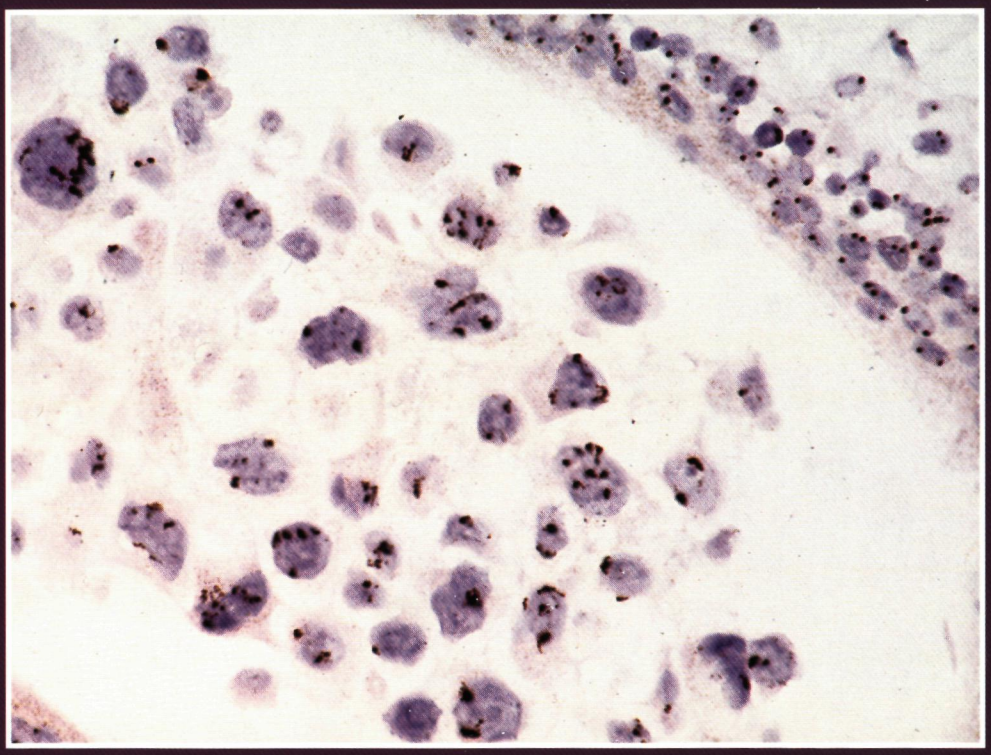
DNA ANALYSIS OF HYDATIDIFORM MOLE

A DNA Cytometric and Interphase Cytogenetic Study



Christina A. Hulsbergen-van de Kaa

DNA ANALYSIS OF HYDATIDIFORM MOLE

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A DNA Cytometric and Interphase Cytogenetic Study

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op het gebied van de Medische Wetenschappen

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Cover picture: Complete hydatidiform mole. *In situ* hybridization with DNA probe specific for chromosome 1. Trophoblast hyperplasia shows polyploidization (DAB, counterstain with Mayer's hematoxylin).

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Aan mijn ouders

Aan Frank en Alexander

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CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

INTRODUCTION

Hydatidiform mole is the most frequent aberration of the gestational trophoblast. Gestational trophoblastic disease encompasses a heterogeneous group of lesions characterized by proliferation of pregnancy-associated trophoblastic tissue of different malignant potential. They can be divided into lesions with chorionic villi, the hydatidiform moles, and in lesions without chorionic villi, of which choriocarcinoma is the most frequent. Table I shows the WHO classification.

Table I. WHO classification of gestational trophoblastic diseases.

Hydatidiform mole
complete
partial
Invasive hydatidiform mole
Choriocarcinoma
Placental site trophoblastic tumor
Trophoblastic lesions, miscellaneous
exaggerated placental site
placental site nodule
Unclassified trophoblastic lesions

Hydatidiform moles are characterized by cystic swelling of the chorionic villi and variable trophoblastic proliferation. They can be divided into complete mole (CM) and partial mole (PM). In CM the histopathological changes are diffuse and an embryo is absent. In PM the histopathological changes are focal and a (malformed) embryo is usually present. The most important reason for the correct recognition of CMs is the relatively high risk of persistent gestational trophoblastic disease (PGTD), which follows in 10-20% of CM and in about 0.5% of PM (*Bagshawe 1990*). PGTD includes residual or recurrent mole, invasive mole and choriocarcinoma. In invasive mole the chorionic villi invade the

myometrium and blood vessels (Fig. 1), which can result in perforation of the uterus and / or massive hemorrhage as well as in metastatic disease. Choriocarcinoma occurs in 2% of CM but is very rare in PM (*Bagshawe 1990, Lage 1991*). On the other hand, 50% of gestational choriocarcinoma are preceded by CM, 25% by abortion, 22.5% by normal pregnancy and 2.5% by ectopic pregnancy (*Hertig 1956*). It has become possible, by monitoring the circulating levels of human ß-chorionic gonadotropin (ß-hCG), to detect the early development of PGTD. If treated promptly, the prognosis of postmolar PGTD including choriocarcinoma is excellent with an overall survival rate of more than 90% (*Lurain 1982*).

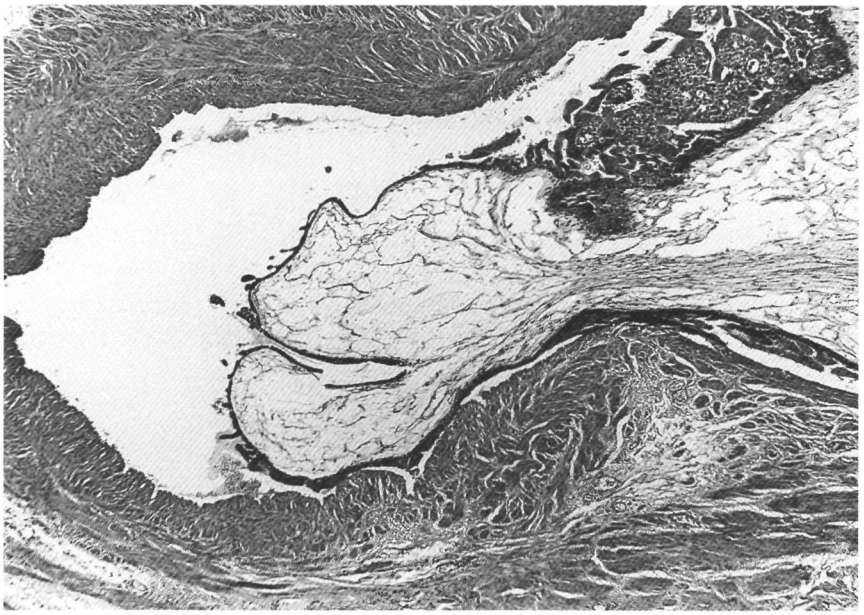

Figure 1. *Invasive complete hydatidiform mole. A chorionic villus invades a blood vessel in the myometrium (H&E, x 50).*

EPIDEMIOLOGY

There is a marked variation in the incidence of molar pregnancies in different regions of the world, varying from 1 : 1000 - 2000 pregnancies in the United States and also in The Netherlands to 1 : 100 in Indonesia (*Bracken 1984*). In The Netherlands, registration of CM, PM and PGTD takes place at the Dutch National Pathology Information System (PALGA), the Central Molar Registration and the Netherlands Studygroup of Trophoblas-

tic Tumors. The mean number of annual registered cases is 125 cases of CM, 50 cases of PM and 25 cases of PGTD.

CLINICAL FEATURES

The risk for hydatidiform mole is increased for women under 20 years and over 45 years (*Bracken 1984*). CM usually present in the first and early second trimester of pregnancy with varying clinical symptoms, such as vaginal bleeding, threatened abortion, marked uterine enlargement, hyperemesis and pregnancy-induced hypertension and occasionally of hyperthyroidism. The diagnosis may be suspected on ultrasonography when cysts are detected without a fetus ("snowstorm"). The ovaries can also be enlarged due to multiple theca-lutein cysts. ß-hCG levels are often markedly elevated.
The symptoms of PM become evident between 8 and 28 weeks, on average a few weeks later than CM, with irregular vaginal bleeding and a suspicion of spontaneous or missed abortion. The uterus is normal or small for gestational age. On ultrasound cysts may be detected together with a fetus, which often has died a few weeks before, although an embryo may not be identifiable if it has died early. Clinical symptoms are less severe and ß-hCG levels generally do not show the marked elevation seen in association with CM.

DIFFERENTIAL DIAGNOSIS

PGTD is common in CM and rare in PM, so that accurate histological diagnosis of molar type is important for prognosis. However, diagnostic accuracy of molar type is poor (*Javey 1979, Messerli 1987, Howat 1993, Conran 1993*). This is due partly to the rarity of hydatidiform mole, partly to changes and confusions in the concept of PM, partly to the impact of early pregnancy intervention based on abnormal ultrasound findings, which forces the pathologist to diagnose hydatidiform mole earlier when histopathologic changes are less pronounced. Hydatidiform mole not only needs to be correctly classified but also to be differentiated from abortion with hydropic degeneration (HA), which show similarities with PM and less pronounced with CM.
Although the risk of PGTD is higher in CM than in PM, at this moment the same clinical follow-up is advised for both entities. This includes clinical follow-up for one year with regular serum ß-hCG monitoring. Because elevations in serum ß-hCG due to PGTD cannot be differentiated from those due to a new pregnancy, the patients have to take contraceptive measures. Since moles are failed pregnancies in patients who wish to

conceive such contraceptive measures should be avoided if not really necessary. Misclassifications of hydropic abortions (HA) as hydatidiform mole leads to unnecessary follow-up with unnecessary contraception and psychological distress. Also, confusion between CM and PM gives incorrect prognosis and the false impression that PGTD is a frequent sequelae of PM.

CYTOGENETICS

Complete hydatidiform mole.

The chromosome constitution of complete moles is usually 46, XX, as has been demonstrated by karyotyping studies after tissue-culture or direct chromosome preparations (*Vassilakos 1977, Szulman 1978 I*). Kajii and Omaha (*1977*) reported that the entire genome was paternally derived (diploid androgenesis). In the majority of cases this can be explained by the fertilization of an "empty" egg, the maternal haploid genome being either lost or inactivated at meiosis, by a haploid sperm that then duplicates its genome without cytokinesis, restoring the diploid chromosome number before mitosis begins (Fig. 2A. *Jacobs 1980*). The result would produce a diploid homozygous mole containing either XX or YY sex chromosomes. The 46, YY cell is nonviable and therefore the 46, XX zygote is the only one that survives to become a mole. In approximately 10% of complete moles a 46, XY chromosome constitution is found, resulting from dispermy, in which an empty ovum is fertilized by two sperm pronuclei, one with an X and the other with an Y chromosome, resulting in a diploid XY heterozygous mole (*Ohama 1981*), or by two sperm each of which carries a X chromosome, resulting in a diploid XX heterozygous mole. The frequency of the total number of heterozygous complete moles has been estimated to be 23-29% (*Fisher 1989*). No morphological differences were noted between homozygous or heterozygous complete moles (*Kajii 1984*). Although the complete mole has exclusively paternally derived nuclear DNA, maternal DNA can still be found in the mitochondria (*Wallace 1982*).

Experiments in mice have shown that the paternal genome appears to be essential for the development of extra-embryonic tissues, whereas the maternal genome is more involved in embryonic development (*Surani 1984, Barton 1984, Surani 1986*). Implanting two paternal pronuclei in an enucleated egg (pure diandry) resulted in exuberant trophoblastic growth and poor development of the embryo, while two maternal pronuclei (pure digyny) gave meager placental development and much better embryonic growth. These experiments indicate that both paternal and maternal genomic imprinting are essential for development to term. These findings are consistent with what is found in a complete mole

in which the conceptus is composed of excessive extra-embryonic tissues (chorionic villi and trophoblast) usually without associated embryonal tissue.

Partial hydatidiform mole.

The partial mole is associated with a triploid diandric conception (*Jacobs 1982, Lawler 1982 and 1991, Vejerslev 1987*): a haploid egg, containing a pronucleus with one copy of maternal DNA, is fertilized by two spermatozoa (dispermy; Fig. 2B) or less frequently by one sperm containing a diploid pronucleus as a result of first meiotic failure (diplospermia; *Jacobs 1982*). The predominance of paternal DNA to maternal DNA results in a relative excess of placental tissue with a growth retarded and malformed fetus (*Doshi 1983*).

In partial moles the sex chromosome complement showed an usual pattern: XXY in 60%, XXX in 37% and XYY in 3% of cases (*Szulman 1981, Boué 1975*). Although all diandric (dispermic) triploids are described to be partial mole by some (*Jacobs 1982, Lawler 1982*), others report a low frequency of diandric triploids without molar changes (*Procter 1984, Vejerslev 1986*).

Hydropic abortions.

About 60 % of first trimester abortions have abnormal karyotypes incompatible with life (*Boué 1976*). Over 60 % of these cases show hydropic degeneration, which is mainly caused by trisomies (50%), triploidy and tetraploidy (12%) (*Honoré 1976*). Of all pregnancies 1-2% are triploid (*Couillin 1978*). In 86% of all triploid conceptions molar changes are found and are believed to be partial mole according to the current definition (*Lage 1992, Szulman 1981*), the remaining 14% being without molar changes (*Szulman 1981*).

A digynic triploidy originates from a normally fertilized abnormal ovum with a diploid pronucleus. The excess of maternal DNA over paternal DNA results in meager placental development, usually without hydatidiform changes and without histological features of triploidy (e.g. trophoblastic inclusion cysts), and with a growth retarded embryo which has a different phenotype than the diandric triploid fetus (*McFadden 1993*). Few cases of digynic triploids are reported to show molar changes, all resulting from first maternal meiotic failure, whereas digynic triploids resulting from second meiotic failure do not show any hydatidiform change (*Jacobs 1982, Uchida 1985*). Digynic triploids are associated with a shorter duration of gestation as compared to diandric triploids (*Jacobs 1982, Lawler 1982*), which may in part also account for the different frequency of hydatidiform changes.

Fig. 2A ***Complete Mole***

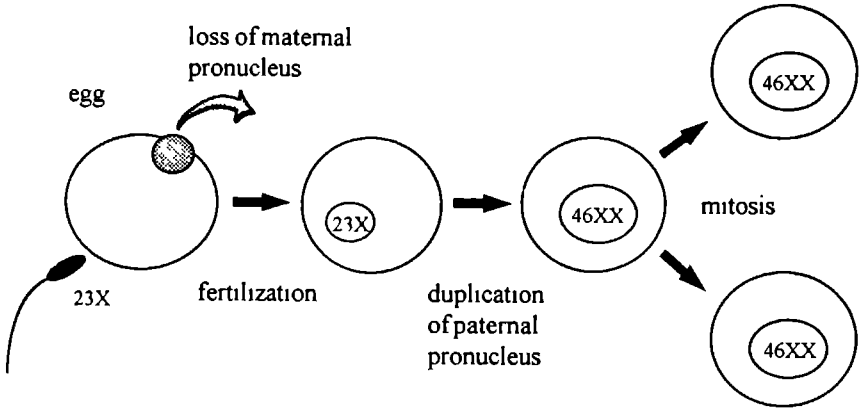

Fig. 2B ***Partial Mole***

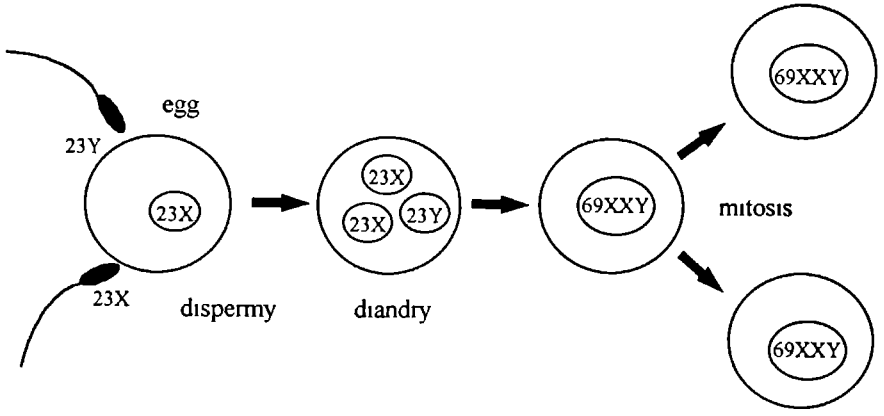

Figure 2. *Schematic representation of the origin of a complete mole (A) and a partial mole (B). A) In a complete mole the ovum has lost its maternal DNA. Fertilization by one sperm which duplicates its DNA or by two sperm (dispermy) results in a diploid, entirely paternal derived genome. B) In a partial mole a normal ovum is fertilized by two sperm, resulting in a triploid genome with a paternal extra set of chromosomes.*

Complete hydatidiform mole

The classical or complete mole is commonly defined as a grossly visible and universal hydatidiform swelling of the chorionic villi in the absence of the embryo/fetus and its amniotic covering. Microscopically trophoblastic hyperplasia is prominent, diffuse and circumferential along the villous surface.

Although this definition is suitable to the fully developed and typical case of complete mole, it certainly does not hold for all cases, especially not for the cases that are obtained for histology early in gestation. There is a wide variation in gross and histological appearance of the complete mole.

Villous edema.

One of the hallmarks of the hydatidiform mole is the villous edema with central cisterns. Hydatidiform mole literally means vesicle mass. This fluid accumulation is a gradual process with none or only a few cisterns in the early specimens and extensive cavitation in almost all villi in the older specimens (Figs. 3 and 4). As in the normal placenta newly formed chorionic villi are constantly produced by the ingrowth of extra-embryonic mesoderm in the trophoblastic columns rendering the secondary villi. The transition to tertiary villi is impaired with or due to an impaired angiogenesis. Fluid gradually accumulates in the villous stroma and leads to cistern formation in the older villi. Therefore, grossly swollen villi can exist next to relatively small villi of apparently normal size, which can lead to an erroneous diagnosis of partial mole. In the complete mole trophoblastic proliferation is excessive and haphazardly orientated along the villous outlines. The formation of new chorionic villi is equally disorientated. Therefore, especially in the younger moles (6-8 weeks), in which edema is not yet fully developed, the villi often have a branching or lobulated aspect. Due to this irregular branching and in older specimens due to the collapse of frankly vesicular villi large folds and invaginations of the villous surface are formed which may appear as large cystic pseudo-inclusions when tangentially cut, but which are quite different from the scalloping villi with small round inclusions of partial moles (see below). In some complete moles there are villi with single cell trophoblastic inclusions similar to those seen in trisomic or triploid abortions, but with more pleomorphic nuclei which appear to have a DNA-polyploid or chromosome-polysomic content.

When edema accumulates and large cisterns are formed the immature mesenchymal cells become more and more compressed to a small peripheral rim with sometimes a sharp inner margin. The stromal cells are spindly and scarcely distributed. Concomitant with the early edema, there is a random cell necrobiosis which is rather characteristic for the

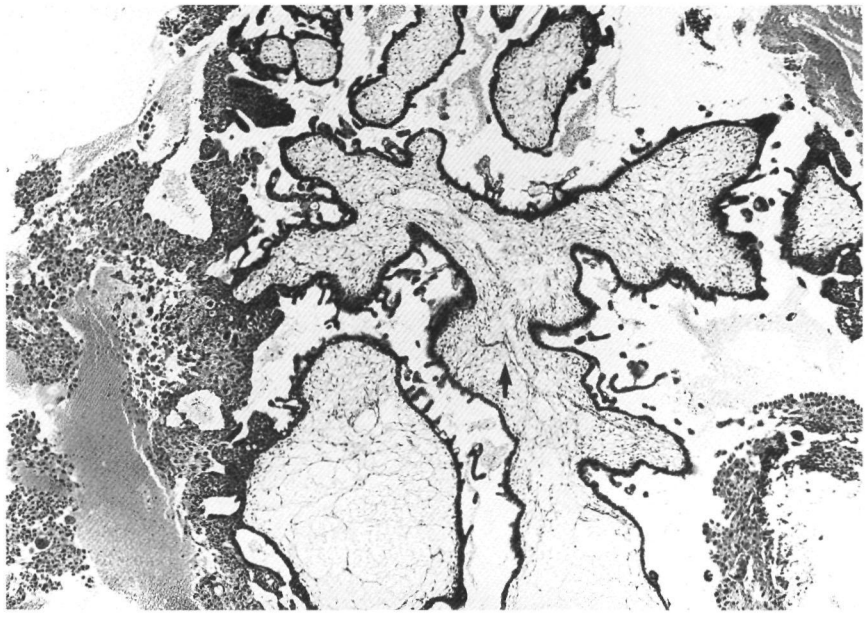

Figure 3. *First trimester complete mole. Newly formed chorionic villi show gradual accumulation of edema. Notice the irregular branching aspect and presence of a capillary (arrow; H&E x 50).*

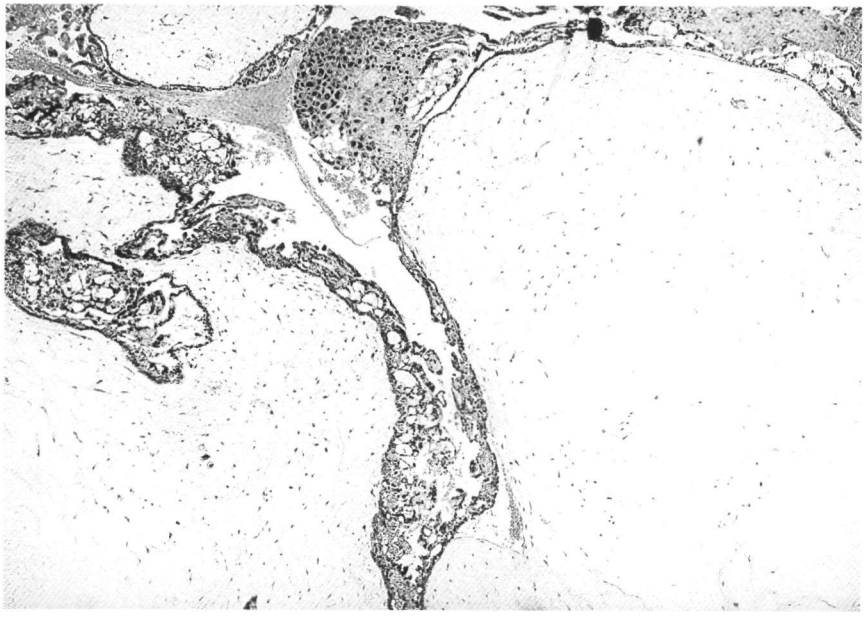

Figure 4. *Second trimester complete mole. The chorionic villi show extensive edema and are surrounded by atypical trophoblastic hyperplasia (H&E, x 50).*

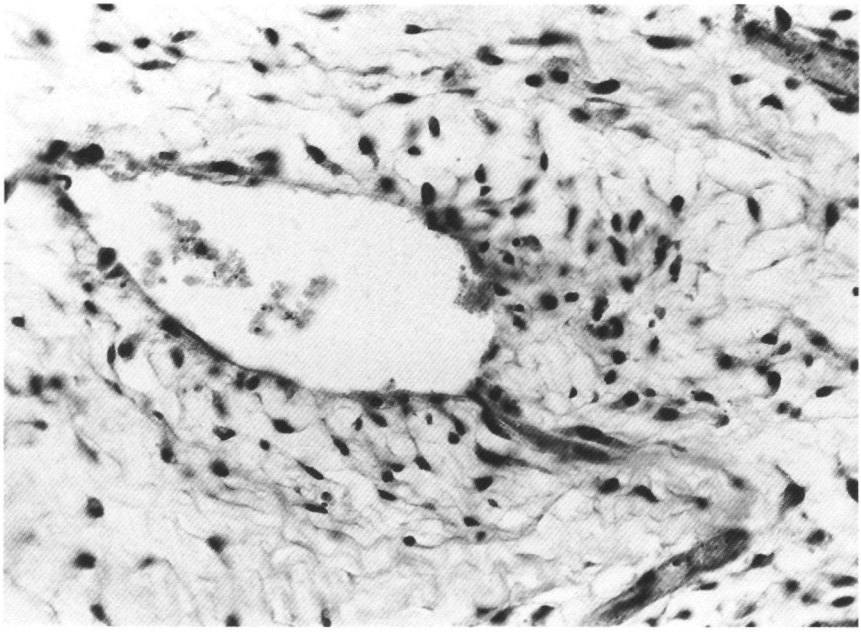

Figure 5. *Complete mole. In the villous stroma karyorrhexis is typically found and capillaries are not uncommon. In the vascular lumen cellular debris (H&E, x 250).*

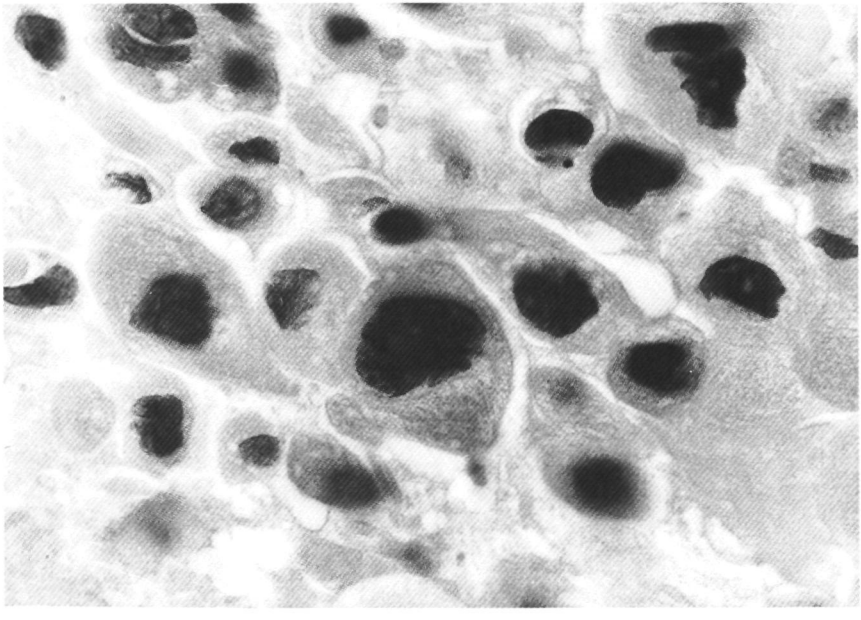

Figure 6. *Complete mole. Hyperplasia of intermediate trophoblast displaying prominent nuclear atypia (H&E, x 500).*

complete moles. Nuclear debris is found randomly distributed in the otherwise well-preserved villi (Fig. 5). Focal calcifications of villous stroma may also be seen. Karyorrhexis being seen next to vital fibroblast is usually not found in partial moles. It should not be confused with karyorrhexis within villous vessels which is found in both types of moles.

Trophoblastic proliferation.

Excessive trophoblastic proliferation is the most important hallmark of complete moles, but the degree of trophoblastic proliferation in an individual mole may show wide variation. In comparison to the normal placenta, in the complete mole this trophoblastic proliferation is abnormal in quantity, quality and spatial orientation. In the normal first trimester placenta the proliferating trophoblast remains at one side of the anchoring villi, usually the polar side. From here towards the decidual tissue, trophoblastic proliferation takes place in an orderly manner. Close to the villous stroma cytotrophoblast proliferates forming a few layers of small cytotrophoblastic cells, which gradually differentiates into intermediate trophoblast towards the implantation site. Syncytiotrophoblastic giant cells are the terminally differentiated trophoblastic cells that line the intervillous spaces. Away from the polar end the chorionic villus is covered by only two layers of trophoblast: the inner layer of cytotrophoblast which still retains proliferative activity, and the outer layer of syncytiotrophoblast which has lost all proliferative activity. In normal pregnancy or abortion only few villi can be found which are circumferentially covered by proliferating trophoblast, probably as a result of tangential sectioning. In complete mole there is a loss of trophoblast orientation and polarity, resulting in proliferative trophoblast surrounding the entire villus in a haphazard way. The trophoblastic proliferation may be marked, affecting most of the villi in a diffuse circumferential way, or only minimal and focal. The trophoblastic proliferation shows a centrifugal growth from the villous surface and may often appear unconnected from the villi in the histological section. The composition of this excessive trophoblast varies in the individual mole. Most often the intermediate trophoblastic component predominates, but in other cases syncytiotrophoblastic component is more prominent with marked vacuolization. In contrast to normal pregnancies, hydropic abortions and partial moles, in complete moles cytologic atypia with nuclear pleomorphism is very pronounced (Fig. 6). This is particularly prominent in the intermediate trophoblast in which the nuclei are enlarged and hyperchromatic, showing irregular outlines and often lobulation. Nucleoli can be prominent. Mitotic figures, including abnormal forms, may be evident. The syncytiotrophoblast often shows little or no atypia, although in some (older) cases nuclear atypia can also be present.

Impaired angiogenesis.

The presence of vessels in a mole is often erroneously interpreted as evidence of this

being a partial mole. In the majority of complete moles some angiogenesis is evidently present mainly in villi with little or no edema (Fig. 5). This de novo capillary formation is usually not very successful. The vessels are small with narrow lumina in which often nuclear and cellular debris is present. Well formed capillaries with wide open lumina are found in 17% of complete moles. In complete moles the capillaries gradually disappear as edema accumulates and cisterns are generated.

Absence of embryo/ fetus and amnion.

By the original definitions (*Szulman, 1978*) the classical or complete mole encompass specimens in which there is no evidence of the existence of the embryo proper or its amniotic covering. Villous capillaries containing nucleated red blood cells should suggest the possibility of a partial mole or twin pregnancy. In rare cases of otherwise typical complete mole we found evidence of vital nucleated red blood cells and/ or amnion, allantois-like structure or yolk sac (Chapter 5). In these cases all chorionic villi were abnormal, including those baring capillaries with nucleated red blood cells, thereby excluding twin pregnancy or partial mole. In most of these cases villous hydrops was not yet very pronounced. In many complete moles in which some villous vessels are still present, the lumens of these capillaries often contained nuclear and cellular debris, suggesting remnants of embryonal / fetal red blood cells (Fig. 5). In the villous stroma the presence of scattered Hoffbauer cells containing hemosiderin pigment support this suggestion. Experimental animal studies in the mouse have shown that when the female pronuclei of eggs were replaced by two paternal nuclei, the biparental androgenetic zygote sometimes developed into a retarded embryo of maximally 6- to 8-somite stage but with extensive trophoblast development (*Surani 1984, Barton 1984, Surani 1986*). The findings of nucleated red blood cells, yolk sac or amnion in the complete mole of humans suggest the development and short existence of a very early embryo that probably perished at the stage of extra-embryonal and embryonal angiogenesis (third week of gestation).

Partial hydatidiform mole.

The partial mole or formerly termed transitional mole is originally defined as a conceptus with a fetus, focal villous hydrops, and focal trophoblastic hyperplasia. There are two populations of chorionic villi: 1) villi that are of normal or relatively small size; and 2) villi that are hydropically enlarged. They were considered as an intermediate form between the common hydropic abortion and the classical mole (*Hertig 1940*).

