. Therefore we should aim to have a genetic referral laboratory in each country.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Author contributions
LM drafted and revised the manuscript and prepared figures.

GJM drafted and revised the manuscript and prepared figures.

CJ drafted and revised the manuscript.

IN revised the manuscript

RF drafted and revised the manuscript.

LS drafted and revised the manuscript and prepared figures.

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Figure Legends

Figure 1. Q-banding of chromosomes showing a triploid karyotype (69,XXY) of a PHM.

Figure 2. hCG normalisation after a triploid PHM without chemotherapy.

Figure 3. (A) MRI in week 16 showed that the myometrium was not invaded deeply. (B) ULS in week 18 revealed no abnormalities in the foetus.

Figure 4. STR genotyping traces from recurrent diploid biparental (A) and recurrent androgenetic (B) moles. A) In the patient's complete hydatidiform moles (CHMs) each locus has peaks of equivalent height, one peak matching the patient (red arrowhead), while the other matches the partner (blue arrowhead), indicating a diploid, biparental origin and a diagnosis of FRHM. B). In many cases the partner's sample may not be available and the presence of alleles that do not match the patient are assumed to be of paternal origin. In this patient's CHMs, a single allele is present at each locus, indicating homozygosity, and the alleles do not match the maternal genotype, indicating that these are alleles of paternal origin (blue arrowhead).

Figure 5. Genotyping of trophoblastic tumours. A) The alleles in the tumour are the same as those in the patient, indicating that the tumour originated from the patient's own cells. B) Paternal alleles (blue arrowhead) identified in the tumour indicating a gestational tumour. Comparing the genotypes of the patient's children to the tumour, revealed a match to the 7-year-old daughter, but the 3-year-old daughter did not have the same paternal alleles (e.g. at *CSF1PO*) and had paternal alleles (e.g. at *D8S1179*) not present in the tumour (black arrowhead).

Figure 6. Detecting circulating tumour DNA from a gestational tumour using deep sequencing of common single nucleotide polymorphisms (SNPs). At 43 of the 100 SNPs where the patient (red) is homozygous (B allele frequencies of 0 or 1), non-patient alleles were detected in the cell free DNA (black), indicating the presence of a gestational tumour.

Figure 7. Ploidy analysis by flow cytometry. DNA histogram of nuclei isolated from FFPE tissue containing a mixture of a POC and decidua, stained with propidium iodide. X-axis shows fluorescence, proportional to DNA content per nucleus. Y-axis shows number of nuclei. One G1 peak and one G2

peak are observed, indicating that the nuclei from the POC have the same ploidy as the nuclei from maternal decidua, i.e. diploidy (41).
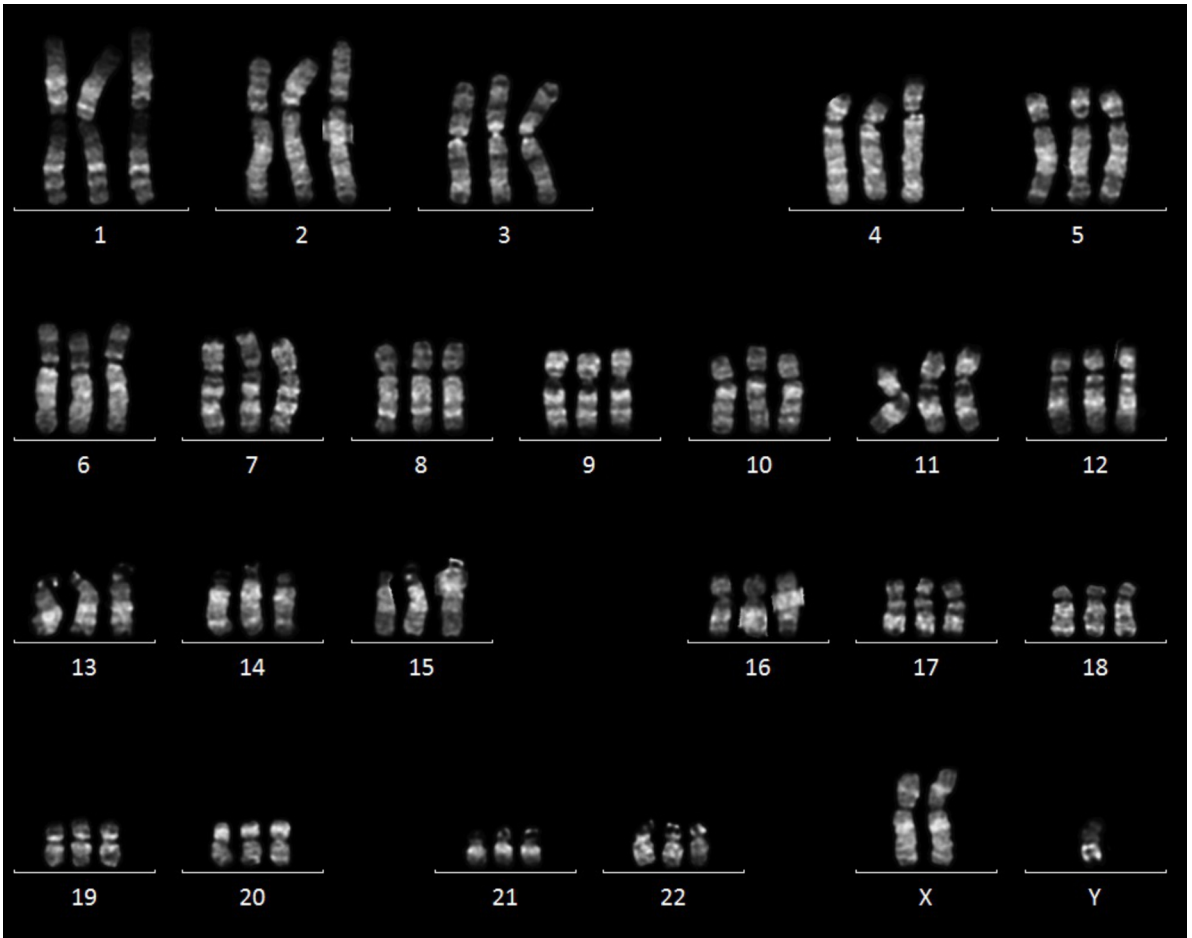
Figure 8. Chorionic villi with p57 immunostaining. Images show representative villi populations with (A) negative p57 immunostaining in both stromal and cytotrophoblast cells; (B) positive "normal" immunostaining in both stromal and cytotrophoblast cells; and (C) discordant immunostaining with negative stromal and positive cytotrophoblast cells. All three types of chorionic villi were present within the same POC.