Genetic studies (*Szulman 1978*) have shown frequent association between focal villous hydrops and triploidy. Later genetic and DNA cytometric studies revealed that under the histological diagnosis of partial mole not only triploid cases but also diploid and tetraploid

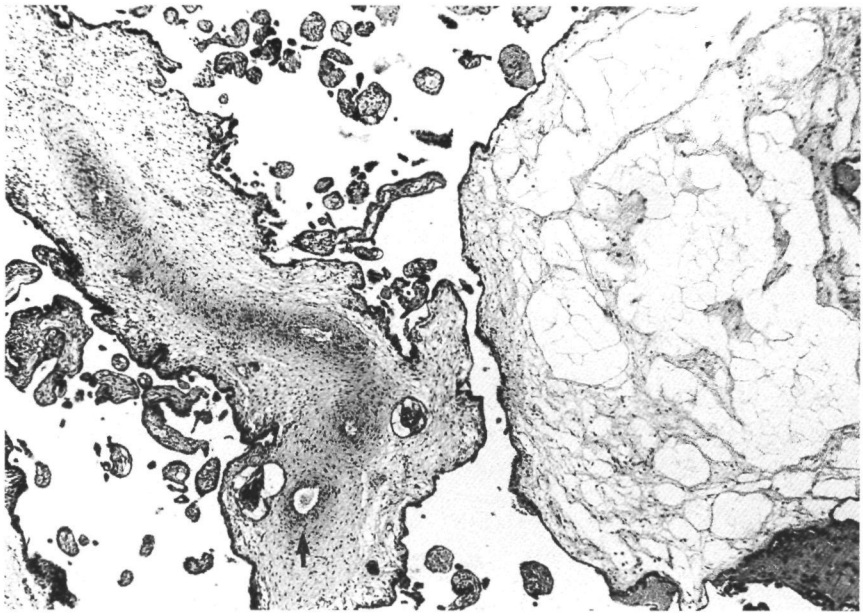

Figure 7. *Partial mole. Villous edema is present in part of the villi (right villus), as well as scalloping villous contours with trophoblastic inclusion cysts (left villus). Blood vessels are well-formed and contain fetal red blood cells (arrow; H&E, x 50).*

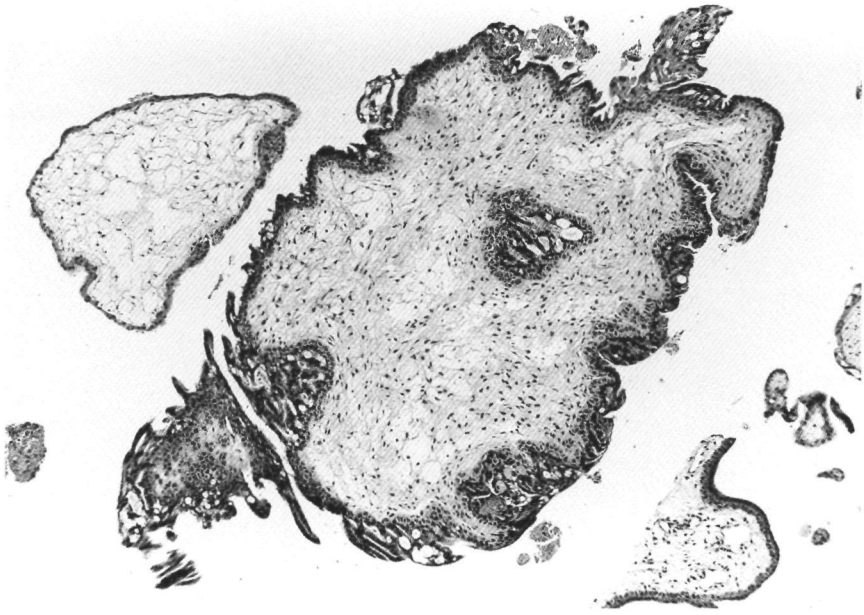

Figure 8. *Partial mole. A chorionic villous is surrounded by an excess of syncytiotrophoblast with vacuolization (H&E, x 100).*

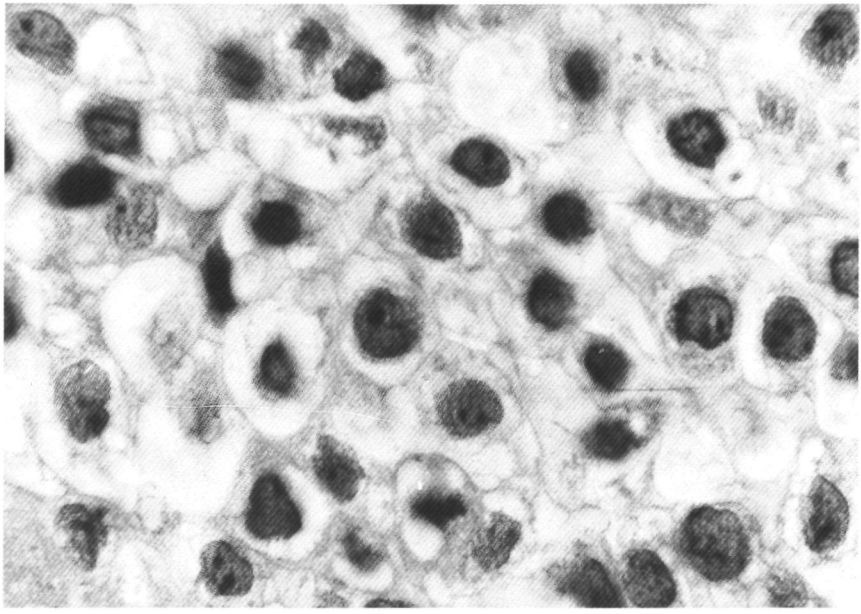

Figure 9. *Partial mole. Hyperplasia of intermediate trophoblast without significant nuclear atypia (H&E, x 500).*

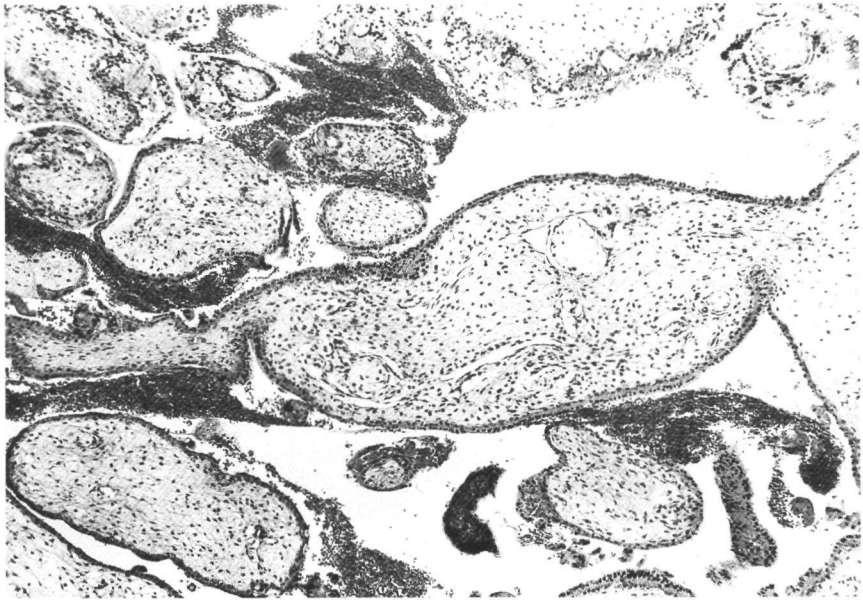

Figure 10. *Partial mole. Angiomatoid vascular changes are not specific to partial moles (H&E, x 50).*

cases were included as well as trisomies. Partial mole, as it was originally defined, appeared to be a gathering of different pathologic conceptuses. Furthermore, the differential diagnosis with complete mole and hydropic abortion is very difficult. The interobserver variability is therefore unacceptably high (50-100%)(*Javey 1979, Messerli 1987, Howat 1993, Conran 1993*). Many cases, initially diagnosed as partial mole, can be revised to complete mole, twin pregnancy with complete mole, or hydropic abortion.

A compilation of different genetic studies has redefined the entity of partial mole. At this moment partial mole is generally believed to be an entity which is mainly seen in triploid diandric conceptuses. These have a number of characteristic histopathological features, but none of these are pathognomonic in itself. Even one of the original diagnostic criteria of hydatidiform mole (trophoblastic hyperplasia) cannot in all cases be convincingly maintained as a conditio sine qua non according to Szulman (*1987*). Although it is logical for a disease entity to be based upon the cytogenetic origin, it must be realized that cases upon which formerly a diagnosis of hydropic abortion was made, are now diagnosed as partial mole. This has consequences for the registered incidence of partial mole and for the follow up of the patients.

The triploid diandric conceptus or partial mole is characterized by the following histological features: 1) variable degree of villous edema; 2) scalloped villous outlines with trophoblastic inclusions; 3) variable degree of trophoblastic hyperplasia; 4) functional angiogenesis; 5) development of an embryo/ fetus.

Villous edema.

As in complete mole, villous edema is always present, but it develops later and to a less extensive degree. Villous cavitation can especially be seen in the second trimester partial moles. The presence of two populations of villi can also in general best be appreciated in the second trimester partial mole (Fig. 7). In the younger cases villous edema is often more gradually present, varying in degree from one villus to the other. Villous edema and cistern formation can be present in villi with well-formed capillaries. When the embryo/ fetus dies in utero, collapse of capillaries takes place with concomitant stromal fibrosis and hyalinosis in the villi which were not yet edematous, in this way accentuating the two villous populations.

Villous contours.

Scalloped villous outlines with multiple invaginations and trophoblastic inclusions (Fig. 7) are major histological diagnostic indications for triploidy, but they are not present in all villi and they are not completely specific since they can also occur in other disorders, and can also be pronounced in some DNA-diploid non-hydropic abortions. If hydropic villous degeneration is not present a diagnosis of triploidy should therefore not be made solely on the presence of these features. Although rarely, these features can also be seen in diploid

hydropic abortions. Scalloping villous outlines and trophoblastic inclusions in partial moles can be present in many villi or only a few and is often more obvious before development of cisterns. The trophoblastic inclusions are generally round and small, either cystic or solid filled with fibrinoid material and syncytiotrophoblast.

Trophoblastic hyperplasia.

In contrast to complete moles in which florid trophoblastic proliferation is the diagnostic hallmark, in partial moles trophoblastic proliferation is minimal in the majority of cases. In comparison to complete mole, in partial mole the trophoblastic proliferation differs both in quantity and quality. In complete mole there is a proliferation of both cytotrophoblast and intermediate trophoblast which are present in increased amounts as compared to normal pregnancies. Syncytiotrophoblast is also increased in quantity but in general this is not predominant. In partial moles the opposite is true. Although it is likely that some cytotrophoblastic proliferation takes place, this becomes usually not visible in an increased number of cytotrophoblastic cells, nor in an increased amount of intermediate trophoblast. Instead, there seems to be a direct differentiation towards syncytiotrophoblastic cells, which are terminally differentiated and mitotically inactive. Along the villous surface small and occasionally larger piling ups of syncytiotrophoblastic cells are visible, often with prominent vacuolization (Fig 8). In some cases there is an increase of intermediate trophoblast, but the nuclear atypia, characteristically found in complete moles is absent in these proliferations (Fig. 9). Most cases of partial mole show only few villi with excessive syncytiotrophoblast, whilst the remainder of the villi show a normal or even attenuated trophoblastic lining. Some cases of triploid hydropic conceptuses pertinently lack trophoblastic hyperplasia. Often the difference with a normal pregnancy or non-triploid abortion is subtle. In practice in most cases it is difficult to differentiate a partial mole from a hydropic abortion on basis of the trophoblastic hyperplasia alone. In both a normal pregnancy as well as in a non-triploid hydropic abortion normal trophoblast, e.g. implantation trophoblast and columns of intermediate trophoblast, often mixed with fibrin, can be prominent and difficult to distinguish from the trophoblastic hyperplasia in partial mole.

Functional angiogenesis

In contrast to complete moles villous capillaries are well-formed with wide open lumina, often filled with embryonal/ fetal red blood cells, indicating functional activity. They may persist after embryonal/ fetal death, but often the erythroblasts degenerate and villous capillaries collapse. In few partial moles angiomatoid vessel changes are found (Fig. 10), but in our experience this is a relatively uncommon phenomenon and not specific to PM. It is, more rarely, also found in complete moles.

Presence of an embryo/ fetus.

Characteristic for the partial mole is the presence of an embryo/ fetus or evidence of its existence (nucleated red blood cells, amnion). The embryo/ fetus is growth retarded and shows many malformations (syndactyly, omphalocele, gastrointestinal, cardiovascular and central nervous system anomalies (*Doshi 1983*). Fetal death occurs usually early in gestation, in the first trimester, but longer fetal survival into the second trimester and even to term is possible (*Lawler 1982*). Although after fetal death capillaries with nucleated red blood cells can persist for a long time, often collapse of capillaries leads to avascular villi. An amnion can be the only evidence of embryonic development. In some cases no such evidence can be found. In these cases differentiation from a complete mole can be difficult.

Hydropic abortions.

The non-molar hydropic abortion is characterized by gross or microscopic villous edema without trophoblastic proliferation or hyperplasia.

Villous edema.

In general the villi show a spectrum of hydropic changes from small non-hydropic often sclerotic villi to large edematous villi (Fig. 11). The median villous size increases in

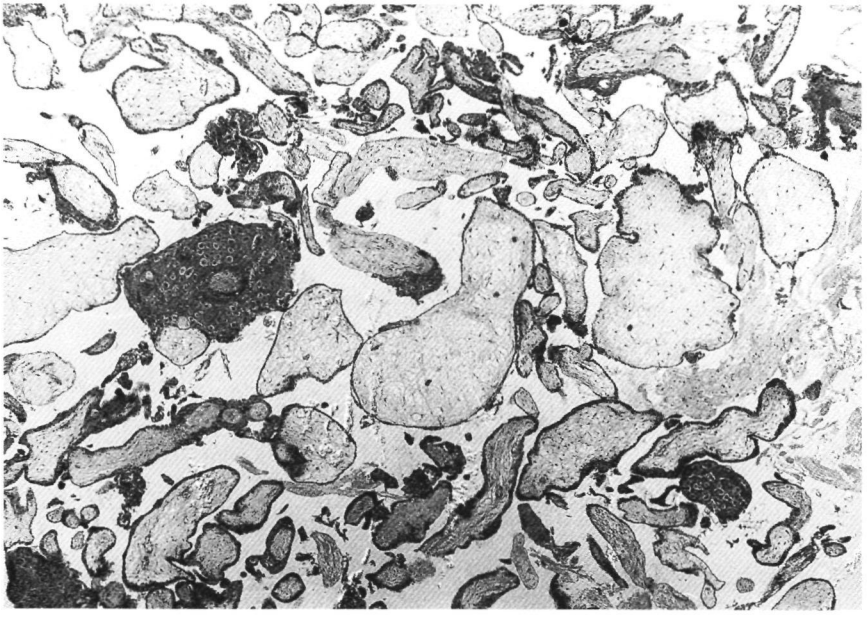

Figure 11. *Hydropic abortion. A gradual scale of edematous changes is found. Extra-villous intermediate trophoblast associated with fibrinoid deposition is present (H&E, x 50).*

hydropic abortion, partial mole and complete mole (0.28, 0.53 and 0.71 respectively; *Lage 1992*), but due to a considerable overlap these figures are not discriminatory. Central cistern formation is usually less pronounced in hydropic abortions and if present it concerns only a few villi, but in some cases large cisterns can occur with dimensions as in complete mole.

Absence of trophoblastic hyperplasia.

The trophoblast can be attenuated, but sometimes it may be abundant as in normal pregnancies. In these cases it is usually polar, characteristic for implantation trophoblast, or associated with fibrinoid deposits in which clumps of intermediate trophoblastic cells are retained (Fig. 11). Single cell trophoblastic inclusions in villous stroma are described in 70% of trisomies (*Boué 1976, Honoré 1976*), but they can also be found in complete and partial moles, although less frequently.

Angiogenesis.

This can be variably present. Villi may be avascular and fibrotic or show residual vessels containing nucleated red blood cells.

Embryo/ fetus.

This is not usually present (blighted ovum), but sometimes remnants or an intact embryo/ fetus are found.

ADDITIONAL TECHNIQUES

Knowledge of the nuclear DNA content of pregnancy products is important for the differential diagnosis of hydropic pregnancies (complete mole, partial mole, hydropic abortion). By karyotyping these different entities can be delineated, but there are some major disadvantages of this technique, such as (1) the necessity of fresh material; (2) the long duration of cell culturing; (3) the selective outgrowth of certain subpopulations (e.g. fibroblasts), and (4) the total loss of histopathologic morphology.

Therefore, for the determination of the nuclear DNA content other techniques were developed which can be performed rapidly on both fresh and paraffin embedded tissues.

DNA cytometry

DNA cytometry may be defined as the quantitative measurement of the total nuclear DNA of individual cells. The use of this technique has rapidly expanded in the last decennium attended by a large volume of publications, especially in cancer journals (*Quirke 1990*). There are two major technical approaches, flow cytometry (FCM) and image cytometry (ICM). For both techniques nuclei are isolated from fresh or paraffin embedded tissues

through disruption and enzymatic disaggregation. The DNA of the nuclei is specifically stained and quantified and compared to normal diploid cells (e.g. lymphocytes), expressed as the DNA index (DI), which is 1.0 for a diploid cell population. In FCM the fluorochrome labeled nuclear suspension is conducted along a laser beam. The excitation of fluorochromes emits light, which generates electrical signals. These are converted into digital signals for computer processing to generate a DNA-histogram. With FCM thousands of nuclei can be measured rapidly, but cells under study cannot be visualized. In ICM the nuclear suspension is cytocentrifuged onto glass slides and then Feulgen stained (*Oud 1984*). The advantage of ICM is that the nuclei can be measured under visual control for DNA-content as well as a series of other nuclear parameters, such as shape, size, texture, etc.

DNA cytometry allows the measurement of the total DNA content of large cell populations, but the sensitivity of the technique is limited in the detection of small cell subpopulations with increased DNA content. Furthermore, tissue morphology is completely lost, making it impossible to relate DNA aberrations to histology.

Interphase cytogenetics

In situ hybridization (ISH) of nuclear DNA with a labeled, chromosome-specific DNA probe allows the direct detection of the chromosome in metaphase spreads as well as in interphase nuclei, the latter being referred to as interphase cytogenetics (*Cremer 1986*). Chromosome-specific DNA probes detect tandemly repeated DNA sequences that are specific for only one chromosome type and are usually located in the pericentromeric region of the chromosome. The chemically or enzymatically modified and labeled DNA probe can be visualized by fluorochromes as well as enzymes (*Raap 1989, Poddighe 1994*). By using differently labeled DNA probes simultaneous detection of two DNA-targets in one nucleus is possible (*Nederlof 1990, Kersten 1994*). The visualized DNA-probe-target can be detected as dot-like signals, reflecting the number of the individual target chromosome.

In our laboratory a procedure has been developed for the application of interphase cytogenetics on paraffin embedded tissue sections (*Hopman 1991*). A major step in the DNA-DNA ISH procedure on interphase nuclei in formalin-fixed paraffin embedded tissue sections, is the breakdown of DNA-nucleohistone complexes to gain access to the target DNA. A reproducible and efficient hybridization reaction was achieved with successive protein-denaturating steps. Dissociation of the DNA-protein complex by heating in 1 M sodium thiocyanate in water at 80°C for 10 minutes improved the efficient removal of nuclear proteins by subsequent pepsin digestion (4 mg/ml in 0.2 N HCl) at 37°C with tuned digestion time (5 - 45 minutes) depending on the fragility of the tissue.

This resulted in the best ISH signals with excellent morphology. Due to tissue sectioning resulting in nuclear truncation the real chromosome copy number is underestimated especially in large nuclei and/or with high copy numbers. An optimal estimation of the maximum copy numbers was found to be achieved in 6 μm thin tissue sections. Depending on the nuclear size and nuclear to cytoplasmic ratio, thinner sections usually increased the underestimation, while thicker sections were difficult to evaluate due to nuclear overlap.

With the developed protocol a fast screening method for estimation of the chromosome copy number and, therefore, for the detection of numerical chromosome aberrations in routinely processed tissues became available. Interphase cytogenetics on paraffin tissue sections has important surplus value as compared to DNA analysis on isolated nuclei (karyometry, DNA cytometry, ISH), since no selection of cells occurs as a result of the isolation procedure, and focal areas with numerical chromosomal aberrations can be recognized and directly correlated to histology.

DNA polymorphism analysis

Detection of small structural chromosomal aberrations is not possible with DNA cytometry or interphase cytogenetics. DNA polymorphism analysis is a more sophisticated technique to study the genome and identify local DNA variations between individuals and to determine parental origin of the genome. This technique can also be applied on DNA extracted from paraffin embedded tissues.

DNA polymorphism analysis is based on the fact that the noncoding DNA of the human genome consists of single copy and repetitive sequences (*Jelinek and Schmid 1982*). This repetitive DNA fraction is found in low, middle and highly repetitive sequences, which occur as clustered and interspersed repeats. Clustered repeats mainly reside in the (peri)centromeric and (sub)telomeric chromosome regions, some of them having structural functions. The interspersed repeats can be divided in short and long interspersed repeats and in the simple sequence motifs (SSMs). These SSMs consist of repeats of short stretches of mono-, di- and trinucleotides. The (CA)n-motif is one of the most common among eukaryotes, is highly abundant, highly polymorphic, and is supposed to be randomly distributed in the human genome (*Tautz and Renz 1984, Smeets 1989, Litt 1990, Weber 1990*). Length variability in these SSMs results in a high degree of allelic variation in a specific gene locus. This can be visualized by combining in vitro DNA amplification using polymerase chain reaction with primers flanking the repeat and high-resolution gel electrophoresis (*Smeets 1989*). The many thousands of structurally similar SSMs in the human genome represent a rich source of highly informative genetic and diagnostic markers.

OUTLINE OF THE THESIS

The aim of this study was to evaluate the applicability and surplus value of DNA cytometric analysis and interphase cytogenetic analysis in the diagnosis of hydatidiform mole, especially with regard to its potential role in the differential diagnosis of complete mole, partial mole and nonmolar hydropic abortion, as well as to its predictive value in the development of persistent gestational trophoblastic disease.

Chapter 2 reports the application of the newly developed protocol of interphase cytogenetic analysis on routinely processed paraffin embedded tissue sections of hydatidiform moles and nonmolar hydropic abortions.

Chapter 3 reports the application of DNA image and flow cytometric analysis on routinely processed paraffin embedded tissues of hydatidiform moles and nonmolar abortions. The differences in the results of DNA flow cytometry and DNA image cytometry are discussed. The results of the application of these techniques are compared with the results of interphase cytogenetic analysis on paraffin tissue sections of some of the cases.

Chapter 4 and 5 describes the investigation of exceptional cases using a combination of DNA cytometry and interphase cytogenetics (Chapter 4) and DNA polymorphism analysis (Chapter 5).

Chapter 6 reports on the predictive value of DNA cytometry and interphase cytogenetics on the development of persistent gestational trophoblastic disease.

Chapter 7 shows an extension of the study with respect to the number of cases studied and comprises the results of the foregoing studies together with an evaluation of the clinical follow-up. Final conclusion are drawn on the applicability and surplus value of DNA cytometric and interphase cytogenetic analysis in the diagnosis of hydatidiform moles. The problem in the differential diagnosis and clinical follow-up of partial moles is discussed.

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CHAPTER 2

INTERPHASE CYTOGENETICS IN PARAFFIN SECTIONS OF ROUTINELY PROCESSED HYDATIDIFORM MOLES AND HYDROPIC ABORTIONS.

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INTERPHASE CYTOGENETICS IN PARAFFIN SECTIONS OF ROUTINELY PROCESSED HYDATIDIFORM MOLES AND HYDROPIC ABORTIONS.

SUMMARY

The differential diagnosis of complete (CM) and partial (PM) hydatidiform moles and hydropic abortions (HA) can be difficult when based on histology alone. Therefore, a more objective approach of chromosome ploidy analysis as detected by in situ hybridization (ISH) was performed on 6 μm paraffin sections of seven cases, originally classified as three CM, two PM, and two HA with a histologic pattern suggestive of triploidy. Probes for repetitive DNA targets in the (peri)centromeric region of chromosomes 1 and X and in the q arm of chromosome Y were used to determine chromosome ploidy and sex chromosome composition. The findings in the three CM were consistent with diploidy: two copies of chromosomes 1 and X and none of chromosome Y. In the two HA with a histologic pattern suggestive of triploidy, three copies of chromosomes 1 and X and none of chromosome Y confirmed triploidy. Two cases originally classified as PM both appeared to have two copies of chromosome 1 with an XX pattern in one case and an XY pattern in the other case, which is consistent with diploidy instead of triploidy. After reviewing, both cases most likely represented CM. We conclude that interphase cytogenetics by ISH on paraffin sections of hydatidiform moles and hydropic abortions enables chromosome ploidy analysis with preservation of histological context. The important advantages of this procedure are: 1) precise discrimination between maternal and trophoblast cells; 2) no risk of selecting certain cell types as a result of the isolation procedure; 3) detection and localization of cell subpopulations with heterogeneous DNA content; 4) determination of the contribution of a single chromosome (e.g. sex chromosome); 5) access to archival material.

Key words: *in situ* hybridization, paraffin sections, hydatidiform mole, placenta, ploidy, chromosome aberrations.

Oedema of chorionic villi is found in complete (CM) and partial (PM) hydatidiform moles and in hydropic abortions (HA). As a rule, CM have a diploid karyotype, 46,XX or 46,XY, while PM are usually described to have a triploid karyotype, 69,XXX or 69,XXY or 69,XYY[1-4]. Triploidy can also be present in HA[3,5-8]. The three categories have a different prognosis and therapeutic approach. In at least 10 percent of CM, persistent gestational trophoblastic disease develops[9,10], with risk of metastases and/or choriocarcinoma. In PM, persistent gestational trophoblastic disease is recognized (0.5 - 6.6 percent)[4,10-13] but it does not occur in HA. In CM, hormonal follow-up and contraceptives are essential for 1 year. This procedure is recommended for PM[10,13], while it is unnecessary in HA.

Histological criteria for classification of hydatidiform moles have been described[1-3]. However, the differential diagnosis based on histological criteria can be difficult, as indicated by a high interobserver variability[14,15]. Determination of DNA-ploidy with the aid of more objective methods may contribute to the differential diagnosis. Karyotyping is an objective approach to detect numerical and / or structural chromosome aberrations. Nevertheless, karyotyping is difficult in a routine setting and carries a potential danger of loss and / or of selection of genetic material[16]. Flow cytometry (FCM) has been used in the diagnosis of hydatidiform moles and abortions[17,18]. However, the method is limited in the detection of heterogeneous cell subpopulations with different DNA contents and gives no information about specific chromosome aberrations. In addition, with both methods the histological context is lost.

Chromosome aberrations at the cellular level can be demonstrated by *in situ* hybridization (ISH) using chromosome specific probes. Since clear results can be obtained not only in metaphase spreads, but also in non-mitotic cells, the procedure was termed "interphase cytogenetics"[19-23]. ISH using specific probes permits the screening of relatively large cell populations as well as the detection of minor cell subpopulations with heterogeneity and imbalance in chromosome copy numbers[22]. In an earlier report[24] and in this paper we introduce a protocol for a non-radioactive ISH procedure on paraffin embedded tissue sections, as a method to determine the copy number of specific chromosomes. In the present study, seven cases, including three CM, two PM and two HA, were selected on the basis of the original histologic diagnosis. To determine chromosome ploidy and the contribution of the sex chromosomes, DNA probes for the (peri)centromeric regions of chromosomes 1 and X and for the q arm of chromosome Y were used.

Patient material

A total of seven conceptuses were investigated, all obtained following curettage performed after ultrasound diagnosis of suspected molar pregnancy (cases 1, 2, 3, 5 and 6, Table 1) or of missed abortion (cases 4 and 7). The period of gestation varied from 9 to 18 weeks. Embryonal or fetal parts were not found. Gross vesicles could be identified in four cases (1, 2, 3 and 6). The specimens were routinely processed (fixed in phosphate buffered 4% formaldehyde for 12 - 24 h and embedded in paraffin at 58°C) and microscopically classified. A diagnosis of CM was made when diffusely, oedematous villi with central cisterns and excessive trophoblast proliferation were present. Embryonal cells (embryonic tissue or nucleated red blood cells) or amnion cells excluded a CM. A diagnosis of PM was considered when hydropically swollen villi were found intermingled with villi of normal size, while excessive trophoblast proliferation was moderate and focal. Morphologic suggestions of triploidy, such as irregular villous contours and trophoblast inclusion cysts, were often seen. Although embryonal elements are regularly present, these can be missed due to sampling error, and evidence of embryonal cells therefore was not always considered essential for a diagnosis of PM. In HA, varying degrees of villus oedema could be found, sometimes with central cisterns, but without excessive trophoblast proliferation. Scalloped villous outlines and trophoblast inclusion cysts were features suggestive of triploidy.

DNA probes and non-radioactive labeling

The satellite III DNA probe for chromosome 1 (pUC 1.77) recognizes a tandem repeat of 1.77 kb in the (peri)centromeric region (1q12) of chromosome 1[25]. The alphoid DNA probe for chromosome X (pBam X5) and the satellite III DNA probe for chromosome Y (DYZ3) recognize tandem repeats in the centromeric region of chromosome X and in the q arm of chromosome Y, respectively[26,27]. Biotinylation of the probes was performed using Bio-11-dUTP (Sigma, St.Louis, USA) in a nick translation reaction[28,29].

In situ *hybridization*

The reproducibility and validity of a protocol to detect chromosome copy numbers in routinely processed, paraffin-embedded tissue sections by ISH has been tested and described elsewere[24]. A brief description is given here. Paraffin sections (6μm) were mounted on 2.5% glutaraldehyde activated poly-l-lysine (1mg/ml; Sigma) coated slides[30], dried at 56°C overnight, deparaffinized, and treated with 1% H_2O_2 in methanol (30 min), rinsed, and air dried. To permeabilize the tissue sections for penetration of modified

DNA probes and antibodies, the slides were incubated with 1 M NaSCN (Merck, Darmstadt, Germany) at 80°C (10 min), rinsed and digested with pepsin (4 mg/ml in 0.2 M HCL; Sigma P7000) at 37°C (15-40 min), rinsed, dehydrated, air dried and heated at 80°C (30 min).

ISH with chromosome-specific probes took place in a mixture of 60% formamide, 2 x SSC (0.3 M NaCl, 30 mM Na-citrate pH 7.0), 10% dextransulphate (Pharmacia, Uppsala, Sweden), 50 ng/μl herring sperm DNA as carrier DNA and 50 ng/μl bakers yeast as carrier RNA, at probe concentrations of 1 ng/μl hybridization mixture. Denaturation was performed at 80°C (10 min), followed by hybridization overnight at 37°C and successive rinsing in 60% formamide, 2 x SSC containing 0.05% Tween-20 (3 x 5 min; Merck) and 0.05 % Tween-20 in phosphate buffered saline (PBS; 2 x 5 min). Immunocytochemistry was performed as previously described[19,30], with mouse anti-biotin (Dakopatts, Glostrup, Denmark), rabbit anti-mouse peroxidase (Dakopatts) and 3,3'-diaminobenzidine (DAB; Sigma), 0.05 % H_2O_2 in PBS containing 0.1 M imidazole to visualize peroxidase activity. The slides were stained with Mayer's haematoxylin and mounted in Permount (Fisher Scientific, New Jersey, USA).

Table I. Results of histologic classification and *in situ* hybridization using specific probes for chromosomes 1, X and Y of hydatidiform moles and abortions.

Case No.	Original histologic diagnosis*	Chromosome copy number in the major fraction of villous stromal and villous lining cells§			Revised diagnosis
		1	X	Y	
1	CM	2	2	0	CM
2	CM	2	2	0	CM
3	CM	2	2	0	CM
4	PM	2	2	0	CM
5	PM	2	1	1	CM
6	HA	3	3	0	HA
7	HA	3	3	0	HA

* CM = complete hydatidiform mole. PM = partial hydatidiform mole. HA = hydropic abortion.

§ Areas of trophoblast proliferations not included. The nuclei of decidual or inflammatory cells from the mother displayed in all cases two signals for chromosomes 1 and X, and none for chromosome Y.

Evaluation of **in situ** *hybridization signals*

Criteria for the evaluation of ISH signals were as follows[24]: a) overlapping interphase nuclei were not counted; b) signals within one nucleus should have more or less the same size and intensity; c) paired ISH spots (split spots) were counted as one signal; d) non-specific signals such as minor binding sites were not counted. Using these criteria, the interobserver variability in the interpretation of ISH signals was less than 10 percent.

In all specimens, maternal tissue (decidua or inflammatory cells) served as internal control of normal diploid XX cells (Fig. 2). Paraffin sections of a transitional cell carcinoma of a male patient and cell suspensions of male lymphocytes were used as Y-chromosome controls.

RESULTS

Evaluation of the ISH method

A strong ISH signal was obtained in all cases with preservation of acceptable nuclear, cytoplasmic and tissue morphology. In general, signals for chromosomes 1 and Y were more intense than signals for chromosome X (Figs. 1 and 2), probably because the alphoid DNA probe for chromosome X recognizes a less highly repetitive DNA sequence than the satellite III DNA probes for chromosomes 1 and Y. Within the same specimen stromal cells and cytotrophoblast cells appeared to be less resistent to pepsin digestion than syncytiotrophoblast cells, which possess smaller nuclei and a more compact chromatin. However, these differences did not influence the analysis of ISH signals. Some specimens had to be hybridized repeatedly under varying conditions because of slight variability in the optimal pepsin digestion time for paraffin embedded tissue in different cases. This proteolytic digestion step is necessary to remove cytoplasmic and nuclear proteins in order to improve penetration of the specific DNA probes as well as antibodies used in the detection steps[22,24]. In all samples, cells with no or only one signal were seen due to nuclear truncation, inherent to the fact that 6 μm thick sections were used.

Applications of ISH on hydatidiform moles and hydropica abortions

The original histologic diagnosis of the seven lesions is shown in Table I. Cases 4, 5, 6 and 7 had morphologic features suggestive of triploidy.

In all cases histologically classified as CM, a constant pattern of two copies per nucleus was found for chromosomes 1 and X in trophoblast cells lining the villi as well as in villous stromal cells (Figs. 1a, and 1b). Chromosome Y was not found.

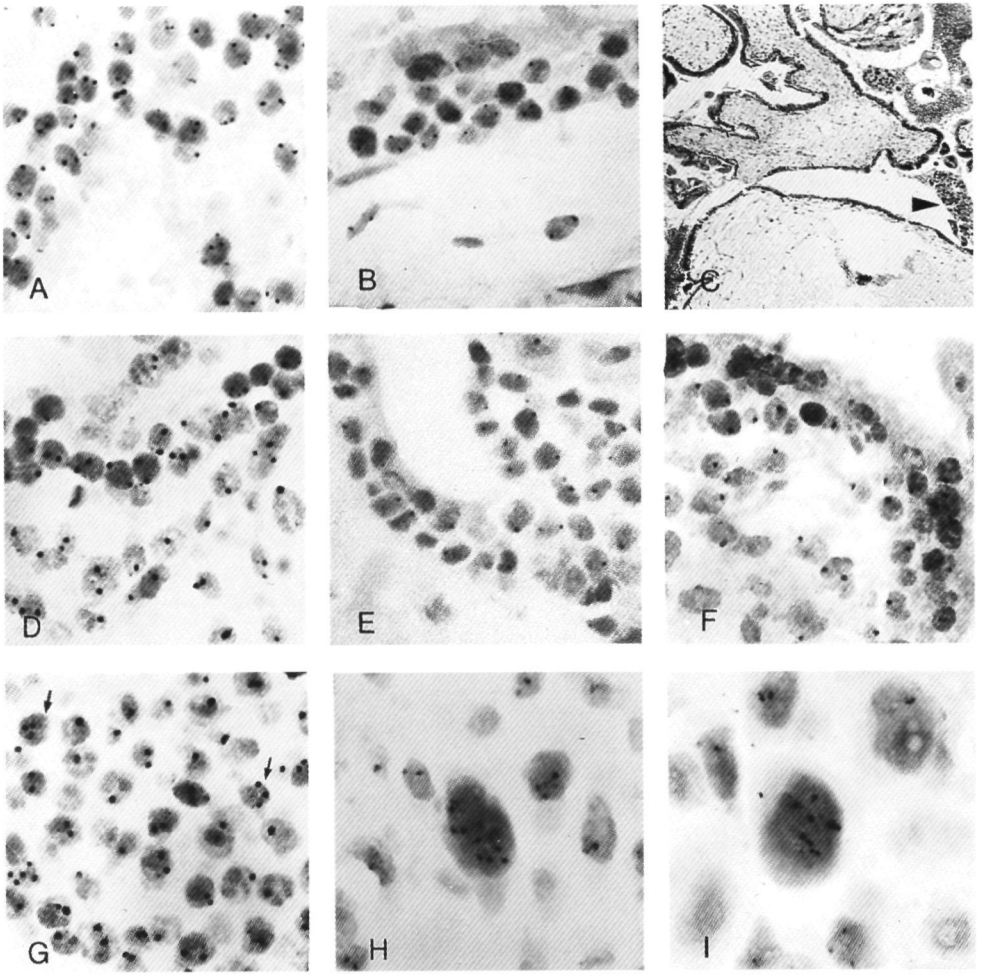

Figure 1. *In situ hybridization results of hydatidiform moles, using biotin labelled probes for chromosomes 1, X and Y, on 6 μm paraffin sections, counterstained with Mayer's Haematoxylin. In the villi of a complete mole (CM, case 3) the majority of nuclei showed two copies of chromosomes 1 (a) and X (b) and none of chromosome Y (not shown). The haematoxylin-eosin stained section (c) of case 5, originally classified as partial mole (PM), showed hydropically swollen villi adjacent to villi of normal size with irregular outlines, and focal atypical trophoblast proliferations (arrow-head). In the villi, two copies of chromosome 1 (d) and one copy of chromosomes X (e) and Y (f) were found. In areas of cytotrophoblast proliferation, nuclei with three or more copies of chromosome 1 were present (g, arrows). In another case of CM (case 1), multiple copies of chromosomes 1 (h) and X (i) were found in the proliferating cytotrophoblast. (Due to truncation of nuclei or limitations in focussing, not all nuclei display the maximum chromosome copy number.)*

In case 4, originally diagnosed as PM with features suggestive of triploidy, two copies of chromosomes 1 and X and none of chromosome Y were found in the main fraction of the cells. In case 5, also originally diagnosed as PM, two copies of chromosome 1 and one copy of chromosomes X and Y were found in the majority of nuclei (Figs. 1d, 1e, and 1f). These data are consistent with a diploid DNA content rather than with triploidy. On re-examination, both cases showed normal villi adjacent to hydropically swollen villi, many of them containing cisterns (Fig. 1c). Trophoblast proliferation with nuclear atypia was focally observed. Irregular villous contours with deep invaginations and trophoblast inclusion cysts, suggestive of triploidy, were present in these cases, but the inclusion cysts could also be explained by collapse and inversion of hydatidiform villi. Embryonic tissue, amnion, and nucleated red blood cells were not found, despite extensive tissue sampling. In case 4, 7 weeks after curettage persistent trophoblastic disease developed without metastases. The β-hCG level was 1300 ng/ml. The patient was treated with methotrexate and is in remission 2 years later. In case 5, signs of persistent trophoblastic disease were not detected after 13 months follow-up.

In some cases, cells with high chromosome copy numbers were observed in sheets of cytotrophoblast proliferation (Figs. 1g, 1h, and 1i). In case 1, these polyploid or aneuploid cytotrophoblast cells showed up to twelve copies of chromosomes 1 or X. ISH signals in these areas, especially in some large nuclei, had a somewhat blurred appearance, while in other areas they were well localized.

In both cases of HA morphologically suggestive of triploidy, no copies of chromosome Y were found. The trophoblast and villous stromal cells showed three copies of chromosomes 1 and X (Figs. 2a, 2b, and 2c). Decidual cells displayed two copies of chromosomes 1 and X (Figs. 2a, and 2b).

DISCUSSION

Karyotyping of hydatidiform moles has established the diploid karyotype of CM (46,XX or 46,XY) and the triploid karyotype of PM (69,XXX or 69,XXY or 69,XYY)[1,3-5]. Also rare cases of diploid PM have been described[1,4,31] as well as CM with trisomy 2[32]. Moreover, recently one case of tetraploid CM and five cases of tetraploid PM were reported[33-35] on the basis of karyotyping studies.

Recent investigations[19-22,36] have demonstrated that probes to repetitive DNA sequences located predominantly in the (peri)centromeric region of a particular chromosome displayed their targets as distinct spots in interphase nuclei when hybridized and detected under standardized conditions. In this way, numerical chromosome aberrations could be

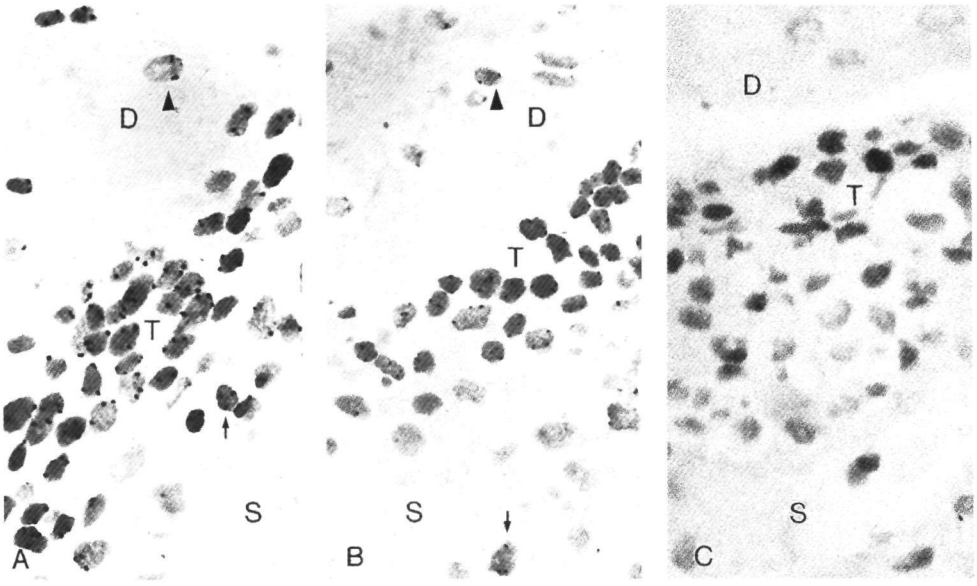

Figure 2. *In situ hybridization results of a hydropically degenerated abortion with histological features suggestive of triploidy (HA; case 6) using biotin labelled probes for chromosomes 1, X and Y on 6μm thick paraffin sections, counterstained with Mayer's Haematoxylin. Three copies of chromosomes 1 (a) and X (b) were found in villous stromal cells (S, arrow) and trophoblast cells lining the villi (T), while in decidual cells (D, arrow-head) two copies were present for both chromosomes. No signals were found for chromosome Y (c).*

detected by ISH in cell suspensions of solid tumours[22]. Recently we developed a protocol to perform ISH on paraffin-embedded tissue sections from bladder cancer[24]. A good correlation was found between chromosome copy numbers in paraffin sections and in cell suspensions of the same tumour, although truncation of nuclei caused some underestimation of the copy number in paraffin sections. In this paper we have presented data on the application of ISH on paraffin-embedded tissue sections of hydatidiform moles and abortions for the assesment of chromosome ploidy and the determination of sex chromosome constitution. Our results show three cases of CM which appeared to have two copies of chromosomes 1 and X, consistent with chromosome diploidy. In two cases of HA histologically suggestive of triploidy, three copies of chromosomes 1 and X were found, confirming chromosome triploidy.

In two cases initially classified as PM, determination of the copy numbers for chromosomes 1, X and Y excluded chromosome triploidy. In one case of PM, one copy of chromosomes X and Y was found. Although histologically PM was suggested in these cases, in retrospect a diagnosis of CM should have been considered because embryonal tissues were lacking. The incidence of diploid PM reported in the literature varies[1,4,31,37]. In series with relatively high percentages of diploid PM, the presence or absence of embryonal tissues is not always mentioned[37]. Criteria considered to be indicative of PM, such as partial hydatidiform change and histologic features suggestive of triploidy, such as irregular villous contours and trophoblast inclusion cysts, can be misleading, as illustrated by cases 4 and 5. Cases which histologically are suggestive of PM but lack evidence of an embryo and which are diploid, on the basis of cytogenetic and / or flow cytometric analyses, most likely represent true CM. At the other end of the spectrum, where hydropic villous changes with evidence of an embryo are combined with triploidy, but show no obvious proliferation and atypia of the trophoblast, overdiagnosis should be avoided. Many of these cases represent hydropically degenerated abortions. In a recent study[10], the diagnosis of PM was confirmed in only 5 out of 11 cases following histological review and measurement of DNA ploidy. In four cases the diagnosis was revised to CM and in two cases there was no evidence of molar pregnancy. This illustrates the value of the combined use of light microscopy and ploidy analysis in the differential diagnosis of CM, PM and HA.

Interphase cytogenetics of tissue sections has the important advantage over karyotyping and flow cytometry (FCM) that the histological context is preserved and can be used in the evaluation. The necessity of bringing cells into culture for karyotyping increases the chance of selection of certain cell populations or cell types (e.g., villous stromal cells)[16,38], and loss of others, while also relatively few metaphases can be studied. The isolation of nuclei required for FCM causes loss of precise control over the cell types examined. The number of admixed maternal cells can influence the measured DNA-index considerably and obscure the profile of the trophoblast cells, thus limiting the sensitivity for the detection of subpopulations of cells with heterogeneous DNA content. Although with ISH the copy number of all chromosomes cannot be determined at the same time, the use of one or two chromosome-specific probes offers the opportunity to estimate chromosome ploidy with accuracy, since exact discrimination between maternal and trophoblast cells is possible and usually a large number of nuclei can be evaluated. In addition, no selection of cell subpopulations takes place. On the contrary, this method revealed the presence of cell subpopulations with high chromosome copy numbers in areas of cytotrophoblast proliferation. Sarto *et al.*[39,40], in studies on nuclear morphology with Feulgen squash techniques as well as autoradiography demonstrated the existence of

polyploidy and endoreduplication in human trophoblast and hydatidiform moles. The role of these cell subpopulations in the pathophysiology of trophoblastic disease is intriguing.

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CHAPTER 3

DNA CYTOMETRIC AND INTERPHASE CYTOGENETIC ANALYSES OF PARAFFIN-EMBEDDED HYDATIDIFORM MOLES AND HYDROPIC ABORTIONS

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DNA CYTOMETRIC AND INTERPHASE CYTOGENETIC ANALYSES OF PARAFFIN-EMBEDDED HYDATIDIFORM MOLES AND HYDROPIC ABORTIONS

SUMMARY

The combined application of DNA cytometric and interphase cytogenetic analyses was used to find objective criteria for the differential diagnosis of complete hydatidiform mole, partial hydatidiform mole and hydropic abortion. DNA-ploidy and G_0/G_1 exceeding rates were determined using image and flow cytometric analyses on paraffin-embedded tissues of 166 cases: 71 cases of complete mole, 20 cases of partial mole, and 75 cases of abortions. To determine the existence and histological distribution of cell subpopulations with numerical chromosome aberrations, interphase cytogenetic analysis using probes specific for chromosomes 1, X and Y was applied to paraffin tissue sections of 23 cases: 12 cases of complete mole, 3 cases of partial mole, and 8 cases of abortions.

In contrast to previously reported findings that complete moles are diploid, the results of this study showed that complete moles are DNA-polyploid (96%), with high G_0/G_1 exceeding rates and a high frequency of numerical chromosomal aberrations in the trophoblast hyperplasia. The majority of partial moles were DNA-triploid (55%). This study, however, also showed the presence of DNA-polyploid partial moles (30%). Abortions were DNA-diploid (60%) or DNA-triploid (39%).

DNA cytometric analysis, especially image DNA cytometric analysis with determination of the G_0/G_1 exceeding rate, and interphase cytogenetic analysis provide objective measurements which are contributory in the differential diagnosis between complete mole, partial mole and hydropic abortion.

Key words: flow cytometry, image cytometry, *in situ* hybridization, placenta, DNA ploidy

INTRODUCTION

The differential diagnosis between complete hydatidiform mole, partial hydatidiform mole and hydropic abortion can be difficult. This is because most histopathological criteria are based on gradual differences in hydropic degeneration, the size and shape of villi, and the degree of trophoblast hyperplasia. These criteria are subject to inter- and intra-observer variability[1]. Persistent gestational trophoblastic disease requiring chemotherapy does not develop after hydropic abortion, but can occur following complete moles in 10%[2,3] and partial moles in 0.5 - 6.6%[3,4].

Karyotyping studies[5-8] have indicated that complete moles are diploid and partial moles usually are triploid. Hydropic abortions, however, can be also diploid or triploid[8,9]. Furthermore, the technique of karyotyping is tedious and cannot be applied for retrospective studies using paraffin-embedded tissues. Flow cytometric analysis (FCM), on the other hand, allows a rapid determination of DNA-ploidy of gestational products, including cases embedded in paraffin[9-12]. These FCM studies also have indicated that complete moles usually have a DNA-diploid pattern, although Hemming *et al.*[11] have found a nuclear fraction with a high DNA content. Interphase cytogenetic analysis using chromosome-specific probes allows the detection of numerical chromosome aberrations[13-15] and therefore can be used to analyse cell subpopulations with a high DNA content. In a preliminary study using interphase cytogenetics, numerical chromosome aberrations were detected in complete moles[15].

The subject of the present study is the combined application of DNA cytometric techniques (flow and image cytometry) and interphase cytogenetic techniques in complete moles, partial moles and hydropic abortions, using paraffin-embedded tissues. The aim was to find objective criteria to discriminate between these three entities. Therefore we performed 1) an analysis of the DNA-ploidy patterns and 2) an analysis of the cell subpopulations with high a DNA content and their histological distribution.

MATERIAL AND METHODS

Cases studied

Paraffin-embedded gestational products of 166 patients were studied. All specimens were reviewed by at least two pathologists. The cases were classified as complete hydatidiform mole or partial hydatidiform mole according to previously published criteria[6,7,15], and abortions without or with hydropic degeneration. (Hydropic) abortions lacked trophoblast hyperplasia characteristic of hydatidiform moles. Scalloped villous outlines with

invaginations and the presence of trophoblast inclusions were considered to be histological features suggestive of "triploidy" in both hydropic abortions and partial moles[8].
Image cytometric analysis (ICM) was performed in all 166 cases; FCM in 155 cases. In 23 representative cases, interphase cytogenetic analysis was performed, using probes specific for chromosomes 1, X and Y to assess chromosome-ploidy and to establish sex chromosome composition.

Cytometric analysis

The specimen preparation procedure has been described elsewhere[16]. In short, 50 μm thick paraffin tissue sections were cut. As a method for orientation and control of cell and tissue composition hematoxylin & eosin stained 5 μm tissue sections were cut in parallel with the thick section. Placental and maternal decidual tissues were separated from each other and processed separately. The maternal decidual tissue or, if not available, lymphocytes served as internal controls. After deparaffinization and rehydration, the 50 μm thick tissue sections were put in a centrifuge tube and incubated in phosphate-buffered saline (PBS; 137 mM NaCl, 13 mM $Na_2HPO_4.2H_2O$, 3 mM KH_2PO_4, pH = 7.4), with 0.1 % protease (type VII from Bacillus amylolique faciens; Sigma Chemical CO, St.Louis, USA) at 37°C, for 60 min. Incubation was terminated by adding 4 to 5 ml cold (4°C) PBS, and putting the tubes on ice. The nuclei were washed twice with PBS using intermediate centrifugation steps. About 30,000 nuclei were counted with a Coulter Counter Model ZB1 (Coulter Electronics, Dunstable, UK), centrifuged and 200 μl fetal calf serum (Gibco, Paisly, UK) was added. For ICM this nuclear suspension was centrifuged to a glass slide using a cytocentrifuge (10 min at 500 rpm; Shandon, Zeist, The Netherlands), briefly air-dried, and fixed in a mixture of methanol, 37% formaldehyde, and acetic acid (85:10:5 by volume) for 1 h. The remainder of the suspension was centrifuged, fixed in cold (-20°C) 70 % ethanol, and kept at -20°C until use.

Image cytometric analysis

After sedimentation onto a glass slide, specimens were stained with Feulgen-pararosaniline[17]. The DNA content of 200 stained intact nuclei was measured using the CAS 100 System (Cell Analysis Systems, Lombard, Illinois, USA)[18]. Nuclei were measured with a 40x objective and monochromatic light with a wavelength of 564 nm. (Pixel size was 0.50 x 0.39 μm^2). At least 30 rat liver cells (DNA-tetraploid) were measured as an external control for DNA content, while at least 20 decidual cells derived from maternal tissue or, when not available, 20 lymphocytes were used as internal controls. All measurements were performed by the same experienced cytotechnician.

Flow cytometric analysis

The nuclei in the remainder of the suspension were stained for DNA using propidium iodide[19] and measured in a cytofluorograph 50 H flow cytometer (Ortho-Instruments, Westwood, USA). Placental and maternal decidual nuclear suspensions were run separately.

Histogram interpretation

For ICM, the DNA content of nuclei was expressed as DNA index (DI), defined as the nuclear integrated optical density of a nucleus divided by the median integrated optical density of the control sample (nuclei from decidual cells or lymphocytes)[20]. Histograms were visually classified as DNA-diploid, DNA-polyploid or DNA-aneuploid, according to the following definitions. In a *DNA-diploid* pattern, a distinct G_0/G_1 peak was found in the diploid (2C; DI = 1.0 $\pm$ 0.1) region with a small proportion of cells in S and G_2/M (4C) phases. In a *DNA-polyploid* pattern, distinct peaks were present in the diploid (2C; DI = 1.0 $\pm$ 0.1) and tetraploid (4C; DI = 2.0 $\pm$ 0.2) regions, or in the diploid, tetraploid, and octaploid (8C; DI = 4.0 $\pm$ 0.4) regions. DNA-ploidy patterns were considered *DNA-aneuploid* in all cases with scattered DI distributions, or uni-, bi-, or multimodal DI distributions, that were non-diploid or non-polyploid. In this group, a separate entity was defined as a *DNA-triploid* pattern, characterized by a distinct G_0/G_1 peak in the triploid (3C; DI = 1.5 $\pm$ 0.15) region, and a small proportion of cells in S and G_2/M (6C; DI = 3.0 $\pm$ 0.3) phases.

For ICM, the nuclear fraction with a DNA content exceeding the G_0/G_1 peak (the *G_0/G_1 exceeding rate*) was calculated for gestational products with a DNA-diploid or DNA-polyploid pattern using the 2.5c exceeding rate (DI > 1.25), and for gestational products with a DNA-triploid pattern using the 3.5c exceeding rate (DI > 1.75). The mutual differences in G_0/G_1 exceeding rates between two groups were studied with a Student-*t*-test. The given *P* values are the two-tailed *P* values obtained from Student-*t*-test.

In the DNA histogram obtained with FCM, the first peak was interpreted as the diploid peak (G_0/G_1). Histograms were then visually classified as DNA-diploid, DNA-polyploid, DNA-aneuploid, or DNA-triploid. Maternal decidual tissue was run separately and the resulting histograms served as internal control. In addition, samples exhibiting DNA-triploidy invariably showed some admixture of maternal diploid cells (e.g., leucocytes) which also served as an internal control.

Interphase cytogenetic analysis

DNA-probes and non-radioactive labeling

The satellite III DNA probe for chromosome 1 (pUC 1.77) recognizes a tandem repeat of

1.77 kb in the (peri)centromeric region (1q12) of chromosome 1[21]. The alphoid DNA probe for chromosome X (pBam X5) and the satellite III DNA probe for chromosome Y (DYZ3) recognize tandem repeats in the centromeric region of chromosome X and in the q arm of chromosome Y, respectively[22,23]. Biotinylation of the probes was performed using Bio-11-dUTP (Sigma, St.Louis, USA) in a nick translation reaction[24].

In situ *hybridization*

The paraffin tissue blocks which were used for cytometric analyses were also taken for interphase cytogenetic analyses. The *in situ* hybridization procedure (ISH) in paraffin-embedded tissue sections has been described elsewhere[15,25].

Evaluation of ISH signals

In each specimen, at least 500 nuclei were evaluated for the number of chromosome-specific ISH signals. Criteria for the evaluation of ISH signals ("spots") were as follows:[15,25] a) overlapping interphase nuclei were not counted; b) signals within one nucleus should have more or less the same size and intensity; c) paired ISH spots (split spots) were counted as one signal; and d) aspecific signals, such as minor binding sites, were not counted. In all samples, cells with no or only one signal were seen due to nuclear truncation, inherent to the fact that 6 μm thin tissue sections were used. In all specimens maternal tissues (decidua or lymphocytes) were present which served as internal controls of normal diploid XX cells. As Y-chromosome controls, paraffin sections of a placenta and of a cell suspension of lymphocytes, known to contain a copy of this chromosome, were used.

RESULTS

Light microscopic classification

The 166 gestational products were classified histopathologically as shown in Table I.

Cytometric analysis

DNA histograms obtained with both ICM and FCM were both taken into consideration in the interpretation to obtain the final results, summarized in Table I. The results of ICM and FCM were grossly similar in the vast majority of cases. Similar results were found for all DNA-diploid and DNA-triploid cases. In the DNA-polyploid cases, the second peak at DI=2 was in 40% of cases much more obvious in the ICM histograms than in the FCM histograms, the latter being difficult to interpret as DNA-polyploid in 12% of cases.

Table I. DNA-ploidy in hydatidiform moles and abortions using image cytometric and flow cytometric analyses.

Histological Diagnosis:	Total	DNA-ploidy pattern:			
		Diploid cases	Triploid cases	Polyploid cases	Aneuploid cases
	N	n *(%)*	n *(%)*	n *(%)*	n *(%)*
Abortions	**75**	**44** *(59)*	**29** *(39)*	**1** *(1)*	**1** *(1)*
Without Hydropic Degeneration	26	22 *(85)*	3 *(11)*	1 *(4)*[1]	-
With Hydropic Degeneration	14	11 *(79)*	3 *(21)*	-	-
With Hydropic Degeneration *and* Histological Features suggestive of "Triploidy"	35	11 *(31)*	23 *(66)*	-	1 *(3)*[2]
Partial Hydatidiform Moles	**20**	**2** *(10)*	**11** *(55)*	**6** *(30)*	**1** *(5)*[3]
Complete Hydatidiform Moles	**71**	**1** *(1)*	**1** *(1)*[4]	**68** *(96)*	**1** *(1)*[3]
Total	**166**	**47**	**41**	**75**	**3**

1 tubal pregnancy with prominent implantation trophoblast (see also table III)
2 uni-modal DNA-aneuploid, DI = 1.25
3 ICM multimodal DNA-aneuploid, FCM DNA-polyploid
4 no fetal parts, fals-positive diagnosis of complete mole instead of partial mole

Table II. G_0/G_1 exceeding rates in hydatidiform moles and abortions as determined by image cytometric analysis.

Histological Diagnosis:	G_0/G_1 exceeding rate:					
	Diploid and polyploid cases			Triploid cases		
	cases	mean	SD	cases	mean	SD
	n	%		n	%	
Abortions	**45**	**19**	**11**	**29**	**12**	**9**
Without Hydropic Degeneration	23	18	12	3	5	4
With Hydropic Degeneration	11	22	7	3	20	7
With Hydropic Degeneration *and* Histological Features suggestive of "Triploidy"	11	15	11	23	12	9
Partial Hydatidiform Moles	**8**	**43**	**15**	**11**	**22**	**9**
Complete Hydatidiform Moles	**69**	**61**	**11**			

Small DNA-peaks near DI=4 were found in 56% of the ICM histograms and were obvious in only two cases of the FCM histograms. In cases of discrepancy, the results of ICM were used.

The G_0/G_1 exceeding rates as determined from the ICM DNA histograms are given in Table II and in Figure 1.

Abortions

In 44 out of all 75 cases of abortions, a DNA-diploid pattern was found (Fig.2A; G_0/G_1 exceeding rate 19 $\pm$ 11%). The only case that showed a DNA-polyploid pattern (G_0/G_1 exceeding rate 54%; Fig.1) was a tubal pregnancy with abundant implantation trophoblast but without hydropic degeneration.

A minority of the cases without histological features suggestive of "triploidy" showed a DNA-triploid pattern, while in 31% of the cases *with* histological features suggestive of "triploidy" a DNA-diploid pattern was found, indicating that the light microscopical features cannot be fully relied upon for the diagnosis of triploidy.

Partial Hydatidiform Moles

Of the 20 cases of partial mole, a DNA-triploid pattern was found in 11 cases (55%; Fig.2E). The G_0/G_1 exceeding rates of the DNA-triploid partial moles were higher than those of DNA-triploid abortions ($P=0.004$), but due to a great overlap the G_0/G_1 exceeding rate could not be used to discriminate between DNA-triploid partial moles and DNA-triploid hydropic abortions (Fig.1). Nuclear atypia was minimal in DNA-triploid partial moles. In the 6 cases of partial mole with a DNA-polyploid pattern, however, G_0/G_1 exceeding rates were high (40 - 60%) and nuclear atypia prominent, both features within the range of that of complete moles (see Fig.1 and below).

Embryonal tissues or amnion were not found in two cases of DNA-triploid partial moles and in two cases of DNA-polyploid partial moles. In the latter two cases, in retrospect, a complete mole could not have been excluded because of the absence of embryonal tissues.

Complete Hydatidiform Moles

The majority of complete moles, 68 out of 71 cases (96%), showed a DNA-polyploid pattern (Fig.2I; G_0/G_1 exceeding rate 61 $\pm$ 11%). There was one case with a DNA-diploid pattern (G_0/G_1 exceeding rate 28%; Fig.1). In this case, the amount of trophoblast hyperplasia in the paraffin tissue block available for cytometric analysis was small. In general, the G_0/G_1 exceeding rates were significantly higher in complete moles than in abortions ($p<0.00001$; Fig.1). A G_0/G_1 exceeding rate $\geq$ 37% could be used as a threshold to determine DNA-polyploidy.

In one case, originally diagnosed as complete mole, a DNA-triploid pattern was found. Because of the absence of embryonal tissues or amnion, it is most likely that this case was erroneously classified as complete mole instead of partial mole.

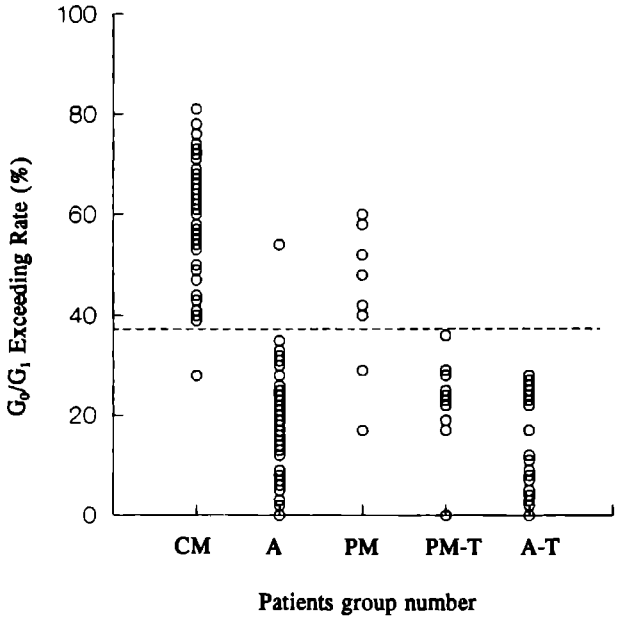

Figure 1. *Graphical distribution of the G_0/G_1 exceeding rates, determined by image cytometric analysis, in hydatidiform moles and abortions. (CM = DNA-diploid/polyploid complete moles; A = DNA-diploid/polyploid abortions; PM = DNA-diploid/polyploid partial moles; PM-T = DNA-triploid partial moles; A-T = DNA-triploid abortions).*

Interphase cytogenetic analysis

ISH using chromosomes 1, X and Y-specific probes gave good interpretable results in all 23 cases studied (Table III).

Abortions

In the cases of abortions, a chromosome-disomic pattern (consistent with a diploidy) or a chromosome-trisomic pattern (consistent with a triploidy) was found in all cell types, i.e., villous trophoblast and villous stromal cells (Fig.2B), as well as implantation trophoblast (Fig. 2C). Only few extravillous cytotrophoblast cells (less than 10%) showed respectively more than two or three copies of one chromosome (Table III). Nuclear atypia was absent in these areas (Fig.2D).

Partial Hydatidiform Moles

In 2 of the 3 cases of partial mole, a chromosome-trisomic pattern was present in both villous and extravillous hyperplastic trophoblast (Fig.2F and G), the latter showing only mild nuclear atypia (Fig.2H). In one of the 3 cases of partial mole, the villi showed a chromosome-disomic pattern but the extravillous hyperplastic trophoblast cells showed a relatively high fraction (28%) of nuclei with multiple chromosome copies and a prominent nuclear atypia. Embryonal tissues (nucleated red blood cells) and an amnion were present. Cytometric analysis showed DNA-polyploidy in this case.

Complete Hydatidiform Moles

In the 12 cases of complete mole, a chromosome-disomic pattern was seen in the villous trophoblast and in the villous stromal cells (Fig.2J). Only sporadically was a nucleus with more than two copies of a chromosome found in these areas. The extravillous hyperplastic trophoblast, however, especially the intermediate and cytotrophoblast, showed a high fraction (16 - 41%) of nuclei with multiple copies for the chromosomes tested (1, X or Y), with copy numbers often in the range of 3 - 4, but not infrequently up to 10 or 12 (Fig.2K). No differences were found between the chromosomes tested (1, X or Y). In these extravillous trophoblast cells, prominent nuclear atypia was present with lobulation and hyperchromasia of the nuclei (Fig.2L).