Figure 9. Absence of maternal *TH01* allele in placental villi with abnormal morphology. Paternal alleles in the placental villi are marked with a blue arrowhead and maternal alleles are marked with a red arrowhead. At *D19S433* the origin of the alleles could not be determined.

Table 1. Typical genetic constitutions of gestational trophoblastic diseases

	Ploidy	Parental contribution[1]	Risk of GTN	Related cases
Partial hydatidiform mole (PHM)	Triploid	MP1P2[2]	Very low (<1.0%)	Case 1.1, 1.2, 1.3, 7.1
Complete hydatidiform mole (CHM)	Diploid	P1P1 or P1P2[3]	High (13–16%)[4]	Case 7.2, Case 10
Mosaic/chimeric mole	Diploid	MP/P1P1 or MP/P1P2	High(?)	Case 9.1, 9.2
Familial recurrent biparental hydatidiform mole (FRHM)	Diploid[5,6]	MP[5,6]	High	Case 3.1
GTN	Reflects the genetic constitution of the causative conceptus		NA	Case 4, 5, 6

[1]M: A maternal genome set; P: A paternal genome set; P1P1: Two identical paternal genome sets; P1P2: Two different paternal genome sets. [2] Very rarely: MP1P1. [3]Approximately 15% of CHMs are dispermic. [4] After CHM the risk of GTN increases with age (4). [5] Recurrent HMs with the parental contribution MP often are caused by biallelic variants *NLPR7* or *KHDC3L* in the patient. [6] Very rarely triploid MMP.
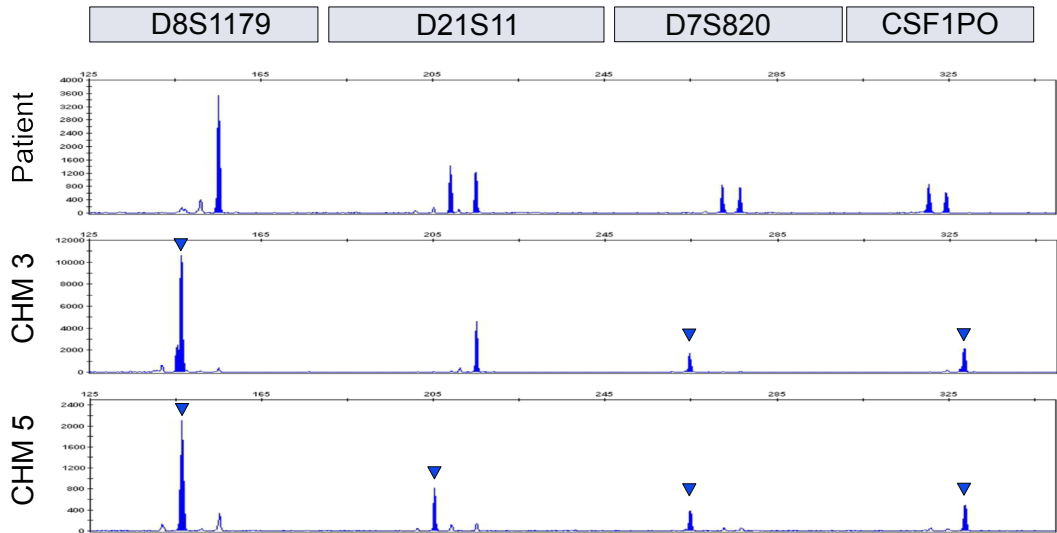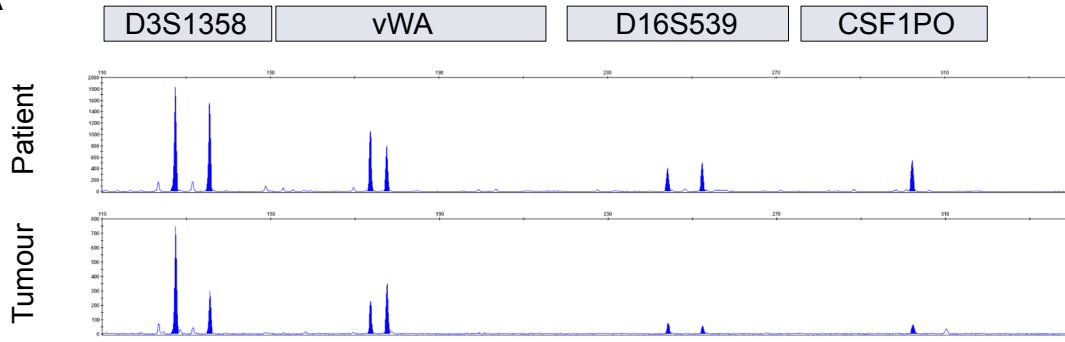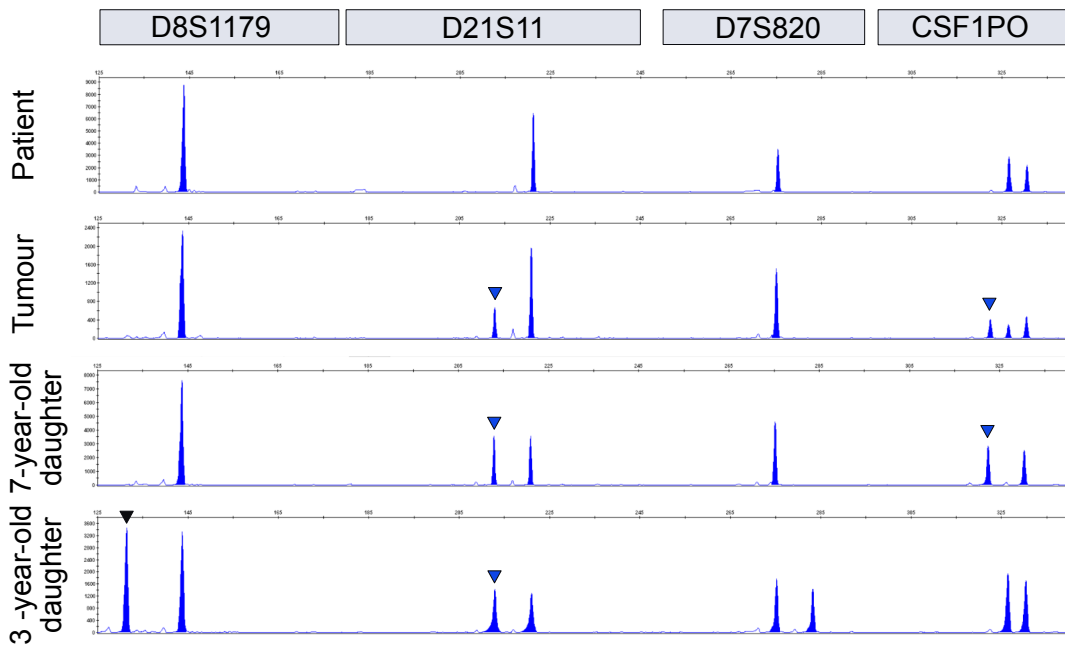
A.

B.

A

| D3S1358 | vWA | D16S539 | CSF1PO |

Patient

Tumour

B

| D8S1179 | D21S11 | D7S820 | CSF1PO |

Patient

Tumour

7-year-old daughter

3-year-old daughter