Figure 2. *DNA histograms obtained by image cytometric analysis of nuclei prepared from paraffin-embedded tissues and stained with Feulgen-pararosanilin (A, E, and I), and results of in situ hybridization (ISH) using biotin-labelled probes for chromosome 1 on 6 μm paraffin sections counterstained with haematoxylin (B, C, F, G, J and K) of cases of abortion (A-D), partial mole (E-H), and complete mole (I-L). DNA histograms: (A) DNA-diploid abortion and (E) DNA-triploid partial mole with small peaks at DI=2 and DI=3 respectively, representing G_2/M phase; (I) DNA-polyploid complete mole with prominent peaks at DI=1 and 2 and few nuclei at DI=4. ISH results: In the case of abortion, two copies of chromosome 1 were found in the main fraction of nuclei in villous trophoblast (T) and stromal cells (arrow; B), as well as in the implantation trophoblast (C) in which nuclear atypia was absent (D). In the case of partial mole, three copies of chromosome 1 were found in the villous trophoblast and stromal cells (F), as well as in the trophoblast hyperplasia (G) in which nuclear atypia was minimal (H). In the case of complete mole, the villi showed a chromosome-disomic pattern (J), but in the trophoblast hyperplasia, frequently up to four copies (arrow) or less frequently multiple copies (double arrow) of chromosome 1 were found (K). In these areas, nuclear atypia was prominent (L). (Not all nuclei display the maximum chromosome copy number due to truncation of nuclei and limitations in focussing at microphotography).*

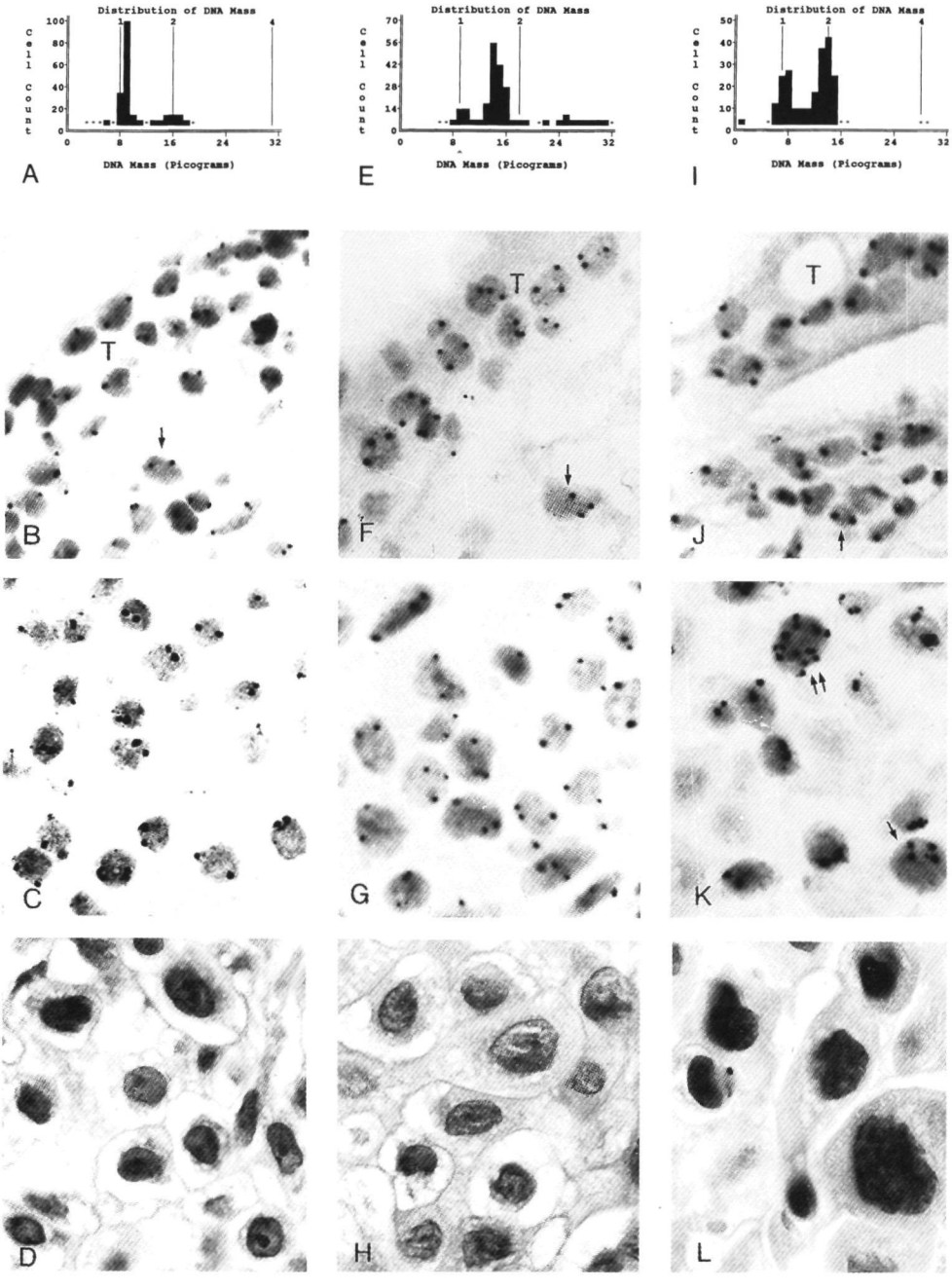
Distribution of DNA Mass
Cell Count
DNA Mass (Picograms)
A
Distribution of DNA Mass
Cell Count
DNA Mass (Picograms)
E
Distribution of DNA Mass
Cell Count
DNA Mass (Picograms)
I
T
B
T
F
T
J
C
G
K
D
H
L

Table III. Chromosome-ploidy in hydatidiform moles and abortions using interphase cytogenetic analysis with probes specific for chromosomes 1, X and Y.

Histological Diagnosis:	Interphase Cytogenetic Analysis: *Chromosome Copy Number per Nucleus in*			Cytometric Analysis: *DNA-ploidy*	
	Chorion villi: 1 X/Y	Extravillous trophoblast[1]: >2 copies (%)	>3 copies (%)	Pattern of Ploidy	G_0/G_1 Exceeding Rates (%)
Abortions					
Without Hydropic Degeneration	disomy, XX	0.4		diploid	25
(N=5)	disomy, XX	5		diploid	27
	disomy, XX	1		diploid	34
	disomy, XY	6		diploid	8
	disomy, XY	5		polyploid	54[2]
With Hydropic Degeneration *and*	disomy, XY	-[3]		diploid	5
Histological Features suggestive	trisomy, XXX		-[3]	triploid	9
of "Triploidy"	trisomy, XXX		3	triploid	2
(N=3)					
Partial Hydatidiform Moles	trisomy, XXX		3	triploid	19
(N=3)	trisomy, XXX		2	triploid	22
	disomy, XX	28		polyploid	49
Complete Hydatidiform Moles	disomy, XX	28		polyploid	56
(N=12)	disomy, XX	26		polyploid	61
	disomy, XX	26		polyploid	63
	disomy, XX	19		polyploid	60
	disomy, XX	37		polyploid	64
	disomy, XX	20		polyploid	73
	disomy, XX	41		aneuploid	73
	disomy, XX	24		polyploid	43
	disomy, XX	16		polyploid	81
	disomy, XX	27		polyploid	50
	disomy, XY	33		polyploid	69
	disomy, XY	32		polyploid	64

[1]: implantation trophoblast or trophoblast hyperplasia
[2]: tubal pregnancy with prominent implantation trophoblast.
[3]: small amount of implantation trophoblast (less than 500 nuclei)

In this study, we applied DNA cytometric analyses and interphase cytogenetic analysis to find objective criteria that contribute to the differential diagnosis of complete mole, partial mole and abortion with hydropic degeneration.

Virtually all cases of *complete mole* were DNA-polyploid using DNA cytometric analysis. To analyse the histological distribution of cell subpopulations with a high DNA content, we applied interphase cytogenetic analysis to paraffin sections of complete moles. This technique allows the analysis of the different cell types of trophoblast, e.g., villous stromal cells, villous trophoblast, and extravillous trophoblast[15]. In cases of complete mole, the villous trophoblast cells as well as the villous stromal cells usually displayed a normal chromosome-disomic pattern. In the extravillous trophoblast hyperplasia, characteristic for complete moles, however, the cells displayed multiple copies of the chromosomes tested. This cannot be explained by duplication of ISH signals for individual chromosomes in the S and G_2/M-phases of the cell cycle as was shown in a previous study[26]. Therefore, these findings imply that in complete moles cell subpopulations with numerical chromosome aberrations exist. This links up with the DNA-polyploid pattern found with DNA cytometric techniques. It might be suggested that the DNA-diploid cell population is mainly derived from the villous components of complete moles, while the DNA-tetraploid and DNA-octaploid fractions are derived from extravillous trophoblast hyperplasia. It is also in these trophoblast hyperplasias that prominent nuclear pleomorphism with lobulation and hyperchromasia is found. A cell biological explanation of our findings could be the process of endoreplication[27-29]. This process of endoreplication normally also occurs in the extravillous trophoblast at the implantation site of normal first-trimester pregnancies[27,29]. In the present study also, using interphase cytogenetic analysis, in the abortions few cells (less than 10%) showed more than two copies of a chromosome, consistent with a low percentage of polyploid cells. In hydatidiform moles, this frequency of polyploidization appears to be significantly increased.

Our results are in contrast with previous karyotyping and flow cytometry studies of hydatidiform moles, which have indicated that complete moles are diploid[6,7,10,12]. An explanation might be that in the process of karyotyping using metaphase spread preparations, as a result of selection during tissue culture only one cell subpopulation is detected[30], e.g., villous stromal cells. Furthermore, in this study in which both image cytometric analysis (ICM) and flow cytometric analysis (FCM) were used, the DNA-polyploid pattern was more obvious in the ICM-derived DNA histograms as compared to the FCM-derived DNA histograms, which might explain the limited attention given to this phenomenon in the previous FCM studies[10,12]. Using FCM, the percentage of admixed

maternal cells (leukocytes, decidual cells) is unknown and can be much higher as compared with ICM, in which these maternal cells are measured separately. As a result these ICM DNA histograms give more information about the DNA content of the trophoblast cells proper. Our results are in agreement with the FCM studies on hydatidiform moles published by Hemming *et al.*[11], who observed a relatively high tetraploid peak on FCM which they interpreted as a high proliferative fraction. In our study, the combined application of DNA cytometric analysis and interphase cytogenetic analysis showed that the increased DNA content is not only a result of a high G_2/M phase, but can also be explained by the existence of cell subpopulations with a high frequency of numerical chromosome aberrations.

In the cases of *abortions*, a DNA-diploid or DNA-triploid pattern was found. Only one case of "abortion", a tubal pregnancy with abundant implantation trophoblast, showed a bimodal DNA-polyploid pattern. Interphase cytogenetic analysis of this case showed that this DNA-pattern was only due to a high G_2/M phase and not to a pathologically increased percentage of polyploid cells, as is the case in complete moles. In the differentiation between complete moles and hydropic abortions, the G_0/G_1 exceeding rate can be used as an objective value to discriminate between DNA-polyploidy and DNA-diploidy. The G_0/G_1 exceeding rates were significantly higher in the cases of complete mole (61%) when compared with the cases of hydropic abortions (19%; $P<0.00001$). Therefore it is concluded that DNA cytometric analysis and interphase cytogenetic analysis are of great value in the differential diagnosis between complete moles and hydropic abortions.

In the majority of *partial moles*, a DNA-triploid pattern was found. This finding of triploidy was confirmed in the two cases of partial mole analysed with interphase cytogenetics. These findings are in agreement with previous studies[7,8,9-12]. The G_0/G_1 exceeding rates were less than 37% and grossly within the range of those found in DNA-triploid abortions. Therefore, the discrimination between DNA-triploid partial moles and DNA-triploid hydropic abortions can only be made in the absence of trophoblast hyperplasia in the latter, but the difference is one of degree and therefore subjective. Indeed, it is often impossible to differentiate between "atypical" trophoblast hyperplasia in DNA-triploid partial moles and implantation trophoblast in hydropic abortions. Further research is needed to reveal the true nature of DNA-triploid partial moles, particularly for their potential for the development of persistent trophoblastic disease. In this study, cases of partial mole with a DNA-polyploid pattern were also found. Interphase cytogenetic analysis performed in one case showed cytogenetic heterogeneity in the extravillous trophoblast, as was also found for the cases of complete mole. It is doubtful whether these DNA-polyploid partial moles are true partial moles. It is possible that they represent twin pregnancies, with one of the two gestational products being a complete hydatidiform

mole[31]. If this is so, the risk of persistent trophoblastic disease will be correspondingly high.

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CHAPTER 4

COMPLETE HYDATIDIFORM MOLE IN TWIN PREGNANCY : DIFFERENTIATION FROM PARTIAL MOLE WITH INTERPHASE CYTOGENETIC AND DNA CYTOMETRIC ANALYSES ON PARAFFIN EMBEDDED TISSUES.

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COMPLETE HYDATIDIFORM MOLE IN TWIN PREGNANCY : DIFFERENTIATION FROM PARTIAL MOLE WITH INTERPHASE CYTOGENETIC AND DNA CYTOMETRIC ANALYSES IN PARAFFIN EMBEDDED TISSUES.

SUMMARY

Six cases of hydatidiform mole associated with normal chorionic villi and a normal embryo/fetus (in five cases) were investigated with interphase cytogenetic and DNA cytometric analyses for diagnostic purposes. DNA probes specific for the pericentromeric regions of chromosomes 1 and X and for the long arm of chromosome Y were used. In four cases a dizygotic twin pregnancy could be proven. In these cases, the histologically normal chorionic villi showed an XY DNA-diploid pattern, consistent with a normal male conceptus, and the molar chorionic villi a XX pattern. In the other two cases an identical sex chromosomal pattern was found in the normal and in the molar villi (XX/XX and XY/XY respectively). In all six cases the molar placental tissues showed prominent trophoblastic hyperplasia with DNA-polyploidy, consistent with a complete hydatidiform mole. In two cases persistent gestational trophoblastic disease developed.

It is emphasized that twin pregnancies composed of a normal conceptus and a complete mole have a relatively high risk for the development of persistent trophoblastic disease and therefore, should be carefully differentiated from triploid partial moles with a relatively low risk of persistent gestational trophoblastic disease. These case reports indicate that additional interphase cytogenetic and DNA cytometric analyses are useful in this differential diagnosis.

Key words: complete mole, partial mole, interphase cytogenetics, DNA ploidy

INTRODUCTION

Hydatidiform moles consist of two main entities, complete moles and partial moles[1,2]. Complete moles are of diploid androgenetic origin[3] with a high frequency of polyploidization[4] and absence of fetal parts. The characteristic histopathological changes of hydatidiform moles, villous edema and trophoblastic hyperplasia, are uniformly present. In partial moles these changes are focal, affecting only some of the chorionic villi. A fetus is present. They are usually triploid with predominance of paternal genomic contribution (diandry)[5]. Partial moles, however, appear to be a heterogeneous group, since diploid cases also have been reported[6].

Partial moles have to be differentiated from dizygotic twin gestations consisting of a complete mole and a normal conceptus. Based on morphology alone a diagnosis of twin pregnancy with a complete mole is very difficult, especially in cases of early gestation. Karyotyping can be useful for the diagnosis of twins, but fresh tissues are required and it is limited to unlike-sexed twins and the possibility of outgrowth by contaminating maternal cells cannot be ruled out. Analysis of cytogenetic marker polymorphism performed in fresh tissues has been successfully used in the diagnosis of twins with a diploid androgenetic complete mole[7-12]. In the present study we made use of interphase cytogenetic analysis, which is a rapid method that can be performed in paraffin embedded tissue sections and therefore is of great value in routine practice[4,13,14]. Six cases were investigated in which the histopathological features were suggestive of a twin pregnancy with a complete mole. In four cases a diagnosis of dizygotic twin pregnancy with complete mole (XX) and normal male conception (XY) could be proven. DNA cytometric analysis was used for further analysis of the differences in polyploidization in the molar and nonmolar components.

MATERIAL AND METHODS

Case reports

Case 1. A 30-year-old gravida III, para II, presented at 9½ weeks' gestation with recurrent vaginal bleeding. Ultrasonography revealed a vital pregnancy with many blood clots in utero. Continued blood loss necessitated termination of the pregnancy at 13 weeks gestation. Suction curettage harvested a macroscopically normal embryo with crown-rump length of 2.5 cm, and a large amount of membranous placental tissues. The material was routinely processed and a provisional diagnosis of partial hydatidiform mole was made by the local pathologist, who subsequently referred the case to our university hospital where

national mole registration takes place. The ß human chorionic gonadotropin (ß-hCG) titer was not measured at that time, but only three weeks later after the definitive diagnosis was made by the reference pathologists. The ß-hCG level then was 216 ng/l and returned to normal within 5 weeks and remained normal for one year of follow up.

Case 2. This was the first pregnancy of a 25-year-old woman. She had an irregular menstrual cycle. Within one month after her last period she complained of fatigue, nausea and irregular vaginal bleeding, later followed by abdominal pains. Gynaecological examination revealed a painfully enlarged uterus corresponding with a pregnancy duration of 16 weeks. Ultrasonography showed a molar pregnancy. An embryo was not found. Curettage was performed, 8 weeks after her last menstrual period. The local pathologist confirmed the diagnosis of hydatidiform mole. The ß-hCG levels one day and one week after curettage were 5300 and 640 ng/l. respectively. Eighteen days after curettage she had again heavy blood loss and an enlarged uterus. The ß-hCG value was risen to 960 ng/l. A second curettage was performed, yielding ample amount of molar tissues. The ß-hCG levels subsequently returned to normal within 2.5 months and remained normal thereafter (3 years follow up). The histological specimens of both curettages were referred.

Case 3. A 36-year-old woman was treated for primary infertility with ovulation induction therapy. She conceived after clomiphene and hCG therapy. The first pregnancy had resulted in the birth of a healthy girl. A second pregnancy was induced in the same way. During this pregnancy she had some vaginal bleeding and progressive nausea and vomiting. At ultrasonography a hydatidiform mole was looked for but could not be detected. Routine bloodtests revealed impaired liver functions. Pathology of the gallbladder was suspected and at 18 weeks gestational age a cholecystectomy was performed for cholecystolithiasis. Two days later she developed the clinical appearance of partial placental ablatio and came into immature labour. She gave birth to a normal male fetus. A manual removal of the placenta was necessary. In the placenta a sharply demarcated area of hydatidiform molar changes was macroscopically and microscopically present. The local pathologist referred the case under the diagnosis of partial hydatidiform mole. The ß-hCG levels (initially postpartum 35,000 ng/l) normalized within three months and remained normal after seven months of follow up.

Case 4. This case has been published previously[15]. A 31-year-old female was treated for primary infertility. She became pregnant after ovulation induction with human menopausal gonadotrophin and hCG and subsequent gamete intra-Fallopian transfer of four oocytes.

At 4 weeks an intact twin pregnancy was diagnosed. The pregnancy evolved uneventfully until 18 weeks when vaginal bleeding occurred. Ultrasonography revealed cystic changes of part of the placenta, suggestive of hydatidiform mole. Serum ß-hCG was 327 150 IU/l. At 25 weeks intra-uterine infection induced immature labour and two karyotypically normal male (XY) fetus were delivered. The placenta was manually removed and appeared to be bichorionic - biamniotic. Part of it had the aspect of hydatidiform mole, with XX karyotype. The case was referred with the diagnosis of triplet pregnancy with complete mole. Serum ß-hCG levels normalized within 10 weeks.

Case 5. The third pregnancy of a 30-year-old women resulted in the birth of a healthy girl (2900 grams) at 38.5 weeks amenorrhoea. At 12 weeks gestation she had had some vaginal bleeding. At 19 weeks bilateral multilocular ovarian cysts were diagnosed, which showed a spontaneous involution postpartum. During the pregnancy, at ultrasonography, there had been no signs of molar changes of the placenta. After birth, however, the placenta showed a well demarcated area with the aspect of hydatidiform mole. The local pathologist considered partial mole or twin gestation and referred the case to our university hospital. The child lived and did well. With the mother, there were no signs of persistent trophoblastic disease.

Case 6. A 32 year old woman, gravida III, para II, presented with vaginal blood loss and hyperemesis at 11 weeks gestation. Ultrasound revealed an intact intra-uterine pregnancy next to an empty gestational sac with placental changes suspect for a hydatidiform mole. Suction curettage was performed, yielding a fragmented embryo, normal placental tissue and a large molar mass. The referring pathologist made a diagnosis of twin pregnancy with complete mole. After an initial decline of the serum ß-hCG (from 700,000 ng/ml to 2000 ng/ml), a progressive rise to 12,000 ng/ml occurred three weeks after curettage. There were no indications of metastases. Methotrexate therapy was started and four courses have been given until now, upon which the ß-hCG serum levels have shown a steady regression to normal. The patient is still under follow up.

Interphase cytogenetic analysis

Interphase cytogenetic analysis was performed on 6 μm thin paraffin embedded tissue sections. The following chromosome specific DNA probes were used: the satellite III DNA probe for chromosome #1 (pUC 1.77), the alphoid DNA probe for chromosome X (pBam X5) and the satellite III DNA probe for chromosome Y (DYZ3), recognizing tandem repeats in the (peri)centromeric region (1q12) of chromosome #1[16], in the centromeric region of chromosome X[17] and in the q arm of chromosome Y[18], respective-

ly. Biotinylation of the probes was performed using Bio-14-dATP (BRL: Gaithersburg, U-SA) according to the suppliers instructions.

The *in situ* hybridization procedure (ISH) in paraffin embedded tissue sections was performed as previously described[4,13,14], with minor modifications in the immunohistochemistry step: mouse anti-biotin (1:100 in PBS-tween with 5% non fat dry milk (NFDM); Dakopatts, Glostrup, Denmark) was followed by biotin labeled horse anti-mouse (1:200 in PBS-tween, 5% NFDM; Vector, Burlingame, Canada) and avidin-biotin complex (1:100 in PBS-tween, 5%NFDM; Vector).

DNA cytometric analysis

The paraffin tissue blocks which were used for interphase cytogenetic analyses were also taken for DNA image cytometric analyses. Intact nuclei were isolated from 50 μm thick paraffin tissue sections, as preciously described[4]. Normal appearing chorionic villi and hydatidiform degenerated molar villi were separately processed, as well as maternal decidual tissue, which served as internal control for normal diploid cells. The DNA content of 100 - 200 pararosaniline-Feulgen stained intact nuclei of the normal and of the molar chorionic villi was measured using the CAS 100 System (Cell Analysis Systems, Lombard, Illinois, USA)[19]. At least 30 rat liver cells (DNA-tetraploid) were measured as an external control for DNA content, while at least 20 decidual cells were used as internal control. The DNA-histograms were visually classified according to the previously described criteria[4]. A brief summery is given here. A *DNA-diploid* pattern consisted of a distinct G_0/G_1 peak in the diploid (2C; DI = 1.0 $\pm$ 0.1) region with a small proportion of cells in S and G_2/M (4C) phases. A *DNA-polyploid* pattern showed distinct peaks in the diploid (2C; DI = 1.0 $\pm$ 0.1) and tetraploid (4C; DI = 2.0 $\pm$ 0.2) regions, or in the diploid, tetraploid and octaploid (8C; DI = 4.0 $\pm$ 0.4) regions. The nuclear fraction with a DNA content exceeding the first G_0/G_1 peak was calculated for gestational products with a DNA-diploid or DNA-polyploid pattern using the 2.5c Exceeding Rate (DI > 1.25).

RESULTS

Histopathology

In all six cases normal and severely abnormal chorionic villi were present, partly intermingled, especially in the younger pregnancy products which were obtained by suction curettage (cases 1 and 2), partly well demarcated in larger clusters of normal and abnormal, molar placental tissue (Fig.1a). In the older pregnancies already at the macroscopic level there was a clear demarcation between normal part and molar part of

the placenta. Transitional chorionic villi were not apparent. In the villous stroma of the normal chorionic villi capillaries filled with embryonal / fetal erythroblasts were regularly found. Villous edema was absent except for case six in which few normal villi showed slight hydropic changes. In one of the younger pregnancies (case 1) implantation trophoblast was variably present, but atypical trophoblastic hyperplasia was not found. The abnormal chorionic villi, however, showed diffuse hydatidiform changes and abundant trophoblastic hyperplasia with prominent nuclear atypia. Capillaries were either absent or very poorly developed, without nucleated red blood cells. In the younger pregnancies (cases 1, 2 and 6) the molar tissue exceeded the normal placental tissue by far in amount. In case 2, after the first curettage, molar tissue only was sampled for histologic examination and normal placental tissues were not identified at that moment, but they were found in small amounts intermingled with the molar tissue after the second curettage. An embryo had never been found in this case.

Table I. Results of interphase cytogenetic and DNA image cytometric analysis.

case	weeks of gestation	Interphase cytogenetic analysis				DNA cytometric analysis	
		chorionic villi *XY pattern*		extravillous trophoblast *% ISH-polyploid* nuclei*		*2.5c Exceeding Rate (%)*	
		normal	mole	normal	mole	normal	mole
1	13	XY	XX	7.5	47.5	36.5	85.0
2	8-12	XY	XX	-**	20.1	-***	64.0
3	18	XY	XX	4.4	18.3	7.5	78.0
4	25	XY/XY	XX	-**	33.0	14.0	70.4
5	38.5	XX	XX	-**	28.0	0.0	51.5
6	11	XY	XY	-**	24.0	15.0	56.5

* percentage of nuclei with more than two ISH signals
** no extravillous (implantation) trophoblast
*** no separate processing of normal villi possible, only mole tissue could be measured

Interphase cytogenetic analysis

Interphase cytogenetic analysis using DNA probes specific for the sex chromosomes revealed a XY pattern in the embryo of case 1 as well as in the normal appearing chorionic villi of cases 1, 2, 3, 4 and 6 (Fig.1b and c). A XX pattern was found in the

molar chorionic villi of cases 1,2,3 and 4, and a XY pattern in case 6 (Fig.1d and e). In case 5 a XX pattern was found in both normal and molar chorionic villi (Table I). In all cases the normal chorionic villi showed a chromosome disomy in the implantation trophoblast (Fig.1f) with only sporadic polysomic cells, mainly in areas with fibrinoid deposits. In contrast, the extravillous trophoblastic hyperplasia of the molar placenta displayed a high frequency of nuclei with multiple chromosome copies (Fig.1g; Table I).

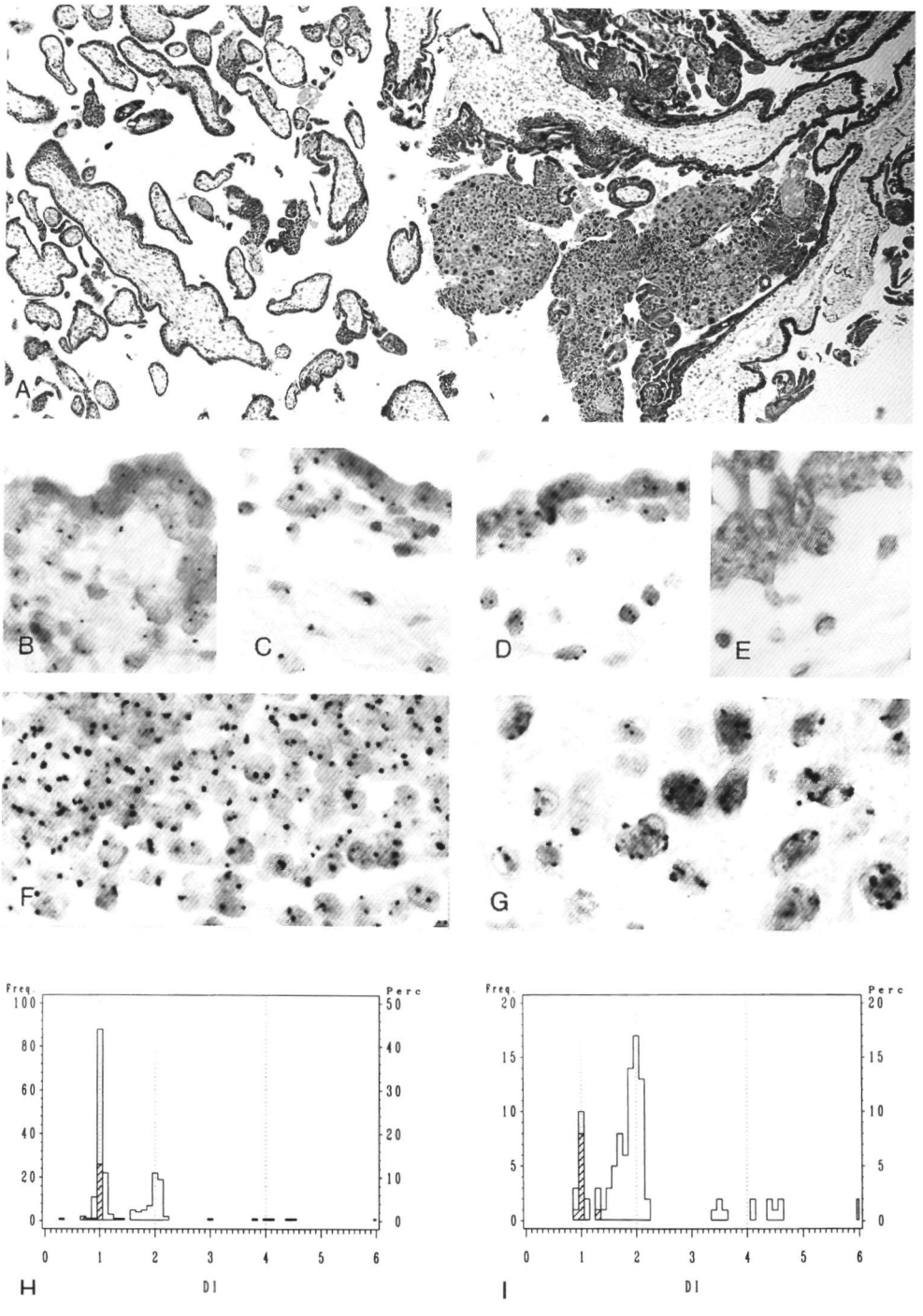
DNA cytometric analysis

Normal and molar placental tissues could be separately processed in cases 1, 3, 4, 5 and 6. In case 2 the normal villi were too intimately intermingled with the molar tissue to be separated. There was, however, ample molar tissue free from normal chorionic villi and this was measured for DNA content. In cases 1, 3, 4, 5 and 6 the normal placental tissue was found to be DNA-diploid with relatively low 2.5c exceeding rates (less than 40%; Fig. 1h; Table I). In all six cases the molar tissue showed a DNA-polyploidy with high 2.5c exceeding rates (Fig. 1i and Table I).

DISCUSSION

Hydatidiform changes in a placenta with a coexisting fetus can be subdivided into three groups: 1) partial hydatidiform moles which are usually triploid and associated with an abnormal fetus; 2) hydropic degeneration of (part of) a diploid or less frequently triploid placenta; and 3) twin pregnancy, including a normal diploid conceptus and an in origin diploid complete hydatidiform mole. In the presented six cases twin pregnancy was suspected on basis of the clear demarcation of normal and molar chorionic villi.

Figure 1. *Case 1. a). Light microscopy displaying normal chorionic villi (left) adjacent to molar chorionic villi (right) with extensive trophoblast proliferation (and villous edema which is not shown; x 30). b-g). Interphase cytogenetic analysis using biotinylated probes specific for chromosomes 1, X and Y, counterstained with Mayer's haematoxylin (x 500). The normal chorionic villi show one ISH-signal per nucleus for chromosome X (b) and Y (c), whilst the molar villi show two ISH-signals per nucleus for chromosome X (d) and none for chromosome Y (e). The implantation trophoblast of the normal villi, in general, shows not more than two ISH-signals for chromosome 1 (f), in contrast to the extravillous trophoblast proliferation of the mole which reveals many nuclei with more than two ISH-signals (g). h). DNA-histogram of the normal placenta showing DNA-diploidy. i) DNA-histogram of the molar placenta showing DNA-polyploidy.*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Dizygosity could be proven in four of the six cases with interphase cytogenetic analysis revealing a male embryo (case 1) and XY normal placenta and XX complete hydatidiform mole.

The complete hydatidiform moles were DNA-polyploid with high 2.5c exceeding rates, a finding that is fully in agreement with our previous report on complete moles showing a high frequency of polyploid cells in the extravillous trophoblast[4]. In cases 5 and 6 dizygosity could not be proven, because normal and molar placental tissues showed an identical sex chromosomal pattern. The DNA-ploidy patterns, however, were similar to the other three cases, showing DNA-diploidy in the normal placenta and DNA-polyploidy in the mole. Therefore, it is very likely that these cases also represent twin pregnancies with complete hydatidiform mole. Another rare, recently described[20] possibility is the coexistence of a normal fetus and placenta with a morphologically complete mole, but cytogenetically resulting from a single gestation with both maternal and paternal DNA contributions. The DNA-polyploidy present in our cases is consistent with the morphological aspect of complete mole with prominent and atypical trophoblastic hyperplasia. Uniparental disomy might be an explanation for these cases. The occurrence of persistent gestational trophoblastic disease in the described case[20] as well as in one of our cases confirms the for a complete mole expected high risk as compared to the triploid partial mole with a relatively low risk[21].

About 31 cases of hydatidiform mole with coexistent fetus have been reported in which, on morphological criteria, the possibility of a twin pregnancy was suggested[9,22-28]. In an additional twelve recently published cases the existence of a dizygotic twin pregnancy with an androgenetic hydatidiform mole could be confirmed with cytogenetic marker polymorphism studies[7-12,29]. In this study we used interphase cytogenetic analysis with DNA probes specific for the sex chromosomes. Although direct prove of dizygosity is limited to unlike-sexed twins, this ISH technique can rapidly be performed on paraffin embedded tissue sections with the important advantage of preservation of histological architecture, so that also in small areas chromosomal aberrations can be detected and related to morphology. This was particularly important in case 2, in which normal and molar chorionic villi could not be separately processed. Using the ISH technique, triploid as well as diploid partial moles can be differentiated from twin pregnancy with complete mole, even from twins of similar sex, on basis of the high frequency of polyploid cells in the molar component.

The importance of recognition of a twin pregnancy with a complete mole component and its differentiation from a triploid partial mole and also from a hydropically degene-rated placenta, is the differential risk of subsequent malignant changes. This will not occur in hydropically degenerated placentae, but follows at least 10-20% of complete moles. The

risk of persistent trophoblastic disease following a partial mole is considered to be low, although the reported frequency varies from 0.5 %[21] to 5.5 %[30]. Not all reported cases of partial moles with subsequent persistent gestational trophoblastic disease, however, were triploid; some cases were diploid[21,30,31]. In these cases, the possibility of a twin pregnancy with complete mole should be excluded. In case 2 the criteria of persistent gestational trophoblastic disease were met because the ß-hCG level was rising again after initial decrease. Although usually this is an indication to start methotrexate therapy, in this case a second curettage was performed followed by a decrease in ß-hCG level. In case 6 persistent gestational trophoblastic disease developed for which methotrexate therapy was given. In fact, in 15 out of the 49 published cases in which twin gestation with complete mole was diagnosed persistent gestational trophoblastic disease developed[9,11,26,28,29], which is in accordance with the high frequency of malignant change in complete hydatidiform mole. Therefore, careful diagnosis and follow up of dizygotic twin molar pregnancies is needed. Although clinical follow up is advised for partial moles as well, the significant difference in relative risk of persistent gestatio-nal trophoblastic disease has implications for the prognosis in individual patients.

It is important to notice that 2 of our 6 cases and 5 of the cases in the literature[9,12,24,25] were pregnancies induced by ovulation induction. There are only a few reports describing hydatidiform molar pregnancies after ovulation induction[24]. As complete moles result from the fertilization of an abnormal egg, it is possible that induction resulting in multiple ovulations also increases the risk of such abnormal eggs and, therefore, the risk of twin pregnancy associated with a complete mole.

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CHAPTER 5

EARLY EMBRYONAL TISSUES DO NOT EXCLUDE A DIAGNOSIS OF COMPLETE HYDATIDIFORM MOLE.

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EARLY EMBRYONAL TISSUES DO NOT EXCLUDE A DIAGNOSIS OF COMPLETE HYDATIDIFORM MOLE.

SUMMARY

The presence of early embryonal tissues, such as nucleated red blood cells (NRBC), amnion or yolk sac, is generally considered to exclude a diagnosis of complete mole (CM). In a series of 195 cases of hydatidiform mole, eleven cases were retrieved with the histopathology of CM and the presence of embryonal tissues. Using DNA image cytometry, all eleven cases showed a bimodal DNA-polyploid pattern, compatible with a diagnosis of CM. DNA-triploidy was not found, excluding a diagnosis of partial mole (PM). Interphase cytogenetics on paraffin tissue sections demonstrated the presence of polyploidization in extravillous trophoblast, which is a prominent feature in CM. Identical sexes were found in the villi and in the embryonal tissues of each case, making the possibility of dizygotic twin pregnancy with CM very unlikely. Progression towards persistent gestational trophoblastic disease occurred in 4 out of the 11 cases (36%), a percentage that is also expected for CM. Three cases were analyzed with polymorphic CA-repeat markers and in two of them no maternal contribution was found for 4 out of 6 markers and 2 out of 5 markers respectively, indicating androgenesis.

We conclude that formation of early embryonal tissues is possible in CM, and not necessarily indicate PM, and that these tissues can develop in the absence of maternal DNA.

Key words: complete mole, embryonal blood cells, yolk sac, amnion, ploidy, *in situ* hybridization, CA-repeat analysis

INTRODUCTION

Complete hydatidiform moles (CM) are generally defined as abnormal pregnancy products, consisting of a placenta with (gross) hydropic swelling of the chorionic villi and trophoblastic proliferation, *in the typical absence* of development of an embryo and its amniotic covering[1,2,3]. CM have a diploid karyotype[1,2], but show DNA polyploidization in the extravillous trophoblast[4-6]. The nuclear DNA of CM is entirely paternal in origin (androgenesis) due to loss of maternal DNA from the fertilized ovum[7]. Experimental animal studies have shown that maternal DNA is essential for the development of an embryo[8-10], and these results have been used to explain the absence of embryonal development in CM. Therefore, finding evidence of embryonic development is considered to be a good discriminant between CM and partial moles (PM), or to indicate twin pregnancy with CM[3,11,12]. PM have a diandric triploid genome[13,14] due to dispermic fertilization of a normal egg with maternal DNA, resulting in development of an (abnormal) embryo. In PM, the chorionic villi show focal edema and trophoblastic excess, and villous capillaries often contain NRBC.

In a recent study[12], however, Paradinas *et al.* have found NRBC and amnion in 8.7% and 16.7% of 149 cases of CM, respectively. These findings suggest that embryonic development may be possible in CM, despite their androgenetic origin.

The present study was designed to determine the occurrence and frequency of CM with NRBC, amnion or other embryonal tissues in our material. In a series of 195 cases of hydatidiform mole, of which the histology was reviewed, eleven cases were retrieved of hydatidiform moles with the histology of CM, and the presence of early embryonal tissues. Additional techniques were performed in order to substantiate the diagnosis of CM, and to exclude the possibilities of PM or twin pregnancy with CM. DNA image cytometry was performed to determine DNA-ploidy and 2.5c exceeding rate (2.5c ER). Interphase cytogenetic analysis on 6 μm thin paraffin tissue sections was applied to detect polyploidization in extravillous trophoblast, and to determine the sex chromosome composition of chorionic villi and early embryonal tissues. CA-repeat analysis using six polymorphic markers was performed in three cases in order to confirm the hypothesis that embryonal tissues can be formed in androgenetic CM.

MATERIAL AND METHODS

Patient material

The eleven cases described in this study were part of a larger series of 195 hydatidiform

moles. These cases were collected and analyzed with DNA image cytometry at the University Hospital Nijmegen, where national registration of hydatidiform moles and persistent gestational trophoblastic disease (PGTD) takes place. The group consisted of 41 cases of PM and 154 cases of CM, of which 7 cases were part of a twin pregnancy, and 11 cases showed a histopathology that was comparable with CM, but of which the presence of embryonal tissues did not fit with this diagnosis. On behalf of these findings the eleven cases were initially classified as PM.

Clinical follow-up after molar evacuation included serum ß-hCG titers measured weekly until normal for three consecutive weeks and then monthly for one year. PGTD was diagnosed when ß-hCG levels persisted at a plateau and/or raised for at least three consecutive weeks.

DNA image cytometric analysis

In all 195 cases, including the eleven cases described in this study, DNA image cytometry was performed as described previously[5]. In five of the eleven cases enough maternal decidual tissue was present to be processed separately as internal control for normal diploid cells. In the other six cases maternal leukocytes were used as alternative. Briefly, intact nuclei were isolated from 50 μm thick paraffin tissue sections. After deparaffinization and rehydration, the 50 μm sections were incubated with 0.1 percent protease in PBS at 37°C for 1 hr. After rinsing the nuclear suspension was centrifuged to a glass slide and stained with pararosaniline-Feulgen. The DNA content of 200 stained intact nuclei was measured using the CAS 100 System (Cellular Imaging Systems, Beckton and Dickinson, Leiden, The Netherlands). At least 30 rat liver cells (DNA-tetraploid) were measured as an external control for DNA content, while at least 20 decidual cells or leukocytes were used as internal control. The DNA-histograms were classified according to the criteria given in Table I. The nuclear fractions with a DNA content exceeding the first, 2C, G_0/G_1 peak, were calculated as the 2.5c exceeding rate (2.5c ER)[5].

Interphase cytogenetic analysis

In all eleven cases, the *in situ* hybridization (ISH) procedure on 6 μm thin formalin-fixed and paraffin embedded tissue sections was performed as described previously[5]. Centromere-associated DNA probes for chromosome 1 (pUC 1.77)[15], chromosome X (pBam X5)[16], and chromosome Y (DYZ3)[17] were labeled by nick-translation with biotin-14-dATP according to the suppliers instructions (BRL: Gaithersburg, USA). For the demonstration of the hybridized DNA probes the horseradish peroxidase labeled avidin-biotin-system (ABC) method was applied using the Elite ABC-kit (Vector Laboratories, Burlingame, CA), and the probe was visualized with diaminobenzidine (DAB)/H_2O_2[18].

Table I. Criteria for the classification of DNA-ploidy histograms.

Classification	Major peak(s) (DI)*	Minor peak(s) (DI)*
DNA - diploidy	1.0 ± 0.1	2.0 ± 0.2
DNA - tetraploidy	2.0 ± 0.2	4.0 ± 0.4
DNA - polyploidy	1.0 ± 0.1, 2.0 ± 0.2	4.0 ± 0.4
DNA - aneuploidy	uni /multimodal, but not 1, 2 or manifold	
DNA - triploidy	1.5 ± 0.15	3.0 ± 0.3

* DI = DNA index

CA - repeat analysis.

In three of the eleven cases enough decidual tissue devoid of trophoblastic invasion was left and separable from chorionic villi to perform CA-repeat analysis. One case of DNA-diploid abortion was used as control. Paternal tissues (blood) were not available because all cases were archival and referred from other laboratories. Ten consecutive 10 μm sections were cut, dewaxed in xylene, and rehydrated by passage through graded ethanols. Maternal decidua and molar tissue was collected using a dissecting microscope and put into separate Eppendorf tubes. DNA was extracted using a standard lysis solution (0.1 mM Tris-HCl, 25 mM EDTA, 0.5% sodium dodecyl sulphate, pH 8.4), incubated for 5 days at 37^0 C, and extracted in phenol chloroform and precipitated in cold ethanol. The concentration of the extracted DNA samples was assayed quantitatively using a TKO-100 dedicated minifluorometer (Hoefer Scientific Instruments, San Francisco, USA). The length of the DNA fragments was about 200 - 500 basepairs.

PCR analysis of six CA-repeat markers (DXS178[19], COL4A5[20], D15S11[21], D9S15[22], ANGIOTENSIN[23] and D3S11[24]) was performed on 1 μg DNA from molar and maternal tissue under conditions as described by the authors[19,24]. Primers were fluorescently labeled and the PCR fragments were analyzed on an automated sequencer, using the Genescanner software (Perkin Elmer/ABI, Foster City, USA).

RESULTS

Histopathology.

All eleven cases showed the histopathological features characteristic for CM. Villous edema was variably present. In nine cases large edematous lakes were present in nearly all chorionic villi, while in two cases (cases 5 and 7) edematous changes were less developed, especially in the newly formed villi, displaying only few edematous lakes in the older villi. The amount of extravillous trophoblast was in excess of normal and showed prominent nuclear atypia (Fig. 1A and B). The villous stroma showed karyorrhexic degeneration in all cases. Villous capillaries were sparse, often in rudimentary form, but sometimes well developed. In all cases capillaries were found containing nuclear and cytoplasmic debris. Intact or vital embryonal nucleated red blood cells (NRBC) were abundant in well formed capillaries in three cases (cases 5, 7 and 11; Fig. 1C) or were found sporadically in the slitlike lumen of rudimentary formed capillaries in three other cases (cases 1, 6 and 10; Fig. 2B). The NRBC showed a progressive degree of degeneration corresponding with the degree of karyorrhexis and edema in the chorionic villi. Scattered siderophages were found along some capillaries. In all cases the chorionic villi with capillaries containing NRBC showed molar changes (edema, stromal karyorrhexis, trophoblast hyperplasia). Normal chorionic villi, indicative of twin pregnancy, were not found. An amnion or yolk sac was found in eight cases (Fig. 2A, 2C and 2D; Table II). In case 10 an embryonal tubular structure was found in one of the larger villouslike structures, possibly chorionic plate (Fig.3A).

Persistent gestational trophoblastic disease followed in 4 of the 11 cases (cases 1,6,9,11).

DNA image cytometry.

Using DNA image cytometry a bimodal DNA-polyploid pattern was found in all eleven cases (Fig. 4; Table II). The DNA-histograms showed a prominent DNA-diploid G_0/G_1-peak and DNA-tetraploid G_0/G_1-peak, the latter varying in height and width, and a small

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Figure 1. Histology (A-C, H&E stain) and ISH results (D-F) of case 5. Villous edema (A x40) with stromal karyorrhexis (C x300) and trophoblast hyperplasia with prominent nuclear atypia (B x300) were characteristic of complete mole. In the villous stroma capillaries contained both vital and degenerated nucleated red blood cells (NRBC; C). In the atypical extravillous trophoblast nuclei showed polysomy for chromosome 1 (D; arrow; x300). In the villous stromal cells and NRBC no ISH signals for chromosome Y (E, x300), and two ISH signals or less for chromosome X (F, x300) were found.

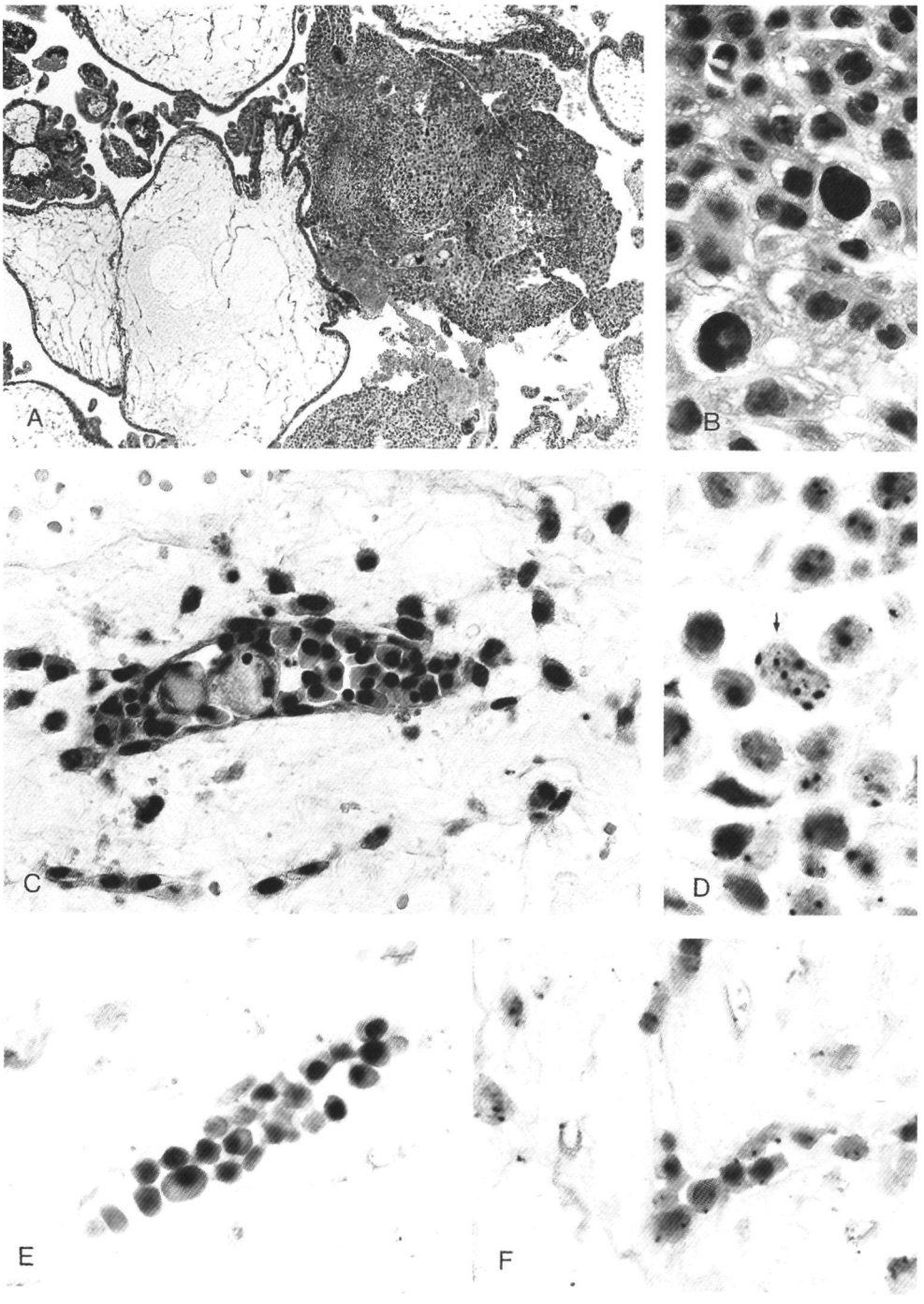
A
B
C
D
E
F

Table II. Clinical data, histopathological features and results of molecular DNA analyses.

case	pregnancy duration	embryonal tissues	DNA-ploidy, 2.5c ER	sex chromosomes in villi and embryonal tissues	parental origin of DNA	PGTD
1).	unknown	NRBC	polyploidy, 54	XX - XX	nd	+
2).	10	amnion	polyploidy, 49	XX - XX	paternal	-
3).	unknown	amnion, yolk sac	polyploidy, 53	XX - XX	nd	-
4).	unknown	amnion	polyploidy, 81	XX - XX	nd	-
5).	8	NRBC	polyploidy, 48	XX - XX	paternal	-
6).	9	NRBC, amnion yolk sac	polyploidy, 48	XX - XX	nd	+
7).	12	NRBC, amnion yolk sac	polyploidy, 68	XX - XX	maternal/paternal	-
8).	8	amnion, yolk sac	polyploidy, 55	XY - XY	nd	-
9).	12	amnion	polyploidy, 78	XY - XY	nd	+
10).	9	NRBC, yolk sac, embryonal tubular structure	polyploidy, 54	XY - XY	nd	-
11).	12	NRBC	polyploidy, 60	XY - XY	nd	+

2.5c ER = 2.5c exceeding rate NRBC = nucleated red blood cells
PGTD = persistent gestational trophoblastic disease nd = not done

DNA-octaploid peak of G_2/M-phase cells of proliferating tetraploid cells. The 2.5c ER varied from 48 to 81%, with a mean value of 59% (SD 11.2). This bimodal DNA-polyploid histogram was consistent with a diagnosis of CM, because such a DNA histogram was found in 97% of the remaining 143 cases of CM of the total series. None of these eleven cases showed a DNA-triploidy as we found in 89% of the 41 cases of PM (data not yet published).

Interphase cytogenetic analysis.

All eleven cases showed ISH-diploid chorionic villi and ISH-polyploid extravillous trophoblast (Fig 1D). Due to nuclear truncation, not all nuclei in the tissue sections

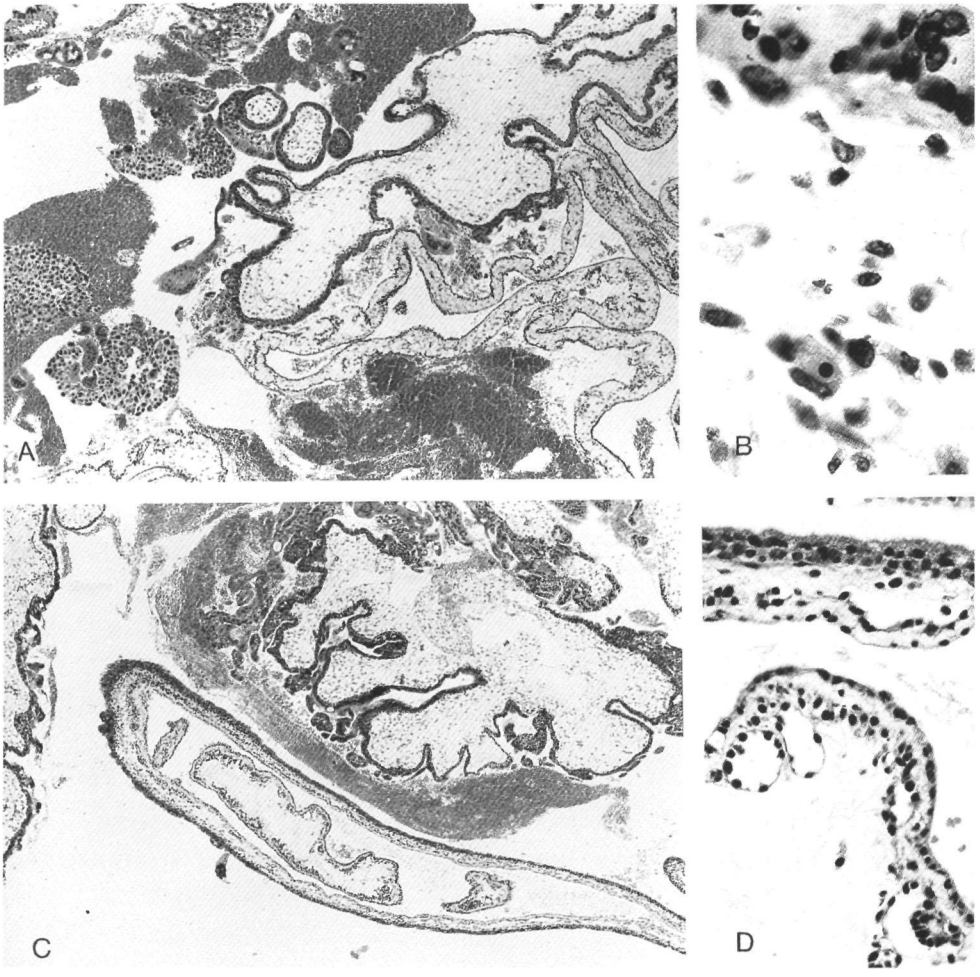

Figure 2. *H&E stained sections of case 1, illustrating the presence of an amnion (A; x 40) and sporadic NRBC in rudimentary capillaries (B; x 300), and of case 8, showing the formation of a yolk sac (C x 40; D x 150).*

displayed the maximum number of ISH signals. In the chorionic villi, two ISH signals for chromosome 1 and X (in XX cases) were found in 68% of the nuclei, in 28% less than two ISH signals and in only 4% more than two ISH signals. In the extravillous trophoblast more than two ISH signals were found in 20.8% (SD 7.9, range 7.1 - 32.0).

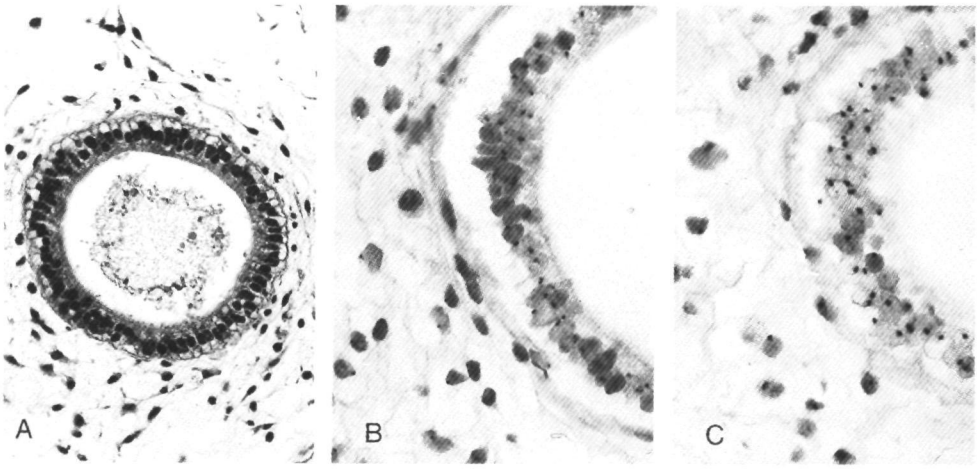

Figure 3. *Histology (A, H&E stain, x150) and ISH results (B,C, x300) of case 10. The embryonal tubular structure shows an identical sex chromosome composition as the surrounding mesenchyme of the chorionic villi: one copy for chromosome X (B), and one copy for chromosome Y (C).*

Nuclei with more than two ISH signals were found in areas with an increase in nuclear diameter, polymorphism and hyperchromatism.

ISH using DNA-specific probes for chromosomes X and Y showed that, in each case, the chorionic villi displayed the same sex as the embryonal tissues of that case, e.g. NRBC, amnion, or yolk sac, or embryonal tubular structure (case 10) (Table II and Fig. 1E and F, and Fig. 3B and C). In the seven twin pregnancies with CM, which were present in the total series of 195 hydatidiform moles, identical sexes in the normal chorionic villi and embryonal tissues, and in the molar villi were found in only 3 cases, the remaining four cases displaying unlike sexes[11] (and unpublished data).

CA - repeat analysis.

DNA from molar and corresponding maternal tissue was isolated in three of the eleven cases (cases 2, 5 and 7 from Table II). In case 5 no maternal alleles were visible for 4 out of 6 markers, and in case 2 for 2 out of 5 markers (Fig. 5A and B). In the latter case, the mole was heterozygous for a non maternal and "maternal" allele for 2 markers. Because no paternal tissue was available, we could not confirm whether the "maternal"

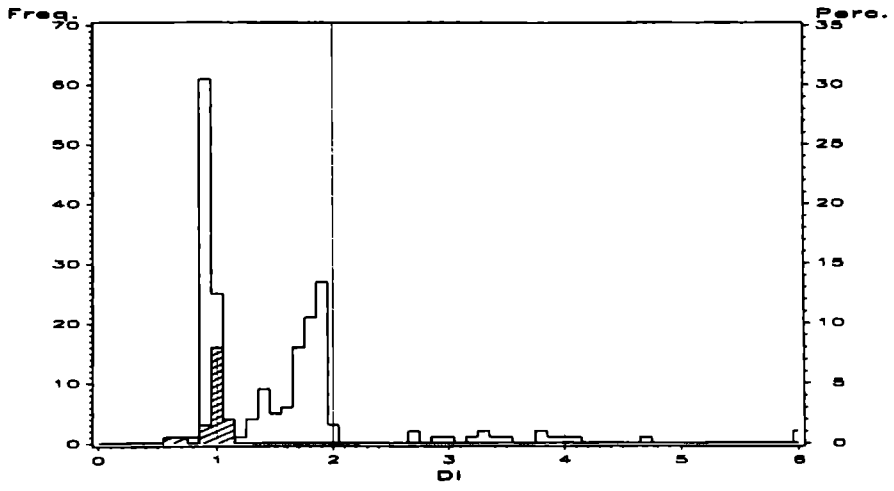

Figure 4. DNA image cytometry showed in all cases a polyploid DNA-histogram with prominent DNA-diploid and DNA-tetraploid peaks and a small DNA-octaploid peak.

alleles were also present in the father, in which case heterozygosity had probably originated from dispermy of an empty egg. Most likely these moles were of paternal origin. In contrast, in the third mole (case 7) and in the abortion maternal alleles were present for all markers that could be unambiguously scored, respectively 6 and 4 (Fig. 5C and D). The probability that all of these alleles were paternal is, based on the population frequency of the alleles, less than 1 in 1,000.

DISCUSSION

In this study, eleven cases were described with a histopathology characteristic of CM and presence of early embryonal tissues (NRBC, amnion, yolk sac, embryonal tubular structure). In these cases, the possibility of a PM or twin pregnancy with CM was excluded for the following reasons. In the first place, the histopathology of these eleven cases was not typical for a PM or twin with CM. Their histopathology showed the three main hallmarks of CM: 1) diffuse edema, albeit of varying degree, 2) stromal karyorrhexis, and 3) excessive trophoblastic proliferation with prominent nuclear atypia, which was also evident in the implantation site trophoblast. In PM, edema is focal, with large cystic

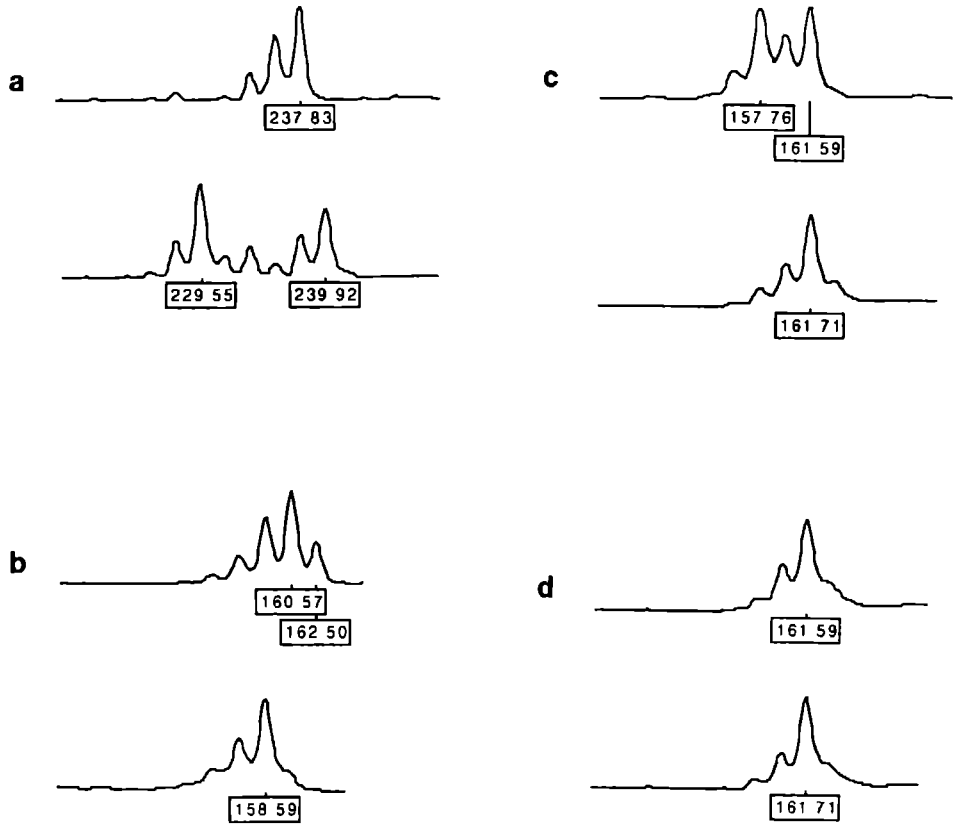

Figure 5. *CA-repeat analysis of maternal (upper part pannel) and molar tissue (lower part panel). The length of the CA-repeat alleles is given in nucleotides below the highest peaks. Marker DXS178 is shown for case 2 (panel a) and marker COL4A5 for case 5 (panel b). Molar and maternal alleles are different. For marker D9S15 alleles are shared between chorionic villi and maternal tissue of the control case (abortion; panel c) and of the mole case 7 (panel d).*

villi next to fibrous villi. Excess of trophoblast is also focal, often vacuolated syncytiotrophoblast, and without prominent nuclear atypia. The villi in PM show scalloping of their contours with trophoblastic inclusions, features that were absent in the eleven cases. In two cases (cases 5 and 7) the edematous cavitation was focal, but the chorionic villi

showed a cellular aspect with beginning edema and karyorrhexis as is described for very early CM[25]. In twin pregnancy with CM, a mixed villous population of normal and molar villi is usually found, with capillaries containing NRBC in the normal chorionic villi and not in the molar villi[11]. In our cases normal chorionic villi were absent and the NRBC were present in chorionic villi which showed all characteristics of molar villi.

In the second place, the DNA-profile of these eleven cases, as obtained from DNA image cytometric and interphase cytogenetic analyses, displayed DNA-diploid villi and polyploidization of extravillous trophoblast, which is also typical for CM and not for PM, which usually are DNA-triploid[1,2,5,6]. Twin pregnancies with CM are dizygotic, with 50% chance of identical sexes of the CM and the normal pregnancy. In a series of seven twins with CM we found identical sexes in 3 cases (43%)[11](and unpublished data). In the present study, all eleven cases showed identical sexes in the molar villi and NRBC, amnion or yolk sac, or embryonal tubular structure. If these cases were twin pregnancies the statistical chance that all eleven cases showed an identical sex in both villi and embryonal tissues is very small ($0.5^{11} \approx 5 \times 10^{-4}$) and therefore not very likely.

The percentage of persistent gestational trophoblastic disease (PGTD) in the present small series was 36%, corresponding better with the literature data about the incidence of PGTD after CM, than with the much rarer incidence of PGTD after PM[26].

In our series of 147 cases of CM (twin pregnancies excluded), the frequency in which embryonal tissues were found in CM was 7.5%. The presence of NRBC was found in 4.1%, amnion in 4.8%, yolk sac in 3.4%, and embryonal tubular structure in 0.7%. These percentages are lower than the results recently published by Paradinas *et al.*[12], who found in a series of 149 CM, the presence of NRBCs in 13 cases (8.7%) and presence of amnion in 16 cases (16.7%). Embryonal tissues, which were not further specified, were found in two cases (1.3.%). These differences in frequencies might be explained by statistical variation, or because the investigators did not exclude the possibility of twin pregnancy with CM. The overall frequencies of early embryonal tissues in CM is relatively low, and is probably even lower, because selection bias of rare cases being more likely to be send in to reference centers cannot be excluded. The low frequency in which early embryonal tissues are found in CM can be explained by either a low frequency in which any embryonal development takes place, or by a short existence of the developed embryonal structures. The combination of many vital NRBC in well formed capillaries in villi with incipient edema and nuclear debris in slit-like capillaries in the more advanced edematous villi probably indicates that when the molar changes progress, the NRBC disappear. Capillaries containing cellular debris and less frequently presence of siderophages, indicating breakdown and phagocytosis of blood cells, are found in many CM. These findings demonstrate that in CM nucleated red blood cells are frequently

formed, but degenerate in an early stage (early in pregnancy and/or shortly after they have been formed), and therefore are rarely found by the pathologist. The role of the duration of the pregnancy is unclear. We found abundant NRBC at 8 weeks of gestation (case 5) as well as 12 weeks of gestation (cases 7 and 11). Paradinas *et al.*[12] found a shorter mean pregnancy duration in cases of CM with NRBC and amnion (11.5 wks) than in cases without embryonal tissues (12.2 wks), but the difference was not significant. Keep *et al.* in their study of four cases of very early complete mole (6.5 to 11 wks gestational age) found no NRBC[25].

Although the results of experimental animal studies[8-10], which have shown that paternal and maternal genomes have complementary roles during embryogenesis in the mouse, are used to explain the absence of embryonal development in CM, the finding of NRBC, amnion, yolk sac and other embryonal structures in CM is not incompatible with these results. These experimental animal studies showed that paternal imprinting of the genome is necessary for the normal development of the placenta, whilst maternal genomic imprinting is essential for normal embryogenesis. In these experiments the reconstituted eggs with two maternal pronuclei implanted developed into normal, albeit retarded embryos with poor membranes and trophoblast. If two paternal pronuclei were implanted excessive trophoblast was found and either no embryo was formed, or a tiny retarded embryo (4-6 somites) and relatively substantial yolk sac in some cases, indicating that early embryogenesis can take place. The results of our study and those of the previously reported findings by Paradinas *et al.*[12] seemed to confirm that early embryogenesis also occurs in human CM. The results of CA-repeat analysis showed that in two cases (cases 2 and 5) no maternal contributions for several markers were found, indicating androgenesis. Therefore, the possibility of an androgenetic CM with the presence of NRBC and amnion was considered to be proven, at least in these two cases. In case 7, however, maternal DNA was found to be present in the placental tissues. Possible explanations for this might be contamination of maternal DNA due to the presence of trophoblast cells in the decidua, which had been missed, or that the case represents a rare case of CM with paternal and maternal contributions to the DNA, originating from paternal uniparental disomy[27,28], with homology for a specific domain or for one or more complete chromosomes of paternal origin, resulting in molar development, a possibility that has yet to be further investigated.

From this study we conclude that 1) in CM NRBC, amnion or yolk sac, and even other embryonal tissues can be formed, and not necessarily indicate PM, and 2) that embryonal tissues can develop in the absence of maternal DNA.

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CHAPTER 6

PERSISTENT GESTATIONAL TROPHOBLASTIC DISEASE : DNA IMAGE CYTOMETRY AND INTERPHASE CYTOGENETICS HAVE LIMITED PREDICTIVE VALUE.

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PERSISTENT GESTATIONAL TROPHOBLASTIC DISEASE : DNA IMAGE CYTOMETRY AND INTERPHASE CYTOGENETICS HAVE LIMITED PREDICTIVE VALUE.

SUMMARY

DNA flow cytometry has shown a wider spectrum of DNA content in complete hydatidiform mole (CM) than the originally reported diploidy. Conflicting results have been published about the relationship of DNA content and the occurrence of persistent gestational trophoblastic disease (PGTD). In the present study, 71 cases of CM and 4 cases of partial mole (PM) accompanied by PGTD and 100 cases of CM without PGTD were evaluated with DNA image cytometry for differences in DNA-ploidy pattern, expressed as the 2.5c and 5c exceeding rates (2.5c ER, 5c ER). A pilot study of 20 cases of each group was performed using interphase cytogenetics to detect differences in the frequency of numerical chromosomal aberrations and in sex chromosome composition. For this purpose, DNA probes specific for the pericentromeric regions of chromosomes 1 and X and for the long arm of chromosome Y were incubated on 6-μm paraffin tissue sections.

The results showed no differences between CM with or without PGTD; DNA-polyploidy occurred in 99% and 98% of cases respectively; the 2.5c ER and 5c ER were 62.6 and 62.4, and 6.5 and 6.0, respectively. The frequency of numerical chromosomal aberrations as detected by interphase cytogenetics was 23.4 and 22.8 %. An XY pattern was found in 3 of 20 cases of CM with PGTD and in 4 of 20 cases of CM without PGTD. The four cases of PM showed a DNA-ploidy pattern identical to that of a CM. For this reason, they would be better reclassified as CMs, despite the presence of nucleated red blood cells or amnion.

Although nuclear atypia and corresponding increased DNA content is pronounced but variable in CMs, the occurrence of PGTD is not related to variations in quantitative DNA content nor to gross heterology or homology in sex chromosomes.

Key words: hydatidiform mole, persistent trophoblastic disease, DNA ploidy, image cytometry, *in situ* hybridization

Persistent gestational trophoblastic disease (PGTD) requiring chemotherapy develops in 10-20% of patients with a complete hydatidiform mole (CM)[1,2]. Choriocarcinoma is included in this group. Before current chemotherapy regimens and ß-hCG monitoring, choriocarcinoma was diagnosed in 2 to 19% of patients with a CM; 50% of gestational choriocarcinoma were preceded by a CM, the other 50% by abortions or normal pregnancies[3,4]. Today, a diagnosis of postmolar choriocarcinoma is rarely made, because PGTD is detected early as a result of ß-hCG monitoring[2], which makes re-evacuations for a histological confirmation of choriocarcinoma unnecessary. If treated promptly, the prognosis of postmolar PGTD, including choriocarcinoma, is excellent, with an overall survival rate of more than 90%[5]. The risk of PGTD after a partial mole (PM) is less established as compared with a CM, but it has been estimated to occur in 0.5% of patients with PM[6]. It is recommended that patients with a CM and those with a PM be given the same clinical follow-up, with regular ß-hCG assays and the postponement of pregnancy.

For pathologists, the histologic distinction between a CM and a PM and their differentiation from hydropic abortion (HA) is not always easy. To assess prognostic and therapeutic implications in the individual patient, it is important that the pathologic classification is as accurate as possible. Previous studies[7,8] using karyotyping delineated the pathologic and cytogenetic differences between these entities. CMs have a diploid karyotype, with the entire nuclear genome being derived from the father[9]. PMs are triploid, with one maternally and two paternally derived sets of DNA[1]. HA usually have an overall diploid DNA but with a variety of individual chromosomal aberrations.

As a powerful aid in the differential diagnosis, DNA flow cytometric analysis is now widely being used. In comparison with karyotyping, however, DNA flow cytometric studies show a wider spectrum of nuclear DNA in CMs then the previously detected diploidy[10-14]. A higher incidence of PGTD was initially reported in DNA-aneuploid CMs[15], but this has not yet been confirmed by other studies[11,13,16]. In all studies, DNA flow cytometric analysis was the applied technique. In one previous study[12], however, it was found that in the detection of cell subpopulations with increased DNA content, DNA flow cytometric analysis is less sensitive than DNA image cytometric analysis (ICM) and interphase cytogenetic analysis. Using interphase cytogenetic analysis, one could establish that the aberrant cell subpopulations with high DNA content (polyploidization) were mainly located in the extravillous trophoblast, with the chorionic villi being uniformly diploid.

In this study DNA ICM was applied to 175 cases of hydatidiform mole, and interphase

cytogenetic analysis to a subgroup of 44 of the 175 cases for additional evaluation of the DNA ploidy and numerical chromosomal aberrations in extravillous trophoblast, with special interest in differences that could predict PGTD. For this purpose, the parameters were 1) the ratio of the cell subpopulations with increased DNA content to the DNA-diploid cell population, expressed as the 2.5c and 5c exceeding rates (ERs) using DNA ICM; and 2) the degree of numerical chromosomal aberrations in the cell subpopulation with increased DNA content using interphase cytogenetic analysis.

MATERIAL AND METHODS

Patients

For this study, 75 cases of hydatidiform mole (71 cases of CM and 4 cases of PM) that were accompanied by PGTD were compared with 100 cases of CM without PGTD. The cases were obtained from the files of the Central Molar Registration of The Netherlands at the University Hospital of Nijmegen, which provides national registration of hydatidiform moles and PGTD and supraregional laboratory service for ß-hCG monitoring, and from the files of the Netherlands Studygroup of Trophoblastic Tumors, in which discussions and decision making concerning follow-up and treatment take place at a national level. Clinical follow-up after molar evacuation included serum ß-hCG titers measured weekly until normal for 3 consecutive weeks and then monthly for 1 year. PGTD was diagnosed when ß-hCG levels persisted at a plateau and/or rose for at least 3 consecutive weeks. The cases of hydatidiform mole with PGTD were selected on the occurrence of PGTD irrespective of the initial or review diagnosis. The cases of hydatidiform mole without PGTD were selected on the review diagnosis of CM.

From all of the cases, the histologic slides were reviewed according to the criteria of Szulman and Surti[7,8] before the DNA analyses.

DNA image cytometric analysis

DNA ICM was performed on all cases, as previously described[12]. Briefly, intact nuclei were isolated from 50-μm-thick paraffin tissue sections. Maternal decidual tissue was processed separately and served as the internal control for normal diploid cells. If decidual cells were not available, maternal lymphocytes were used as the alternative. After deparaffinization and rehydration, the 50-μm sections were incubated with 0.1 % protease (type VII *Bacillus amylolique faciens*; Sigma Chemical Co., St. Louis, USA) in PBS at 37°C for 20 min. Incubation was terminated by adding 4 to 5 ml of cold (4°C) PBS and putting the tubes on ice. After rinsing with PBS, 30,000 nuclei were counted

with a Coulter Counter Model ZB1 (Coulter Electronics, Dunstable, UK). After centrifugation, 200 μl fetal calf serum (Gibco, Paisley, UK) were added, and this nuclear suspension was centrifuged to a glass slide using a cytocentrifuge for 10 min at 500 rpm (Shandon, Zeist, The Netherlands), air-dried, and fixed in a mixture of methanol, 37 % formaldehyde, and acetic acid (85:10:5 by volume) for 1 hour. The nuclei were then stained with pararosaniline-Feulgen. The DNA content of 200 stained and intact nuclei of trophoblast and villous stromal cells were selectively measured using the CAS 100 System (Cellular Imaging Systems, Becton Dickinson, Leiden, The Netherlands)[17]. Leukocytes and decidual cells were not measured as diagnostic cells. At least 30 rat liver cells (DNA-tetraploid) were measured as an external control for DNA content, and at least 20 decidual cells were used as internal control.

The DNA histograms were classified as follows. A *DNA-diploid* pattern consisted of a distinct G_0/G_1 peak in the diploid (2C; DNA index (DI) = 1.0 $\pm$ 0.1) region with a small proportion of cells in S and G_2/M (4C) phases, defined by a 2.5c ER of less then 40%[12]. A *DNA-polyploid* pattern showed distinct peaks in the diploid (2C; DI = 1.0 $\pm$ 0.1) and tetraploid (4C; DI = 2.0 $\pm$ 0.2) regions with a 2.5c ER of 40% or more, or in the diploid, tetraploid and octaploid (8C; DI = 4.0 $\pm$ 0.4) regions. The 2.5c ER and the 5c ER were determined from the nuclear fractions exceeding the first, 2c G_0/G_1 peak with a DI greater than 1.25 and the 4c peak with a DI greater than 2.5, respectively. A 2.5c ER of 40% or more appeared to be a reliable parameter for the discrimination between DNA-diploidy and DNA-polyploidy[12].

Interphase cytogenetic analysis

Interphase cytogenetic analysis was performed on 6-μm-thin paraffin-embedded tissue sections in 44 of the 175 cases: 20 cases of CM with PGTD, all four cases of PM with PGTD, and 20 cases of CM without PGTD. The chromosome specific DNA probes used were the satellite III DNA probe for chromosome 1 (pUC 1.77), the alphoid DNA probe for chromosome X (pBam X5) and the satellite III DNA probe for chromosome Y (DYZ3), recognizing tandem repeats in the (peri)centromeric region (1q12) of chromosome 1[18], in the centromeric region of chromosome X[19], and in the q arm of chromosome Y[20], respectively. Biotinylation of the probes was performed using Bio-14-dATP (BRL: Gaithersburg, USA), according to the suppliers instructions.

The *in situ* hybridization procedure (ISH) in paraffin-embedded tissue sections was performed as previously described[12,21], with minor modifications in the immunohistochemical step: mouse anti-biotin (1:100 in PBS-tween with 5% non fat dry milk (NFDM); Dakopatts, Glostrup, Denmark) was followed by biotin labeled horse anti-mouse (1:200 in PBS-tween, 5% NFDM; Vector, Burlingame, Canada) and avidin-biotin complex (1:100

in PBS-tween, 5%NFDM; Vector). The frequency of ISH signals for the different chromosomes was evaluated in at least 500 nuclei according to previously reported criteria[12,21].

Statistical analysis

The mutual differences in 2.5c ER and 5c ER as well as in the chromosome copy numbers between the group of CMs with PGTD and the group of CMs without PGTD were studied with Student *t* test.

RESULTS

Histopathologic findings

The initial diagnoses given by the referring pathologists are listed in Table I and compared with the revised diagnoses. Excluding the cases in which no initial diagnosis was made, the interobserver variability was 26% and 35% for the groups of hydatidiform moles with PGTD and without PGTD, respectively. The four cases of PM with PGTD were remarkable in that the histomorphologic characteristics of the chorionic villi and the degree of trophoblastic hyperplasia were identical to those found in CMs, but focal vital

Table I. Comparison of initial histologic diagnosis of the referring pathologist with the revised histologic diagnosis.

Diagnosis after histologic review	Initial diagnosis of referring pathologist: No initial diagnosis	CM (n)	PM (n)	HA (n)	A (n)	Total N
Moles without PGTD						
CM (n)	12	57	25	6	-	100
Moles with PGTD						
CM (n)	5	50	14	1	1	71
PM (n)	-	1	2	1	-	4

CM = complete mole PM = partial mole PGTD = persistent gestational trophoblastic disease
HA = hydropic abortion A = abortion

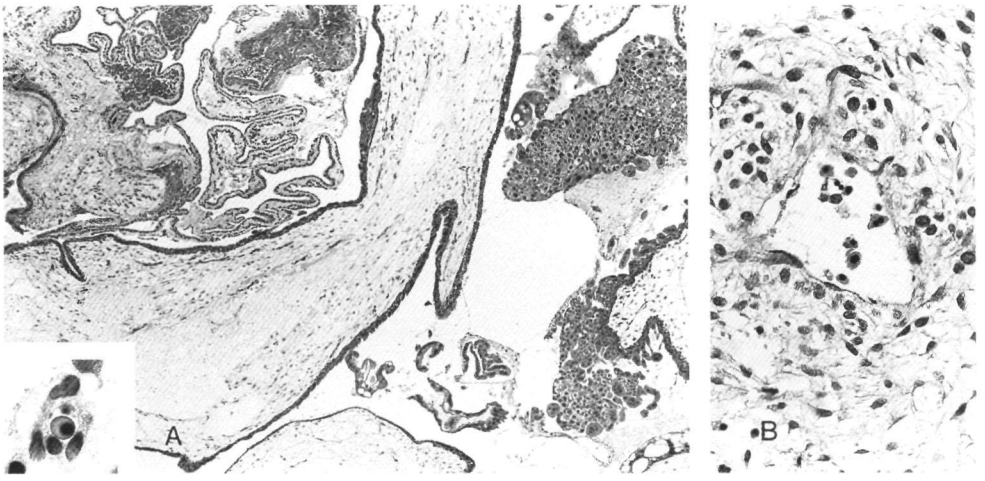

***Figure 1. A)**. Classical histologic aspect of a CM with atypical trophoblastic hyperplasia (right) and stromal edema and karyorrhexis, but with an amniotic structure (upper left) and sporadic NRBCs in stromal capillary (inset (x 700) ; Hematoxylin & eosin, x 33). **B)**. A similar case with NRBCs in some of the stromal vessels (H&E, x 260). The cases were classified as PM.*

nucleated red blood cells (NRBCs) within capillaries of molar villi, amnion, or yolk sac structures could be found (Fig 1), because of which the cases were formerly classified as PM. In CMs, nonfunctional capillaries were often found within the villous stroma. They contained nuclear and cellular debris, and pericapillary hemosiderin deposits could sometimes be found when an iron stain was performed (Fig. 2). The presence of NRBCs is unusual, however, in these ill-formed stromal vessels of otherwise unequivocally molar villi with stromal karyorrhexis and atypical trophoblast hyperplasia. Twin gestation was unlikely on the basis of the histomorphologic characteristics (the absence of normal villi, the presence of NRBCs in molar villi) and results of interphase cytogenetic analysis (see below).

The mean age of the patients was similar in both groups (30 yr), as was the mean duration of pregnancy (12 wk).

DNA image cytometric analysis

In all of the cases, a satisfactory DNA histogram was obtained, except in one case of a CM without PGTD in which the chorionic villi were too degenerated. Of the remaining

99 cases of CM without PGTD, 2 cases were DNA-diploid, whereas all of the other cases (98%) were DNA-polyploid (Fig. 3). Also, in the cases with PGTD, one case was DNA-diploid and the other cases were DNA-polyploid (99%), including the four cases with a previous review diagnosis of PM. There was no significant difference in 2.5c and 5c ERs between the cases with PGTD and the cases without PGTD (Table II).

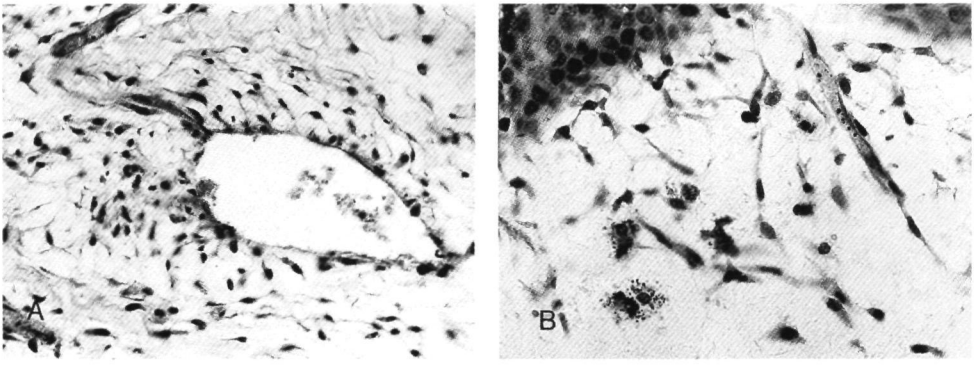

Figure 2. A)*. Stromal vessels in a CM containing cellular and nuclear debris (H&E, x 200).* ***B)****. Hemosiderin-loaded macrophages in the neighborhood of rudimentary stromal capillary in a CM (Perls' iron stain, x 400).*

Interphase cytogenetic analysis

The 20 cases of CM with PGTD on which interphase cytogenetic analysis was performed were representative of the total number of cases (mean 2.5c ER, 63.9 %), as were the 20 cases of CM without PGTD (mean 2.5c ER, 62.3 %). The results are shown in Tables III and IV. In all of the 40 cases, the percentage of nuclei with 3 or more ISH signals in the villi did not exceed 6% (mean, 2.3%; range, 1.1 - 5.5%). This is compatible with diploidy. The extravillous trophoblast hyperplasia, however, showed a higher frequency of nuclei with three or more ISH signals (mean, 23.1%; range, 9.8 - 43.1%; Fig. 4) but without significant differences between cases without or with PGTD ($P = 0.075$).

The four cases of PM with PGTD were also analyzed. The results of DNA cytometric examination (DNA-ploidy, 2.5cER, 5cER) and interphase cytogenetic analysis (ISH-ploidy, fraction of numerical chromosomal aberrations) were comparable with the findings in CMs. (Tables II and III). DNA-triploidy was not found with either method.

Table II. 2.5c and 5c exceeding rates (%) in hydatidiform moles with and without persistent gestational trophoblastic disease (PGTD).

Exceeding Rates	Cases with PGTD		Cases without PGTD
	Complete mole	Partial mole	Complete mole
Total N	71	4	99
2.5c exceeding rate:			
mean	62.6	60.0	62.4
standard deviation	11.2	11.2	12.1
range	31.5 - 89.0	48.0 - 77.9	28.0 - 86.9
5c exceeding rate:			
mean	6.5	5.4	6.0
standard deviation	6.3	2.8	6.4
range	0 - 32.5	1.3 - 8.5	0 - 38.3

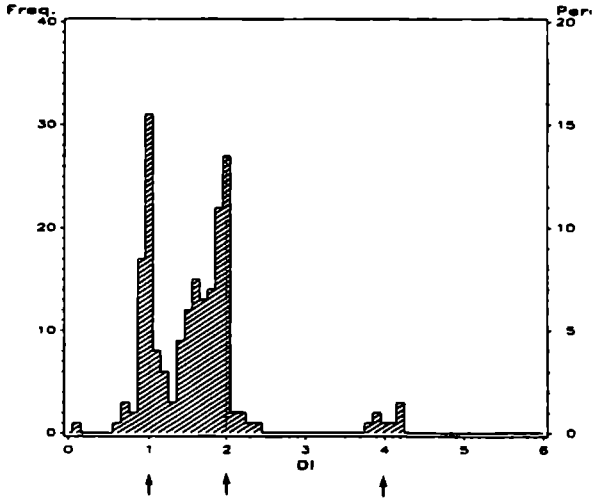

Figure 3. DNA ICM of a CM showing DNA-polyploidy: the DNA-diploid (arrow at DI = 1), DNA-tetraploid (arrow at DI = 2), and DNA-octaploid (arrow at DI = 4) peaks represent different nuclear fractions of the molar tissue.

Table III. Overall results of interphase cytogenetic analysis in extravillous trophoblast of hydatidiform moles with and without persistent gestational trophoblastic disease (PGTD).

Diagnosis	Total	Interphase cytogenetic analysis XY XX	Percentage of nuclei with ISH signals			
	N	*n* *n*	≤2 mean ± SD	≥3 mean ± SD	≥3 range	≥5 mean ± SD
CM - PGTD	20	4 16	77 1 ± 7 8	22 8 ± 7 8	11 1 - 40 7	2 8 ± 2 4
CM + PGTD	20	3 17	76 6 ± 8 3	23 4 ± 8 7	9 8 - 43 1	3 2 ± 3 6
PM + PGTD	4	2 2	80 1 ± 10 3	19 8 ± 10 3	7 1 - 32 3	2 9 ± 3 1

ISH = *in situ* hybridization
SD = standard deviation
CM /+ PGTD = complete mole without/with PGTD
PM + PGTD = partial mole with PGTD

Table IV Mean spectrum of *in situ* hybridization (ISH) signals per nucleus in extravillous trophoblast of hydatidiform moles with and without persistent gestational trophoblastic disease (PGTD).

Diagnosis	Interphase cytogenetic analysis Mean percentage and (*Standard Deviation*) of nuclei with ISH signals									
	0	1	2	3	4	5	6	7	8	>9
CM - PGTD	2 7	23 9	50 6	13 5	6 5	1 5	0 8	0 3	0 2	0 1
	(1 3)	*(5 4)*	*(7 2)*	*(3 6)*	*(2 8)*	*(1 1)*	*(0 6)*	*(0 3)*	*(0 3)*	*(0 3)*
CM + PGTD	3 5	23 0	50 0	13 7	6 5	1 6	0 7	0 4	0 3	0 2
	(2 2)	*(6 4)*	*(9 5)*	*(3 6)*	*(3 1)*	*(1 6)*	*(0 9)*	*(0 6)*	*(0 4)*	*(0 5)*
PM + PGTD	3 2	28 9	48 0	12 3	4 6	1 1	0 9	0 4	0 2	0 3
	(1 7)	*(9 1)*	*(4 8)*	*(5 5)*	*(2 4)*	*(0 8)*	*(1 0)*	*(0 6)*	*(0 3)*	*(0 5)*

CM /+ PGTD = complete mole without/with PGTD
PM + PGTD = partial mole with PGTD

The chorionic villi showed the same sex chromosomal composition as the NRBCs, amnion, or yolk sac (Fig. 5).

Statistical analysis showed no significant correlation between the 2.5c ER using ICM and the polysomic nuclear fraction using ISH.

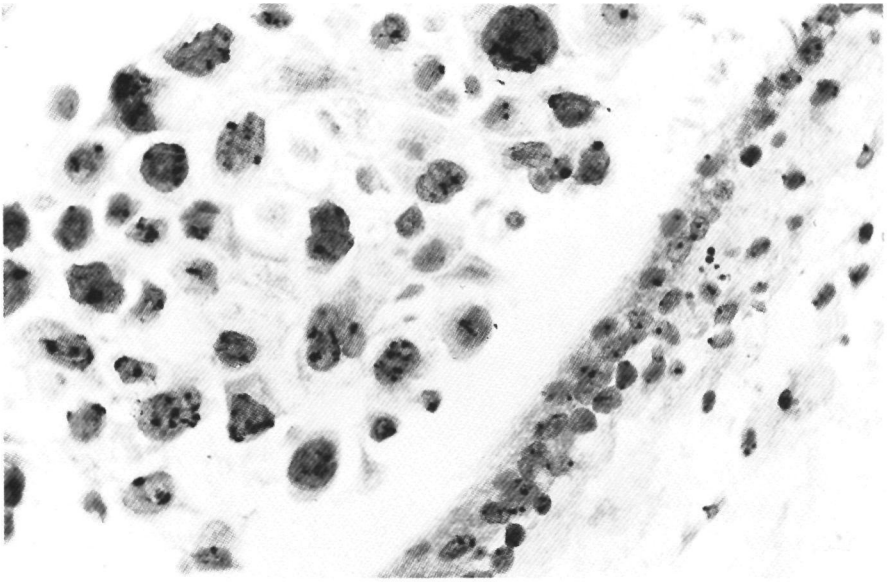

Figure 4. *Interphase cytogenetic analysis on a 6-μm paraffin-embedded tissue section using a biotinylated DNA-probe specific for the pericentromeric region of chromosome 1. The extravillous trophoblast shows prominent nuclear atypia with polysomy, in contrast to the villous trophoblast and stromal cells which show disomy (right; counterstaining with Mayer's hematoxylin, x 300).*

DISCUSSION

PGTD develops in only a minority of patients with a hydatidiform mole. The lack of a reliable predictor of PGTD, however, necessitates clinical follow-up in all patients. Nevertheless, the pathologist must distinguish the low-risk (0.5%) PM from the high-risk (10-20%) CM. The degree of nuclear atypia and the DNA content of the extravillous trophoblast are significantly higher in CMs than in PMs[12], but considerable variability also exists within CMs. Because the potential for malignant progression in hydatidiform moles is thought to be inherent to molar trophoblast and because tumor aggressiveness in general correlates with increase in nuclear atypia and DNA content, it seemed logical to explore the predictive value of histologic grade and DNA content in CMs.

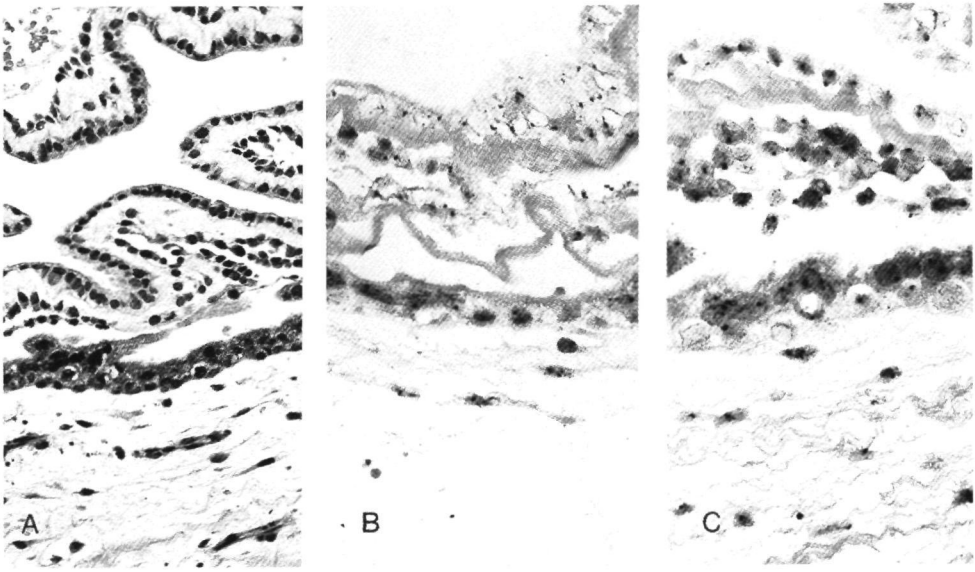

Figure 5. *Same case as shown in Figure 1A.* ***A).*** *H&E stain shows a detail of the amnion (upper part) and adjacent chorionic villus (lower part; x 500).* ***B and C).*** *Serial sections showing interphase cytogenetic analysis on 6-μm paraffin-embedded tissue sections using DNA-probes specific for chromosomes X (**B**) and Y (**C**). Both the villus and the amnion show monosomy for chromosomes X and Y (counterstain with Mayer's hematoxylin; x 500).*

Histologic grade, assessed on the basis of degree of trophoblastic hyperplasia and atypia, had already been suggested to have prognostic significance by Hertig and Sheldon[22] in a six-group grading system and by Hertig and Mansell[3] in a more reproducible three-group grading system. Although some studies[23,24] initially supported these findings, others could not[25,3] and finally the grading system has been abandoned.

DNA content had been related to progressive disease by Sugimori *et al.*[26], using micro-spectrophotometric analysis, which demonstrated a subsequent increase of DNA content in CMs without and with progression toward destructive mole and in choriocarcinoma. They also found a correlation between higher and more widely distributed DNA content and delayed ß-hCG regression curves. Using DNA flow cytometric techniques Martin *et al.*[15] also found a positive correlation between DNA content and the development of PGTD. PGTD developed in 45% of their series of 40 patients, involving 77% of aneuploid cases and only 30% of euploid cases. The correlation of clinical course with ploidy was

significant ($P<0.01$). No association was found with proliferative (mitotic) index ($P>0.05$). Other studies[11,13,16], however, have not confirmed these findings. Hemming *et al.*[16] noticed a relatively high tetraploid DNA-peak in most CMs, which they interpretated as a high proliferative fraction but which more likely represents the extravillous trophoblast with increased polyploidization[12]; Hemming *et al.*[16] could not detect a correlation between this fraction and the occurrence of PGTD. Lage *et al.*[11] and Fukunaga *et al.*[13] found a relatively high frequency of DNA-tetraploid CMs, but there was no increased incidence of PGTD in these cases as compared with their group of DNA-diploid CMs.

All of these studies were based on DNA flow cytometric analysis. A previous study[12], however, found that DNA flow cytometric analysis is less sensitive than DNA ICM and interphase cytogenetic analysis in the detection of aberrant cell subpopulations with increased DNA content. Using interphase cytogenetic analysis, we demonstrated that chorionic villi in CMs are always diploid, whereas the extravillous trophoblast shows extensive polyploidization. Others confirmed this finding[14]. Therefore, in the present study, both of these techniques were used for additional evaluation of the variability in DNA content in CMs and the possible relation to PGTD. Using DNA ICM, however, no such correlation could be found: almost all of the cases were found to be DNA-polyploid with a high 2.5c and 5c ER, which are objective numerical parameters for increased DNA content, but there was no significant difference in the height of the 2.5c and 5c ER between CMs with or without PGTD.

Interphase cytogenetic analysis performed on paraffin-embedded tissues allows the detection of numerical chromosomal aberrations at the nuclear level with conservation of histologic morphology. Therefore, a more specific evaluation of the area of interest (extravillous trophoblast) is possible. Again, however, no correlation could be found between the occurrence of PGTD and the degree of numerical chromosomal aberrations, neither with respect to the number of aberrant nuclei nor to the degree of chromosomal aberrations within the nucleus. Also, with this method, there will be an underestimation of the real degree of numerical chromosomal aberrations, because the nuclei are truncated as a result of tissue sectioning. Therefore, smaller variations could be missed.

On the basis of these results, we conclude that in CMs the degree of nuclear atypia and corresponding increase in DNA content is not correlated with PGTD. The mechanism of tumor progression in trophoblastic disease and somatic neoplasia, therefore, seems to be different. A possible explanation for these negative findings comes from experimental animal studies[27], from which it is known that during formation of the placenta different cycles of placental bed trophoblastic giant cells are formed, with high DNA contents present as a result of endoreduplication. This phenomenon especially occurs in the intermediate trophoblast at the placental implantation site, at which location trophoblast

cells invade the uterus between decidual and myometrial cells. This giant cell transformation is related to terminal differentiation, in which dividing potential is lost. In CMs the atypical intermediate trophoblast might then also represent terminal differentiation, although occurring at an abnormal site (in the extravillous trophoblast) and in abnormal degree (as compared with normal human pregnancy and abortion).

Although no correlation between PGTD and quantitative DNA changes could be found, this does not exclude a possible causative role for qualitative genomic changes. In CMs, all nuclear DNA is derived from the father. The majority of cases are homozygous (XX) because of the replication of the haploid genome of one sperm. In about 16% of cases, heterozygous DNA is found because of dispermy[2]. A sex ratio of 2:1 for XY:XX is expected (YY is nonviable) and corresponds with the reported incidence of XY moles (13%)[28]. Loss of heterozygosity, one of the mechanisms of tumor progression, seems likely and would favor the development of PGTD in homozygous CMs. Different studies[29-31], however, have shown that PGTD, including choriocarcinoma, can also develop in heterozygous CMs, and there have also been suggestions that heterozygous CMs have a higher risk of PGTD than do homozygous CMs[32]. This would imply a higher incidence of XY CMs in patients with PGTD than in patients without PGTD. In the present study, we could not confirm this. The frequency of XY was 15% in the group of CMs with PGTD and 20% in the group of CMs without PGTD, but the difference in the incidence was not statistically significant. Lawler *et al.*[2], too, could not detect a difference in the frequency of requirement for chemotherapy between patients with homozygous or heterozygous CMs.

Also of interest in this study is the finding that four cases, which were originally classified as PMs and which were accompanied by PGTD, showed trophoblastic hyperplasia that was identical to that of CMs with respect to the extent of hyperplasia, the degree of nuclear atypia, the DNA-ploidy and the pattern of numerical chromosomal aberrations. Twin gestation was unlikely on the basis of morphologic characteristics and because in all of our cases, the sex of the molar villi and of the NRBCs, amnion, or yolk sac was identical. If these cases are of androgenetic origin, this would imply that embryonal tissues can be formed in CMs, which would be in agreement with experimental mouse studies[33] as well as with two previous publications by Lage and Young[34] and Paradinas *et al.*[35] in which the occurrence of NRBCs in CMs is mentioned. It seems that this is especially likely in an early CM. Because ultrasonographic examination identifies more cases of CM and identifies them earlier, the pathologist must become accustomed to seeing NRBCs in CMs. Therefore, these cases must be classified as CMs to ensure a proper follow-up for PGTD.

In conclusion, DNA cytometric and interphase cytogenetic analysis have an important

applicative role in differentiating the low-risk PMs, which are DNA-triploid, from the high-risk CMs or from high-risk (and incorrectly classified) PMs, which are DNA-polyploid. In the latter group, the information derived from these techniques has no additional predictive value in the development of PGTD. Therefore, the mechanisms that play the decisive role in the progression of hydatidiform moles remain to be elucidated.

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CHAPTER 7

THE ROLE OF DNA IMAGE CYTOMETRIC AND INTERPHASE CYTOGENETIC ANALYSES IN THE DIFFERENTIAL DIAGNOSIS, PROGNOSIS AND CLINICAL FOLLOW-UP OF HYDATIDIFORM MOLES.

A REPORT FROM THE CENTRAL MOLAR REGISTRATION IN THE NETHERLANDS.

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THE ROLE OF DNA IMAGE CYTOMETRIC AND INTERPHASE CYTOGENETIC ANALYSES IN THE DIFFERENTIAL DIAGNOSIS, PROGNOSIS AND CLINICAL FOLLOW-UP OF HYDATIDIFORM MOLES.

A REPORT FROM THE CENTRAL MOLAR REGISTRATION IN THE NETHERLANDS.

SUMMARY

To assess the value of DNA-ploidy in the differential diagnosis and clinical follow-up of hydatidiform moles, the histopathology, DNA-ploidy and clinical follow-up were compared in 347 cases: 143 complete moles (CM), 52 partial moles (PM), and 152 abortions of which 56 cases were hydropic abortions with histological features of triploidy (HA+T), but lacked trophoblastic hyperplasia. In all cases, DNA image cytometry (DNA ICM) was performed and in 85 of these cases also interphase cytogenetics (ISH).

Using DNA ICM and ISH a bimodal polyploid DNA pattern was present in 97% of CM, 27% of PM and 4% of abortions. All of these cases of PM were reclassified to CM on basis of this DNA pattern and the histopathology, despite the presence of fetal blood cells, amnion or yolk sac. DNA-triploidy was found in 95% of the remaining PM, in 77% of HA+T, and in 14% of the remaining abortions. Reliable differentiation between DNA-triploid PM and HA+T was not possible on basis of the histopathology (trophoblastic hyperplasia) or 3.5c exceeding rates. DNA-diploidy was found in 1% of CM, in 23% of HA+T, and in 78% of the remaining abortions. DNA-tetraploidy was rarely found (1% of CM, 2% of PM, 1% of abortions).

Persistent gestational trophoblastic disease developed in 33% of the bimodal DNA-polyploid cases (all CM), in 1% of DNA-diploid cases (CM), and in 1% of the DNA-triploid cases (PM).

DNA analysis is mandatory in the diagnosis of hydatidiform moles to decide upon clinical follow-up.

Key words: hydatidiform mole, ploidy, in situ hybridization, persistent gestational trophoblastic disease

Hydatidiform mole is defined as an excessive growth of placental tissues with trophoblastic hyperplasia and cystic swelling of the chorionic villi. After evacuation the majority of cases resolves spontaneously, but some persist and show progression towards metastatic disease and/or choriocarcinoma. On the basis of histopathologic and cytogenetic criteria two entities are recognized[1,2], the complete mole (CM) and the partial mole (PM). CM show diffuse villous edema and trophoblastic hyperplasia in the absence of an embryo. CM have a diploid karyotype that is entirely paternally derived (androgenesis, either by duplication of the haploid genome of one sperm (always XX; YY is nonviable) or by dispermy (XX or XY in a ratio of 1:2))[3,4,5]. PM show focal villous edema and trophoblastic hyperplasia and evidence of an embryo. PM have a triploid karyotype with one maternal and two paternal contributions to the genome (diandry)[6,7].

Hydatidiform mole must be differentiated from hydropic abortions (HA). They show mild villous edema without trophoblastic hyperplasia. Various cytogenetic abnormalities can be found, with diploid or triploid karyotypes. HA have no increased risk for persistent gestational trophoblastic disease (PGTD), which implies a major difference in clinical follow-up. Despite well described histopathologic criteria, the differential diagnosis of CM, PM and HA remains to be difficult[8,9].

Several DNA flow cytometric (DNA FCM)[5,9-16] and few DNA image cytometric (DNA ICM) studies[12,14,15] have established the additional value of DNA cytometry in the differential diagnosis, but they have also reported a wider spectrum of DNA-ploidy than the cytogenetic studies. A high percentage of tetraploid CM and few cases of diploid and tetraploid PM were found[11,13]. The value of DNA analysis to predict the risk of PGTD after hydatidiform moles is controversial[10,11,13,17] and up to now no reliable predictor of PGTD is known. Therefore, all patients are recommended clinical follow-up with anticonceptive counseling and serum ß-hCG monitoring for at least one year.

In the present study we have analyzed 347 cases of CM, PM, HA, and non-hydropic abortions, with respect to histopathology and DNA ICM results, and in a selected number of these cases (85) also the results of interphase cytogenetic analysis. Of the 347 cases, 244 cases were referred to the Central Molar Registration of The Netherlands for consultation or review. The goals of this study were as follows: 1) comparison of the submitted histological diagnosis with the review diagnosis; 2) gain insight in the wide variability of DNA-ploidy; 3) evaluation of the additional value of DNA ploidy analysis in the differential diagnosis; and 4) evaluation of the additional value of DNA ploidy analysis in the assessment of the risk of PGTD and consequently in the clinical follow-up policy.

Patient material

During the years 1988 till 1993 the 347 cases were collected at the University Hospital of Nijmegen. Of these cases, 244 cases were obtained from different pathology laboratories throughout the country who referred their cases to the Central Molar Registration of The Netherlands located at the University Hospital of Nijmegen, where national registration of hydatidiform moles and PGTD is provided. This registration includes recording of data on clinical follow up, histological consultation and review and supraregional laboratory service for hCG monitoring. Data on incidences were also obtained from the Dutch National Pathology Information System, a national automated registration of pathological diagnosis in which all pathology laboratories in The Netherlands participate. Clinical follow-up after molar evacuation included serum hCG levels measured weekly until normal for 3 consecutive weeks and then monthly for 1 year. PGTD was diagnosed when hCG levels persisted at a plateau and/or raised for at least three consecutive weeks[18]. All hCG measurements were performed by means of a radioimmunoassay that measures both native and free ß-subunits, as described before[19].

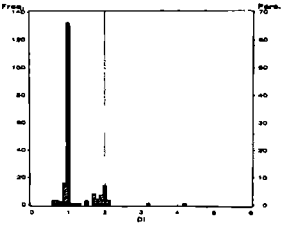
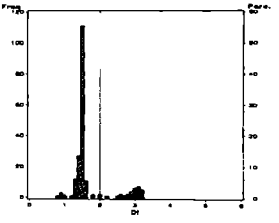
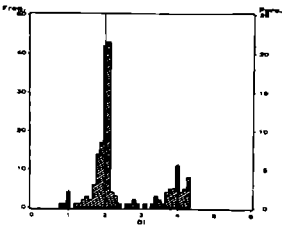
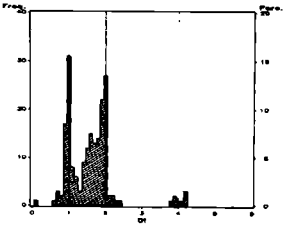
The histological slides of all cases were reviewed according to the criteria of Szulman and Surti[1,2]. A diagnosis of hydatidiform mole was not made if trophoblastic hyperplasia was lacking. If scalloped villous outlines and trophoblast inclusions were present, but villous edema was mild and trophoblastic proliferation not present, a diagnosis of HA with histological features of triploidy (HA+T) was preferred instead of PM.

DNA image cytometry

DNA ICM was performed on all cases, as previously described[12]. A brief description is given here. Paraffin tissue blocks were preferentially selected on the amount of trophoblastic hyperplasia. Intact nuclei were isolated from 50 μm thick paraffin tissue sections using 0.1 percent protease digestion for 20 min. Maternal decidual tissue was processed separately and served as internal control for normal diploid cells. If decidual cells were not available, maternal lymphocytes were used as alternative. The nuclear suspension was centrifuged to a glass slide using a cytocentrifuge (10 min at 500 rpm), air-dried, and fixed in a mixture of methanol, 37 percent formaldehyde, acetic acid (85:10:5 by volume) for 1 h. The nuclei were then stained with pararosaniline-Feulgen, after which the DNA content of 200 intact nuclei of villous stromal cells and of trophoblast were selectively measured using the CAS 100 Image Analysis System (Cellular Imaging Systems, Beckton and Dickinson, Leiden, The Netherlands). At least 30 rat liver cells (DNA-tetraploid) were measured as an external control for DNA content, while at least 20 decidual cells

were used as internal control. The DNA histograms were classified according to criteria as shown in Table I. The nuclear fractions with a DNA content exceeding the 2C G_0/G_1 peak, the 3C G_0/G_1 peak, or the 4C G_0/G_1 peak (G_0/G_1 ER), were calculated as the 2.5c,

Table I. Classification of DNA histograms.

Classification	Major peak *DNA-index*	Minor peak *DNA-index*	Histogram
DNA-diploid	1.0 ± 0.10	2.0 ± 0.20	
DNA-triploid	1.5 ± 0.15	3.0 ± 0.30	
DNA-tetraploid	2.0 ± 0.20	4.0 ± 0.40	
DNA-polyploid, bimodal	1.0 ± 0.10 2.0 ± 0.20	4.0 ± 0.40	

* DI = DNA - index

3.5c, or 4.5c exceeding rates (2.5c ER, 3.5c ER, or 4.5c ER), respectively. A 2.5c ER of 40% or more was used as a discriminator between DNA-diploidy and bimodal DNA-polyploidy[12].

Statistical analysis. The mutual differences in exceeding rates between the groups were analyzed by the Student *t*-test for unpaired observations. Logistic regression analysis was performed on the subgroups of PM with DNA-triploidy and HA with DNA-triploidy in order to assess whether the 3.5c ER could be used as a classifier to allocate individual patients in one of these two groups.

Interphase cytogenetics

In 85 selected cases of which the ICM DNA histogram was available, interphase cytogenetic analysis was performed on 6 μm thin paraffin embedded tissue sections. The 85 cases consisted of 41 cases of CM (40 cases bimodal DNA-polyploid, one case DNA-tetraploid), 7 twins with CM, 21 cases of PM (6 cases DNA-triploid, 14 cases bimodal DNA-polyploid and one case DNA-diploid), 9 cases of HA (6 cases DNA-triploid, one case DNA-diploid, 2 cases DNA-tetraploid), and 7 cases of abortions without hydropic degeneration (6 cases DNA-diploid, one case bimodal DNA-polyploid). The following chromosome specific DNA probes were used: the satellite III DNA probe for chromosome 1 (pUC 1.77), the alphoid DNA probe for chromosome X (pBam X5), and the satellite III DNA probe for chromosome Y (DYZ3), recognizing tandem repeats in the (peri)centromeric region (1q12) of chromosome 1[20], in the centromeric region of chromosome X[21], and in the q arm of chromosome Y[22], respectively. Biotinylation of the probes was performed using Bio-14-dATP (BRL: Gaithersburg, USA) according to the suppliers instructions.

The *in situ* hybridization procedure (ISH) in paraffin embedded tissue sections was performed as previously described[12,23], with minor modifications in the immunohistochemistry step: mouse anti-biotin (1:100 in PBS-tween with 5% non fat dry milk (NFDM); Dakopatts, Glostrup, Denmark) was followed by biotin labeled horse anti-mouse (1:200 in PBS-tween, 5% NFDM; Vector, Burlingame, Canada) and avidin-biotin complex (1:100 in PBS-tween, 5%NFDM; Vector).

Evaluation of ISH signals. In all cases DNA-probes specific for chromosomes 1, X and Y were used to determine ISH-ploidy and sex chromosome composition. The overall ISH-ploidy of the whole specimen was determined in the chorionic villi by counting the number of ISH signals in 500 nuclei of fibroblasts. Due to truncation of nuclei as a result of tissue sectioning at 6 μm not all nuclei showed the maximum copy number. This percentage of nuclei with the maximum copy number decreased with increasing copy number of chromosomes[23,24]. ISH-ploidy was based on the results of all three DNA

probes. A specimen was considered to be diploid if the maximum ISH copy number did not exceed two ISH-signals in more than 10% of the nuclei. In the same manner, in triploid cases the maximum copy number should not exceed three ISH-signals in more than 10% of the nuclei, and in tetraploid cases this maximum ISH copy number should not exceed four ISH-signals in more than 10% of the nuclei. The occurrence of focal numerical aberrations in e.g., extravillous trophoblast was evaluated by counting 500 nuclei in these areas. In otherwise diploid cases, focal polyploidy was considered to be present if in that area more than 10% of the nuclei displayed more than two ISH-signals. Likewise, in triploid cases more than three ISH-signals and in tetraploid cases more than four ISH-signals.

RESULTS

Histopathology

In 219 of the 244 referred cases a submitted diagnosis was provided by the pathologists who had initially seen the cases. The overall agreement between the submitted diagnoses and the revised diagnoses was only 55.7% (Table II). The percentage of agreement for the diagnosis PM was especially low (27.3%). Of the submitted cases of PM, 42.0% were revised to a diagnosis of CM and 30.7% to a diagnosis of nonmolar abortion.

Table II. Comparison between submitted histopathologic diagnosis and revised diagnosis.

Revised Diagnosis	Submitted Diagnosis				
	CM	PM	twin with CM	NM	Total
CM	77	34	0	4	115
PM	12	24	0	8	44
twin with CM	1	3	3	0	7
NM	8	27	0	18	53
Total	98	88	3	30	219

CM = complete mole PM = partial mole NM = nonmolar pregnancy

Histopathological features of CM which frequently resulted in a false diagnosis of PM were found to be: 1) presence of chorionic villi of seemingly normal size, as a result of gradual accumulation of edema in newly formed villi, giving the impression of edema to be focal (Fig. 1A); 2) the presence of irregular branching of chorionic villi giving the appearance of deep invaginations and large inclusion cysts on tangential cut, which are different from the scalloping villous outlines with small inclusion cysts in PM (Fig. 1B); and 3) the presence of capillaries. The presence of rudimentary capillaries in the villous stroma of otherwise typical CMs was found to be a rather frequent phenomenon. In 17% of the cases of CM these capillaries were prominent with lumina containing cellular and nuclear debris suggesting degenerated embryonal red blood cells (Fig. 2A). In 11 cases of otherwise typically CMs nucleated red blood cells, amnion or yolk sac were found (Fig. 2B), on basis of which formally a diagnosis of PM was given, albeit with a side-note that atypical trophoblastic hyperplasia was similar to that found in CM. In our experience, the most reliable criterium for a diagnosis of CM was trophoblastic hyperplasia with prominent nuclear atypia, especially of the intermediate trophoblastic cells (Fig. 3). This was never found in a typical PM. Syncytiotrophoblast with vacuolization was not exclusive for PM. It was also frequently found in CM. The disagreement between the

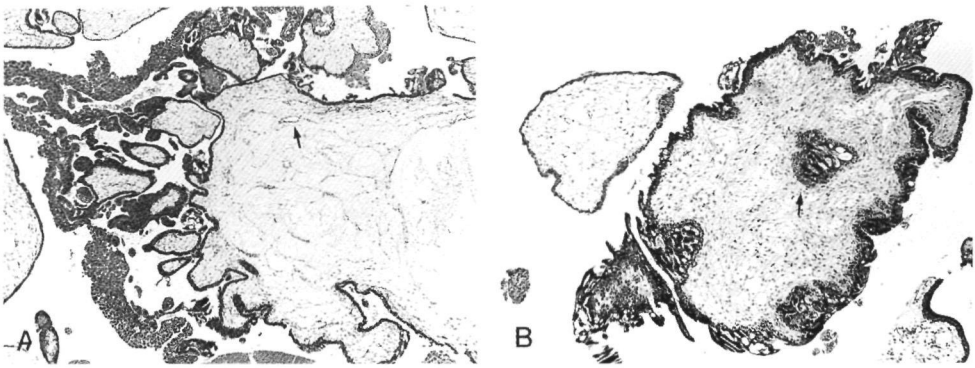


Figure 1. *A) Complete mole (CM) illustrating the formation of new villi, giving a branching aspect with deep invaginations, and progressive edematous degeneration. Trophoblastic hyperplasia is prominent. Note the presence of a capillary (arrow; H&E x50). B) Partial mole showing scalloping of villous outlines with small inclusion cyst (arrow) and excess of vacuolated syncytiotrophoblast (H&E x150).*

diagnoses of PM and hydropic nonmolar abortion was mostly based on the presence or absence of trophoblastic hyperplasia, which was found to be a subjective criterium for a diagnosis of PM.

In seven of the 347 cases a diagnosis of a twin pregnancy with CM was suspected, on basis of the presence of two separate villous populations. One part of the villi were normal with capillaries containing intact nucleated red blood cells, while the other villi were clearly abnormal with stromal edema and karyorrhexis, no capillaries or rudimentary capillaries without embryonal red blood cells, and with markedly atypical trophoblastic hyperplasia. In three cases an embryo was found.

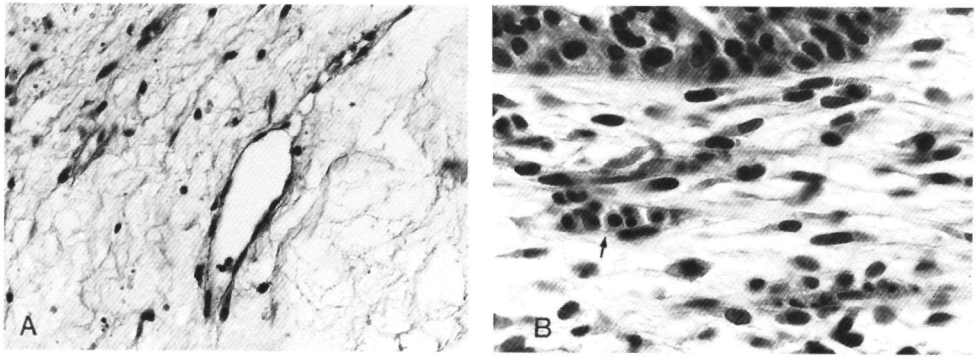


Figure 2. *In CM capillaries can be present, usually containing cellular debris (A, H&E x250) and rarely vital nucleated red blood cells (B, arrow; H&E x400).*

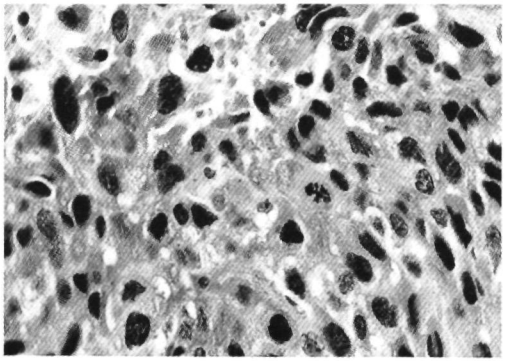


Figure 3. *In CM the trophoblastic hyperplasia shows prominent nuclear atypia (H&E, x350).*

Table III. DNA-ploidy in hydatidiform moles and abortions using image cytometric analysis.

Histological Diagnosis prior to DNA Analysis:	Total	DNA - ploidy pattern:			
		Diploid	Triploid	Tetraploid	Polyploid
		cases	cases	cases	cases
	N	n *(%)*	n *(%)*	n *(%)*	n *(%)*
Abortions	**152**	**88** *(58)*	**56** *(37)*	**2** *(1)*	**6** *(4)*
Without Hydropic Degeneration	48	41 *(85)*	3 *(6)*		4 *(8)*
With Hydropic Degeneration	48	34 *(71)*	10 *(21)*	2 *(4)*	2 *(4)*
With Hydropic Degeneration *and* Histological Features suggestive of "Triploidy"	56	13 *(23)*	43 *(77)*	-	-
Partial Moles	**52**	**1** *(2)*	**36** *(69)*	**1** *(2)*	**14** *(27)*
Gemelli with Complete Mole	**7**	-	-	-	**7** *(100)*
Complete Moles	**136**	**2** *(1)*	**1** *(1)*	**1** *(1)*	**132** *(97)*
Total	**347**	**91**	**93**	**4**	**159**

Table IV. Two-tailed P-values of statistical differences between complete moles, partial moles and hydropic abortions.

Diagnostic group	CM	HA	PM	PM + T	HA + T
CM	-	< 0 0001	0 02	<0 0001	<0 0001
HA			<0 0001	0 87	<0 0001
PM				<0 0001	<0 0001
PM + T					0 001
HA + T					-

CM, PM, HA = complete mole, partial mole and (hydropic) abortion with DNA-di/polyploidy
PM + T, HA + T = partial mole and hydropic abortion with DNA-triploidy

DNA image cytometry

The results of DNA ICM are given in Tables III and IV and Figure 4.

In CM a bimodal DNA-polyploidy was found in 97% of cases, and one case was DNA-tetraploid. The case which appeared to be DNA-triploid was considered as a misclassified case of PM.

In the 7 cases of twin pregnancy with CM, the normal and the molar villi had been separately processed. The normal villi showed a bimodal DNA-diploid pattern and the molar villi a DNA-polyploid pattern similar to that found in the cases of CM.

The majority of PM (69%) showed the expected DNA-triploidy. One case was DNA-tetraploid and histopathologically very similar to the case of DNA-tetraploid CM. Both cases were histologically borderline cases between CM and PM with slight to moderate nuclear atypia of the hyperplastic trophoblast. The G_0/G_1 ER (4.5c ER) was low, 29.5% and 22.0% respectively. Embryonal tissues were found in the case of PM, but not in the case of CM. In 11 of the 14 cases of PM with a bimodal DNA-polyploidy, embryonal tissues (nucleated red blood cells, amnion or yolk sac) were found and these cases were histopathologically classified as PM as mentioned above, with a side-note of atypical trophoblastic hyperplasia as usually found in CM.

In the majority of cases of (hydropic) abortion DNA-diploidy or DNA-triploidy was found. Few cases were DNA-polyploid with a maximum 2.5c ER of 54%. Implantation trophoblast was prominent in these cases, but without the nuclear atypia found in CM. Two cases of HA were DNA-tetraploid. Histopathologically they were not different from the other cases of HA.

Figure 4 gives the range of G_0/G_1 ER (2.5c ER and 3.5c ER) of the DNA-diploid, DNA-polyploid, and DNA-triploid cases. Table IV gives the two-tailed *P* values of statistical differences between the G_0/G_1 ER of the subgroups as depicted in Figure 4. Although a significant difference was found between the 3.5c ER of DNA-triploid PM and the lower 3.5c ER of DNA-triploid HA ($P = 0.001$), there was a considerable overlap between the 3.5c ER of each subgroup. Logistic regression analysis showed that it was not possible to distinguish between these two groups.

Interphase cytogenetics

The 40 cases of CM, all with a bimodal polyploid DNA ICM pattern, showed ISH-diploid chorionic villi and ISH-polyploid extravillous intermediate trophoblastic cells (Fig. 5). The mean percentage of ISH-polyploid nuclei in the 6 μm thin tissue sections was 23% (range 10 - 44%). The majority of this percentage of ISH-polyploid nuclei showed three ISH-signals (14%) or four ISH-signals (7%) nuclei, but a small percentage (3%) of nuclei showed ISH-signals of up to twelve spots. These ISH-signals were often larger in

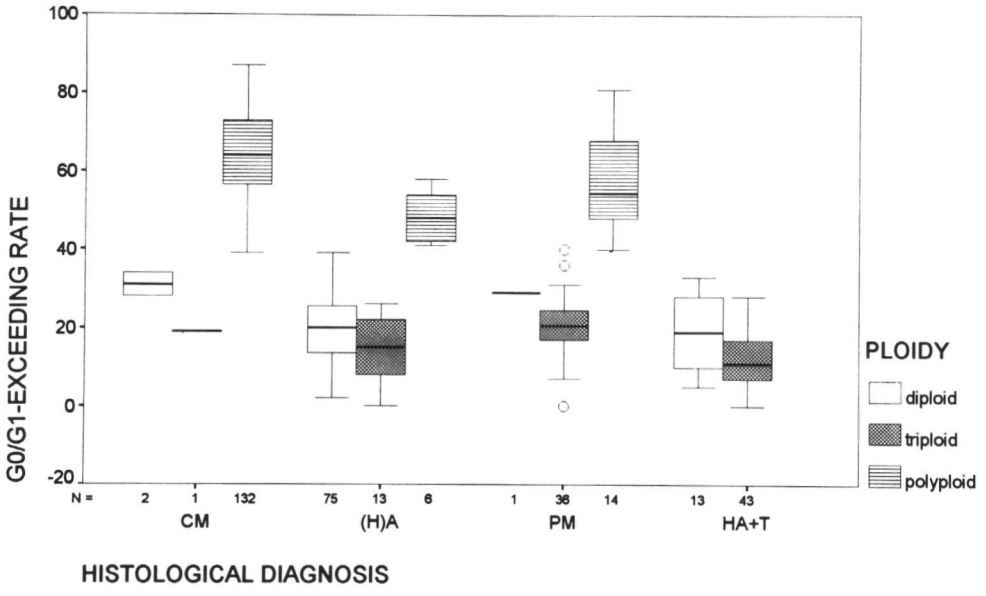


Figure 4. *G_0/G_1 exceeding rates (ER) of DNA-diploid and DNA-polyploid cases (2.5c ER), and of DNA-triploid cases (3.5c ER). The 2.5c ER of the seven twins with complete mole (CM) and the 4.5c ER of the four DNA-tetraploid cases are not shown. The G_0/G_1 ER of the DNA-diploid and DNA-triploid cases are in the same range and did not exceed the value of 40%. In the DNA-polyploid cases the G_0/G_1 ER of the diploid peak was equal or greater than the treshold value of 40%. (HA+T = hydropic abortion with histopathological features of triploidy).*

size and more intensely stained then the ISH-signals in the chorionic villi. ISH-tetraploid villi were not found.

In the 7 twin cases the molar villous population showed the same pattern as CM, with ISH diploid villi and ISH-polyploid extravillous trophoblast, while the normal villi with implantation trophoblast showed a ISH-diploid pattern. There were 3 cases with different sex chromosomes in the normal and molar villi, demonstrating dizygosity.

The six cases of PM and the six cases of HA with both histological features of triploidy and ICM results of triploidy all showed a uniform ISH-triploid pattern in both villi and extravillous trophoblast. Nuclei with more then three ISH-signals were only rarely (4%) found in the latter component, mainly associated with fibrinoid deposits in which nuclei often showed degeneration and disintegration of the ISH spots.

All 14 cases of PM with a bimodal polyploid DNA ICM pattern were investigated and

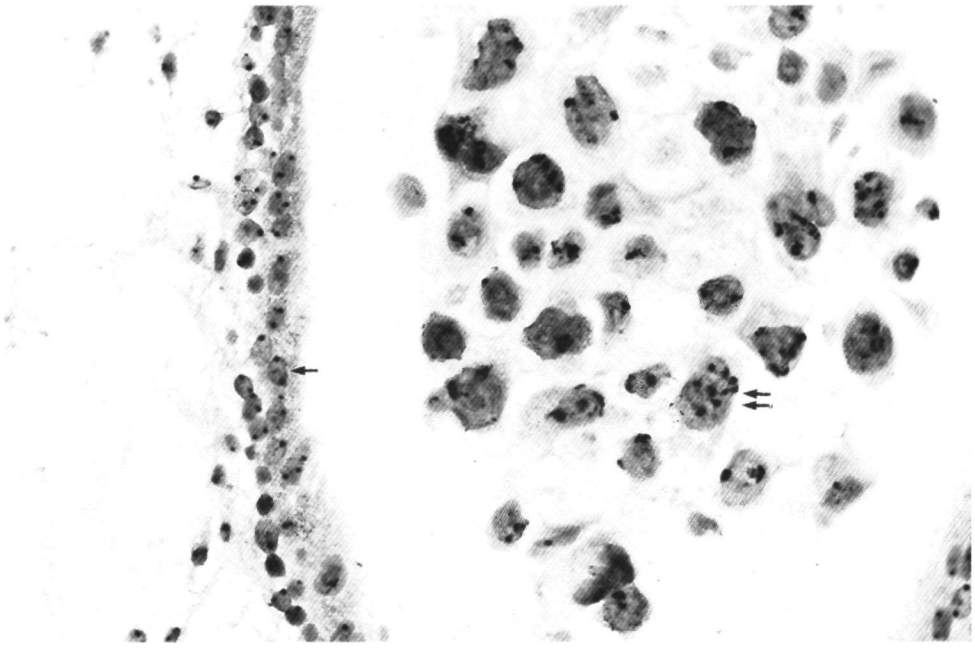


Figure 5. *Interphase cytogenetics on 6 μm thin tissue sections using biotinylated DNA probe specific for the pericentromeric region of chromosome 1 (hematoxylin counterstain). In complete mole the nuclei of the villous trophoblast and stromal cells show disomy (arrow), in contrast to the polysomy found in the markedly enlarged and atypical nuclei of the extravillous trophoblast (double arrow; x400).*

showed the same pattern as CM: ISH-diploid villi and ISH-polyploid extravillous trophoblast. In 11 cases with NRBC, amnion or yolk sac, these structures showed the same sex chromosome composition as the molar villous cells.

In the eight cases of abortion, seven cases with a diploid DNA ICM pattern and one case with a bimodal polyploid DNA ICM pattern (2.5c ER 54%), ISH-diploidy was found in chorionic villi as well as in implantation trophoblast. In the latter areas only few ISH-polyploid nuclei were found (5%).

The cases of CM, PM, and HA in which a tetraploid DNA ICM pattern was found, showed a uniform ISH-tetraploid pattern in both villi and extravillous trophoblast. The sex chromosome composition was XXXY in the CM, XXYY in the PM, and XXXX and XXYY in the HA. Polyploidization in extravillous trophoblast as seen in the DNA-polyploid CM was not found.

Clinical follow-up

Clinical follow up for at least one year was available for all cases of hydatidiform mole, with exception of 8 cases (6 cases of CM and 2 cases of PM) in which patients were lost from clinical follow-up. In the cases of HA with histological and cytometric features of triploidy inquiries for data of clinical follow-up were retrospectively made and obtained in all cases. Table V gives the results on the occurrence of PGTD requiring chemotherapy.

In cases of CM no differences were found between the cases with and without PGTD with respect to histology, DNA-ploidy, 2.5c ER, percentage of ISH-polyploid nuclei in the extravillous trophoblast, or the XX/XY ratio. The case of PGTD after DNA-triploid PM had a 3.5c ER of 19, and showed no differences with the other cases of DNA-triploid PM or HA+T. PGTD developed in 4 out of the 14 bimodal DNA-polyploid cases of PM. All 4 cases showed NRBC, amnion or yolk sac structure.

Pretreatment serum hCG levels were known in 32 patients with CM not followed by PGTD and in 15 patients with CM followed by PGTD. No correlation was found between the hCG level and the 2.5c ER or the occurrence of PGTD.

Table V. Frequency of persistent gestational trophoblastic disease in hydatidiform mole related to DNA-ploidy.

Histological Diagnosis:	Total	Diploid	Triploid	Tetraploid	Polyploid
		cases	cases	cases	cases
	n / N	n / N	n / N	n / N	n / N
Abortions	**0 / 152**	**0 / 88**	**0 / 56**	**0 / 2**	**0 / 6**
Without Hydropic Degeneration	0 / 48	0 / 41	0 / 3		0 / 4
With Hydropic Degeneration	0 / 48	0 / 34	0 / 10	0 / 2	0 / 2
With Hydropic Degeneration *and* Histological Features suggestive of "Triploidy"	0 / 56	0 / 13	0 / 43	-	-
Partial Moles	**5 / 52**	**0 / 1**	**1 / 36**	**0 / 1**	**4 / 14**
Gemelli with Complete Mole	**2 / 7**	-	-	-	**2 / 7**
Complete Moles	**47 / 136**	**1 / 2**	**0 / 1**	**0 / 1**	**46 / 132**
Total	**54 / 347**	**1 / 91**	**1 / 93**	**0 / 4**	**52 / 159**

In the present study the results of DNA ICM in the cases of CM seem quite different from that of all other studies published in the literature up till now (Table VI). The ICM DNA histograms that were found to be characteristic of CM in this study, as well as the differences with data of previous studies, can be explained by the results of the DNA-ISH findings. The bimodal polyploid DNA histogram of CM has a DNA-diploid G_0/G_1 peak due to the presence of ISH-diploidy in the different cell types of the chorionic villi, and a second major, DNA-tetraploid G_0/G_1 peak with S-phase cells and a small DNA-octaploid G_2/M peak due to the presence of proliferating ISH-tetraploid cells in the extravillous trophoblast. In most cases some trophoblastic cells showed more than 4 chromosome copies in these areas, most likely the consequence of doubling of the chromosomes of the tetraploid cells which is not followed by nuclear and cell division (endomitosis or endoreduplication). An increase in spot size also indicates the presence of doubling of the chromatides without division of the chromosome (polyteny). Both mechanisms result in polyploidization. It is difficult to detect such high copy numbers of chromosomes in thin tissue sections, because it has been shown that the probability to find all ISH-spots in a truncated nucleus decreases with the increase of chromosome copies[24]. The ISH-polyploidy found in the extravillous trophoblast corresponded well with the prominent nuclear atypia in these areas, which, in our experience, was the most typical and constant histopathologic feature of CM.

Our interphase cytogenetic studies have disclosed that the bimodal polyploid DNA histogram must be discerned from the unimodal diploid DNA histogram and the unimodal tetraploid DNA histogram. The former is characterized by a major DNA-diploid G_0/G_1 peak with S-phase cells and a small DNA-tetraploid G_2/M peak, and was seen in cases with ISH-diploidy in both the chorionic villi and extravillous (implantation) trophoblast. A good criterion to distinguish the DNA-diploid and the bimodal DNA-polyploid histogram appeared to be a 2.5c ER > 40[12]. The unimodal tetraploid DNA histogram is characterized by missing the major DNA-diploid G_0/G_1 peak of the bimodal DNA-polyploid histogram. All our cases with a tetraploid DNA histogram exhibited ISH-tetraploidy in both the chorionic villi and extravillous trophoblast.

The bimodal polyploid DNA histogram was found in 97% of the histopathologically diagnosed CMs and in all cases histopathologically diagnosed as gemelli pregnancies with CM. Unimodal DNA-diploidy was found in only 1% of our cases of CM, which could be explained by the small amounts of extravillous trophoblast in the paraffin tissue blocks that were analyzed. Unimodal DNA-tetraploidy was found to be rare (1%) in CMs. The bimodal polyploid DNA pattern was probably not always recognized or it was differently

Table VI. DNA classification of hydatidiform moles in different studies.

Study	Complete Moles						Partial moles					
	N	diploid	tetraploid	polyploid	triploid	aneuploid	N	diploid	tetraploid	polyploid	triploid	aneuploid
		%	%	%	%	%		%	%	%	%	%
Hemming et al[10]	49	94	0	-	4	2	32	31	3	-	63	3
Lawler et al [5]	151	85	0	-	1	-	49	2	4	-	86	-
Lage et al[11]	56	50	43	4	2	0	49	2	2	8	82	2
Conran et al[9]	21	90	10	-	0	-	29	0	0	-	100	-
Fukunaga et al[13]	129	71	19	-	0	9	43	5	0	-	95	0
Berezowsky et al[14]	32	53	47	-	0	-	18	11	0	-	89	-
Jeffers et al[15]	14	36	-	50	0	14	15	13	-	0	87	0
Paradinas et al [16]	150	99*	*	-	1	-	150	0*	*	-	97	-
Present study	143	1	1	97	1	-	52	2	2	27**	2	-

* DNA-diploid and DNA-tetraploid cases were taken together in this study

**Cases were reclassified as CM on basis of the DNA cytometry results

interpreted in other studies (Table VI). The results of the classical karyotyping studies, showing that CMs are diploid, can easily be explained by the fact that these studies are based on cultures of fibroblasts, which are derived from the diploid chorionic villi. The results of some DNA FCM studies[5,9-11,13-16], showing variable frequencies of DNA-diploid and DNA-tetraploid CMs, can partly be explained by a variable admixture of maternal cells obscuring the DNA-tetraploid G_0/G_1 peak in cases classified as DNA-diploid, and by incorrectly ascribing the DNA-diploid peak to the maternal cells and the DNA-tetraploid peak to the placental cells in cases classified as DNA-tetraploid. In one of these studies[14] DNA ICM was also performed on nuclear suspensions and on tissue sections. DNA ICM on tissue sections confirmed the presence of tetraploid cells in the extravillous trophoblast next to diploid chorionic villi. In this study and in another study[15] in which DNA FCM and DNA ICM on nuclear suspensions were performed DNA-tetraploidy and DNA-polyploidy were respectively found in 47 and 50% of the cases of CM using both methods. The discrepancy with our study using DNA ICM in which DNA-polyploidy was found in 97% of CMs might be explained by differences in the selection of the paraffin tissue blocks used for DNA analysis and by differences in the isolation procedure of nuclei from these paraffin tissue blocks.

It must be discouraged to interprete a bimodal polyploid DNA histogram in CMs as DNA-tetraploid, because this will give a false impression of a high incidence of DNA-tetraploid CMs. Genuine tetraploid moles have been described with a triple paternal DNA contribution and one copy of maternal DNA[5]. Some of these cases were originally histologically classified as CM, but are in fact PM. Due to a relative larger contribution of paternal DNA as compared to triploid partial moles, the morphology of these tetraploid cases might be in between that of PM and CM. This may also be the case for the risk of PGTD, but this is not yet known. Therefore, correct classification and clinical follow-up is mandatory.

In 27% of the histopathologically diagnosed cases of PM a bimodal polyploid DNA pattern was found. In all of these cases interphase cytogenetic analysis disclosed ISH-diploidy in chorionic villi and ISH-polyploidy in extravillous trophoblast, which showed a corresponding prominent nuclear atypia. On basis of these DNA ICM and ISH findings we concluded that these cases must be considered as misclassified cases of CM, despite of the presence of fetal red blood cells, amnion or yolk sac in 11 cases. In two of these cases the androgenetic origin could be proven in a seperate experiment using CA-repeat analysis (data not shown). From experimental mouse studies[25-27] as well as from our own observations and that from others[28,16], it must be concluded that initial embryonal development takes place in CM and that the presence of capillaries with nuclear debris and the occasional presence of vital NRBC, amnion or yolk sac does not exclude a

diagnosis of CM. In the remaining cases of PM, virtually all cases were DNA-triploid (95%). One DNA-diploid case was on review considered as a misclassified case of HA. Differentiation between PM and HA is extremly difficult, as has been illustrated by different publications[8,9] as well as by our own findings PMs are characterized by histopathologic features that are present in a variable and focal way. If trophoblastic hyperplasia is taken as an indispensable condition for the diagnosis of a hydatidiform mole, a diagnosis of PM can be made in only few cases. Differentiation from implantation trophoblast in HA is often difficult and subjective. If PM and HA with histopathologic features of triploidy are taken together, then the trophoblast is often more hypoplastic than hyperplastic Using DNA ICM a significant difference was found between the 3.5c ER of these two groups ($P = 0\ 001$), but the overlap was too extensive to be of any help in the diagnosis Reliable differentiation between PM and HA with histopathologic features of triploidy is not possible on basis of histopathologic or DNA ICM features. It is possible that both groups have the same cytogenetic background of diandric triploidy and must be considered as PM[28] DNA analysis showed that the histopathological criteria of triploidy cannot be relied upon even when used by pathologists with ample experience. In 10-20% of the cases, DNA-diploidy was found. These patients would have been given clinical follow-up unnecessarily if DNA-analysis was not performed. With ICM, DNA-triploidy was found in 14% of the cases of abortion without histopathological features of triploidy. It is assumed that these cases represent digynic triploids without increased risk of PGTD[29].

In this study PGTD developed in 33% of the bimodal DNA-polyploid cases (all being cases of CM). No correlation was found between the development of PGTD and the degree of DNA-polyploidization, as determined from the 2 5c ER and 5c ER, and from the results of interphase cytogenetics. This is in accordance with the results of most other studies[10,11,13], which were based on FCM Of the DNA-triploid cases only one case progressed to PGTD (1%) This case was diagnosed as PM No differences were found in histopathology or 3 5c ER with the other cases of PM Different data on incidences for PGTD following PM are reported in the literature, varying from 0 5%[30] to 5.5%[11], the investigators assuming 1% of pregnancies to be triploid and 86% of triploids to be PM[28] For The Netherlands with 200,000 pregnancies each year, this means that each year a diagnosis of PM would be made in about 1720 patients, and that 9 - 95 patients would develop PGTD. However, the mean number of registered cases of PGTD each year is 25 (virtually all being CM), which renders the data for development of PGTD after PM given in the literature very unlikely. In these studies, as in our own study, a strong selection bias occurred, because reference centers are involved which are likely to handle more complicated cases. When all cases of hydropic abortion with histological and DNA

cytometric features of triploidy are taken as PM, then the frequency of PGTD is very low (<<0.5%). If DNA-triploidy is not confirmed by DNA analysis many cases will be missed or overdiagnosed. Therefore, a rational base justifying a clinical follow-up policy as in CM (postponement of pregnancy and continuing monitoring of the serum ß - hCG levels after normalization) is lacking. Differences in the risk of PGTD after CM or PM can be important for the individual patient with respect to emotional distress, duration of follow-up and the planning of future pregnancies.

In conclusion we state that: 1) CMs have specific histopathologic features (e.g. atypical trophoblast hyperplasia) and a characteristic bimodal polyploid DNA pattern. Genuine tetraploid hydatidiform moles are rare. 2) PMs have histopathologic features that are focal and non-specific. A DNA-triploid pattern easily differentiates PM from CM, but not from nonmolar HA. If trophoblastic hyperplasia is not considered as an indispensable condition for the diagnosis of PM, many cases of PM have been missed in the past and many cases of nonmolar HA are likely to be overdiagnosed as PM in the future, when DNA analysis is not performed. 3) PGTD must be considered as a rare phenomenon of PM. 4) DNA analysis is mandatory for the correct classification of hydatidiform moles and nonmolar hydropic abortions. 5) For the development of PGTD DNA analysis has no direct predictive value.

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SUMMARY

Hydatidiform mole is a failure of conception, resulting in an excessive amount of placental tissues and an absent or maldeveloped fetus. The histopathological hallmarks are edema of the chorionic villi and trophoblastic hyperplasia. Two entities are delineated, the complete mole (CM) and the partial mole (PM). In CM the histopathological changes are diffuse and an embryo is absent. In PM the histopathological changes are focal and a (malformed) embryo is usually present. Hydatidiform mole has a risk to persist or to progress towards choriocarcinoma (persistent gestational trophoblastic disease, PGTD). For CM this risk is evident (20%), for PM this risk is uncertain. No histopathological or clinical parameters exist which can predict persistence or progression of the disease. As a consequence both patients diagnosed to have CM and patients diagnosed to have PM are given clinical follow-up with contraceptive measures and serum β-HCG monitoring for at least one year.

A correct histopathological classification of hydatidiform moles and differentiation from nonmolar hydropic abortion (HA), which has no risk of PGTD, is important, but proved to be very difficult. This is due partly to the rarity of the disorder and partly to changes in the concepts of PM. Additional techniques to assist in this differential diagnosis are needed. Different cytogenetic studies performed on fresh tissues have shown that CM have an androgenetically derived diploid genome and PM are associated with diandric triploidy. These techniques for different reasons are not suitable for daily routine pathology practice. This thesis describes the application of two techniques, DNA cytometry and interphase cytogenetics, that can be performed on routinely processed paraffin embedded tissues and that can have a role in this differential diagnosis (Chapters 2 to 5) as well as in the prediction of PGTD (Chapters 6 and 7).

In Chapter 1 an overview is given of the relevant literature on the cytogenetic origin of hydatidiform moles as well as a description of the histopathology. In the other chapters varying histopathological aspects are mentioned, but a compilation of the experiences gathered in the progress of this study are given in Chapter 1.

Chapter 2 describes the application of a newly developed protocol of interphase cytogenetic analysis on 6 μm paraffin embedded tissues sections of hydatidiform moles (five cases) and nonmolar hydropic abortions (two cases). Probes for repetitive DNA targets in the (peri)centromeric region of chromosomes 1 and X and in the q arm of chromosome Y were used to determine the copy number of chromosomes and the sex chromosome composition. In all cases the technique was successfully applied and enabled determination of the ploidy with preservation of histological context. Major advantages of this technique were 1) precise discrimination between maternal and trophoblastic cells, 2) no

risk of selecting certain cell types as a result of the isolation procedure (e.g. villous stromal cells in tissue cultures for karyotyping), and 3) detection and localization of cell subpopulations with heterogeneous DNA content. In CM the chorionic villi appeared to have diploid stromal cells and trophoblastic lining cells, whilst the extravillous trophoblast showed numerical chromosomal aberrations.

To find objective criteria in the differential diagnosis of hydatidiform moles and nonmolar hydropic abortions, Chapter 3 describes the combined application of DNA cytometry and interphase cytogenetics. DNA flow cytometry (FCM) and DNA image cytometry (ICM), with determination of G_0/G_1 exceeding rates, were performed on nuclear suspensions isolated from paraffin embedded tissues of 166 cases: 71 cases of CM, 20 cases of PM, and 75 cases of nonmolar abortions. The interphase cytogenetic analysis as initiated in Chapter 2 was extended to 23 cases: 12 cases of CM, 3 cases of PM, and 8 cases of nonmolar abortions. In contrast to previously reported findings that CM are diploid, the results of this study showed that using ICM CM are bimodal DNA-polyploid (96% of cases), with high G_0/G_1 exceeding rates (mean 61%) and, using interphase cytogenetics, display a high frequency of numerical chromosomal aberrations in the extravillous trophoblast (mean 28%), which was interpreted as polyploidization. Histopathologically a corresponding prominent nuclear atypia was found in these areas. Using FCM DNA-polyploidy was not or less obvious in 40% of cases. The majority of PM was DNA-triploid (55%), but bimodal DNA-polyploid cases were found in 30%. The question was raised whether these bimodal DNA-polyploid cases represented true PM or were in fact still CM, e.g. as part of a twin pregnancy. Nonmolar (hydropic) abortions were either DNA-diploid (60%) or DNA-triploid (39%). One case of nonmolar abortion with excessive implantation trophoblast showed also a bimodal DNA-polyploid pattern using ICM, but using interphase cytogenetics no corresponding high percentage of numerical chromosomal aberrations was found in the implantation trophoblast. The results of ICM in this case were interpreted as a high G_2/M fraction.

Chapter 4 and 5 deals with a further investigation of the unexpected finding of bimodal DNA-polyploid cases of PM. In Chapter 4 the hypothesis that some of these cases are twin pregnancies with CM is explored and confirmed. Six cases are described with normal chorionic villi and a normal embryo (in five cases) and abnormal chorionic villi which had histopathological features of CM. Interphase cytogenetic analysis revealed unequal sexes in the molar and normal villi in four cases, demonstrating dizygosity. The trophoblastic hyperplasia of the molar villi showed features characteristic of CM in histopathology (prominent nuclear atypia), using ICM (bimodal DNA-polyploidy) and using interphase cytogenetics (numerical chromosomal aberrations - polyploidization). In Chapter 5 eleven cases are analyzed with the histopathology of CM and the presence of

nucleated red blood cells (NRBC) in capillaries of molar villi, and / or presence of amnion or yolk sac. Normal villi were absent, excluding the possibility of a twin pregnancy with CM. DNA ICM showed a bimodal DNA-polyploid pattern. Interphase cytogenetics confirmed polyploidization in the extravillous trophoblast and showed that in every case sexes were identical in the molar villi and in the NRBCs, amnion or yolk sac. Three of these cases were analyzed with polymorphic CA-repeat markers and in two of them no maternal contribution was found for 4 out of 6 markers and 2 out of 5 markers respectively, indicating androgenesis. It was concluded that formation of NRBCs, amnion or yolk sac is possible in CM and does not necessarily indicate PM.

Chapter 6 is devoted to the question whether DNA ICM and interphase cytogenetics can have any predictive value in the development of PGTD. In this study 71 cases of CM and 4 cases of PM followed by PGTD and 100 cases of CM without PGTD were evaluated with DNA ICM for differences in ploidy pattern expressed as the 2.5c and 5c exceeding rates. Interphase cytogenetics was performed in 20 cases of CM and 4 cases of PM followed by PGTD, and in 20 cases of CM without PGTD to detect differences in the frequency of numerical chromosomal aberrations and in sex chromosome composition. The results showed no differences between CM with or without PGTD: using ICM the 2.5c and 5c exceeding rates were 62.6 and 62.4, and 6.5 and 6.0 respectively; using interphase cytogenetics the frequency of numerical chromosomal aberrations was 23.4 and 22.8%. The XY pattern showed no significant differences. The 4 cases of PM showed a bimodal DNA-polyploidy and were reclassified as CM with NRBCs or amnion. It was concluded that in CM the occurrence of PGTD could not be predicted on basis of variations in quantitative DNA content or sex chromosome composition.

Chapter 7 is partly an extension of the study initiated in Chapter 3 combined with data on clinical follow-up, and partly it summarizes the results of the foregoing Chapters. Histopathology, DNA-ploidy and clinical follow-up were compared in 347 cases: 143 CM, 52 PM, and 152 nonmolar abortions of which 56 cases had histopathological features of triploidy (HA+T), but lacked trophoblastic hyperplasia (a conditio sine qua non for the diagnosis of PM). In all 347 cases DNA ICM was performed and in 85 of these cases interphase cytogenetics. A bimodal DNA-polyploid pattern was present in 97% of CM, 27% of histopathologically classified cases of PM, and 4% of nonmolar abortions. The bimodal DNA-polyploid cases of PM were reclassified as CM with NRBC, amnion or yolk sac. DNA-triploidy was found in 95% of the remaining PM, in 77% of HA+T, and in 14% of the remaining nonmolar abortions. Reliable differentiation between (DNA-triploid) PM and HA+T was not possible on basis of the histopathology (trophoblastic hyperplasia, which appeared to be a highly subjective criterium) or of the 3.5c exceeding rates. DNA-diploidy was found in 1% of CM, in 23% of HA+T, and in

78% of the remaining nonmolar abortions. DNA-tetraploidy was rarely found (1% of CM, 2% of PM, 1% of abortions), a finding that is in contrast with some other large studies published in the literature. PGTD developed in 33% of the bimodal DNA-polyploid cases (all CM), in 1% of DNA-diploid cases (CM), and in 1% of the DNA-triploid cases (PM).

From this study the following conclusions can be drawn: 1) CM constitute an entity with specific histopathological features (e.g. atypical trophoblastic hyperplasia) and a characteristic bimodal DNA-polyploid pattern. The presence of NRBC, amnion, or yolk sac is rare, but does not necessarily indicate PM. If normal chorionic villi and an embryo are present, the possibility of a twin pregnancy with CM must be considered. 2) PM has various histopathological features, that are focal (and consequently not always found, e.g. trophoblastic hyperplasia) and none of which are specific. The DNA-triploid pattern easily differentiates PM from CM, but not from nonmolar HA. If trophoblastic hyperplasia is not considered as a *conditio sine qua non* for the diagnosis of PM, many cases of PM may have been missed in the past. Furthermore, if DNA analysis is not routinely performed, some cases of (DNA-diploid) nonmolar HA are likely to be overdiagnosed as PM in the future. 3) PGTD must be considered as a rare incident after a correctly diagnosed case of PM. 4) DNA analysis is important for the correct classification of hydatidiform moles and nonmolar hydropic abortions. For the development of PGTD DNA analysis has no direct predictive value, but it has an indirect value by assisting in making a correct diagnosis.

De mola hydatidosa is een stoornis in de conceptie, resulterend in een excessieve hoeveelheid placentair weefsel en een ontbrekende of afwijkende foetus. Histopathologische kenmerken zijn oedeem van de chorion vlokken en trofoblast hyperplasie. Er worden twee types onderscheiden, de complete mola (CM) en de partiële mola (PM). Bij de CM zijn de histopathologische veranderingen diffuus aanwezig en ontbreekt een embryo. Bij de PM zijn deze veranderingen focaal en wordt er meestal wel een (afwijkend) embryo gevonden. De mola hydatidosa heeft een kans op persisteren van de afwijking en/of progressie naar choriocarcinoma (persisterende gestationele trofoblast ziekte, PGTZ). Voor CM is deze kans 20%, voor PM is dit nog onduidelijk. Er zijn tot nu toe geen histopathologische of klinische parameters bekend, die het optreden van PGTZ kunnen voorspellen. Daarom krijgen zowel patiënten bij wie een CM is gediagnostiseerd als patiënten waarbij een PM is vastgesteld dezelfde klinische follow-up gedurende tenminste 1 jaar, met contraceptieve maatregelen en serum β-hCG monitoring.

Een goede histopathologische classificatie van de mola hydatidosa en differentiatie van de hydropische abortus (HA), welke geen verhoogde kans heeft op PGTZ, is belangrijk, maar blijkt niet altijd even gemakkelijk te zijn. Dit komt ten dele door de relatieve zeldzaamheid van de afwijking, en ten dele door veranderingen in de opvattingen over PM. Aanvullende technieken die kunnen helpen in deze differentiële diagnose zijn daarom noodzakelijk. Op basis van cytogenetische studies van vers materiaal heeft men kunnen vaststellen dat CM uitsluitend paternaal diploid DNA hebben (androgenesis), en dat de PM geassocieerd is met diandrische triploidie. Om verschillende redenen zijn deze technieken niet routinematig toepasbaar. Dit proefschrift geeft een beschrijving van de toepassing van twee technieken, DNA cytometrie en interfase cytogenetica, die wel uitgevoerd kunnen worden op routinematig bewerkt, paraffine ingebed weefsel en die een rol kunnen hebben in deze differentiële diagnostiek (Hoofdstuk 2 t/m 5) en in de voorspelling van het optreden van PGTZ (Hoofdstuk 6 en 7).

In Hoofdstuk 1 wordt een overzicht gegeven van de relevante literatuur over de cytogenetische origine van de mola hydatidosa en een beschrijving van de histopathologie. In de andere hoofdstukken komen wel diverse onderdelen van de histopathologie aan de orde, maar een samenvoeging van de ervaringen opgedaan tijdens dit onderzoek wordt weergegeven in een overzicht in Hoofdstuk 1.

Hoofdstuk 2 beschrijft de toepassing van een nieuw ontwikkeld protocol voor interfase cytogenetica op 6 μm dunne paraffine coupes, van mola (5 casus) en hydropische abortus (2 casus). DNA probes specifiek voor repetitieve sequenties in het (peri)centromere gebied van chromosoom 1 en X en in de q arm van chromosoom Y werden gebruikt om

het kopie aantal van het chromosoom en de geslachtschromosoom samenstelling te bepalen. In alle gevallen werd de techniek met succes toegepast en kon de ploidy worden vastgesteld met behoud van histologische context. De belangrijke voordelen van deze technieken zijn : 1) nauwkeurig onderscheid tussen maternale en trofoblastaire cellen; 2) geen risico op het uitselecteren van bepaalde type cellen als een gevolg van de isolatie-procedure (zoals b.v. stroma cellen bij weefselkulturen ten behoeve van karyotypering); en 3) detectie en lokalisatie van cel subpopulaties met heterogene DNA inhoud. In CM bleken de chorion vlokken diploide stroma cellen en trofoblastbekleding te hebben en numerieke chromosoomafwijkingen in de extravilleuse trofoblast.

Om te komen tot objectieve criteria voor de differentiële diagnose tussen mola hydatidosa en hydropische abortus wordt in Hoofdstuk 3 een beschrijving gegeven van de gecombineerde toepassing van DNA cytometrie en interfase cytogenetica. DNA flow cytometrie (FCM) en DNA image cytometrie (ICM), met bepaling van de G_0/G_1 exceeding rates, werden daartoe verricht op kernen geïsoleerd uit paraffine materiaal van 166 gevallen: 71 CM, 20 PM, en 75 gevallen van (hydropische) abortus. Het interfase cytogenetisch onderzoek zoals gestart in Hoofdstuk 2 werd uitgebreid tot 23 gevallen: 12 CM, 3 PM, en 8 gevallen van abortus. In tegenstelling tot de tot dan toe verschenen publikaties dat CM diploid zijn, laten de resultaten van dit onderzoek zien dat met ICM een bimodaal DNA-polyploid patroon wordt gevonden bij CM (96%), met hoge G_0/G_1 exceeding rates (gemiddeld 61%), en dat met interfase cytogenetica een hoge frequentie van numerieke chromosomale afwijkingen wordt gedetecteerd in de extravilleuse trofoblast (gemiddeld 28%), welke werd geïnterpreteerd als polyploidisatie. Histopathologisch werd er een sterke kernatypie gevonden in deze gebieden. Met FCM was deze DNA-polyploidie minder of niet duidelijk in 40% van de gevallen. De meerderheid van de PM was DNA-triploid (55%), maar bimodale DNA-polyploidie werd in 30% van de PM gezien. Het is de vraag of deze gevallen echte PM betreffen of toch misschien CM zijn, mogelijk als deel van een tweelingzwangerschap. De gevallen van (hydropische) abortus waren of DNA-diploid (60%) of DNA-triploid (39%). In één casus werd ook een bimodaal DNA-polyploid patroon gevonden met ICM, maar interfase cytogenetisch onderzoek bevestigde geen polyploidisatie in de implantatie trofoblast. Waarschijnlijk betrof het hier een hoge G_2/M fractie.

In Hoofdstuk 4 en 5 wordt verder aandacht besteed aan de onverwachte bevinding van bimodaal DNA-polyploide PM. In Hoofdstuk 4 wordt de hypothese tweelingzwangerschap met CM uitgewerkt en bevestigd. Zes gevallen worden hier beschreven met enerzijds normale chorion vlokken en een normaal embryo (in 5 gevallen) en anderzijds abnormale chorion vlokken met de histopathologische kenmerken van een CM. Interfase cytogenetische analyse toonde in 4 gevallen een dizygotie aan doordat de geslachten in mola en

normale vlokken verschillend waren. De trofoblast hyperplasie van de molavlokken was karakteristiek voor een CM met betrekking tot de histopathologie (sterke kernatypie), het DNA patroon (bimodaal polyploid), en de bevindingen met interfase cytogenetica (numerieke chromosomale aberraties - polyploidisatie). In Hoofdstuk 5 worden 11 gevallen beschreven die de histopathologische kenmerken van een CM hebben, maar waarbij kernhoudende rode bloedcellen (KRBC), amnion of dooierzak werden gevonden, in de afwezigheid van normale chorion vlokken, zodat een tweelingzwangerschap uitgesloten was. Met ICM werd een bimodaal DNA-polyploid patroon gevonden. Interfase cytogenetica toonde in alle gevallen polyploidisatie in de trofoblast hyperplasie en een gelijk geslacht in chorion vlokken, KRBC, amnion en dooierzak. In drie gevallen kon een analyse met polymorfe CA-repeat markers worden verricht, waarmee in twee van deze gevallen geen maternale contributie aan het genoom kon worden vastgesteld voor 4 van de 6 markers en 2 van de 5 markers respectievelijk, hetgeen wijst op androgenese. Op basis van deze bevindingen werd geconcludeerd dat in CM vorming van KRBC, amnion en/of dooierzak mogelijk is, en dus niet per definitie op een PM duidt.

Hoofdstuk 6 behandeld de vraag of DNA ICM en interfase cytogenetica een voorspellende waarde kunnen hebben bij de progressie tot PGTZ. In dit onderzoek werden 71 gevallen van CM en 4 gevallen van PM met PGTZ en 100 gevallen van CM zonder PGTZ geëvalueerd met DNA ICM voor verschillen in ploidy patroon en 2.5c en 5c exceeding rates. Interfase cytogenetica werd uitgevoerd in 20 gevallen van CM en 4 gevallen van PM met PGTZ en in 20 gevallen van CM zonder PGTZ, waarbij onderzocht werd of er verschillen waren in de frequentie van numerieke chromosomale aberraties en in geslachtschromosoom samenstelling. De resultaten toonden aan dat er geen verschillen waren tussen CM met en zonder PGTZ: met ICM werden 2.5c en 5c exceeding rates gevonden van 62.6 en 62.4, en 6.5 en 6.0 respectievelijk; met interfase cytogenetica was de frequentie van numerieke chromosomale aberraties 23.4 en 22.8%. Het XY patroon liet geen significante verschillen zien. De 4 gevallen van PM hadden een bimodaal DNA-polyploid patroon en werden gereclassificeerd als CM met KRBC of amnion. Geconcludeerd werd dat de DNA inhoud en de geslachtschromosoom samenstelling geen voorspellende waarde hadden bij het optreden van PGTZ na een CM.

Hoofdstuk 7 is gedeeltelijk een uitbreiding van het onderzoek zoals dat is gestart in Hoofdstuk 3, gecombineerd met gegevens over klinische follow-up. Gelijktijdig geeft het een samenvatting van de resultaten van de voorafgaande hoofdstukken. Histopathologie, DNA-ploidy en klinische follow-up werden vergeleken in 347 gevallen: 143 CM, 52 PM, en 152 gevallen van abortus waarvan 56 gevallen wel histopathologische kenmerken van triploidie toonden (HA+T), maar geen trofoblast hyperplasie (een conditio sine qua non voor de diagnose PM). DNA ICM werd uitgevoerd in alle 347 gevallen en interfase

cytogenetica in 85 van deze gevallen. Een bimodaal DNA-polyploid patroon werd gevonden in 97% van de CM, in 27% van de histologisch geclassificeerde gevallen van PM en in 4% van de gevallen van abortus. De bimodale DNA-polyploide gevallen van PM werden gereclassificeerd als CM met KRBC, amnion en/of dooierzak. DNA-triploidie werd gevonden in 95% van de resterende PM, in 77% van de HA+T en in 14% van de resterende gevallen van abortus. Een betrouwbare differentiatie tussen (DNA-triploide) PM en HA+T was niet mogelijk op grond van histopathologie (trofoblast hyperplasie bleek een zeer subjectief criterium) of op grond van 3.5c exceeding rates. DNA-diploidie werd gevonden in 1% van de CM, in 23% van de HA+T en in 78% van de overige gevallen van abortus. DNA-tetraploidie werd in een laag percentage aangetroffen (1% van CM, 2% van PM, 1% van abortus), een bevinding die niet in overeenstemming is met andere grote onderzoeken gepubliceerd in de literatuur. PGTZ trad op in 33% van de bimodaal DNA-polyploide gevallen (allen CM), in 1% van de DNA-diploide gevallen (CM) en in 1% van de DNA-triploide gevallen (PM).

Uit de resultaten van dit onderzoek kunnen de volgende conclusies worden getrokken: 1) CM is een entiteit met specifieke histopathologische kenmerken (o.a. atypische trofoblast hyperplasie) en een karakteristiek bimodaal polyploid DNA patroon. De aanwezigheid van KRBC, amnion en/of dooierzak is zeldzaam, maar sluit de diagnose CM niet uit. Indien normale chorion vlokken en/of een embryo aanwezig zijn, dan moet de mogelijkheid van een tweelingzwangerschap met CM overwogen worden. 2) PM heeft verschillende histopathologische kenmerken, die focaal zijn (en dus niet altijd worden aangetroffen, b.v. trofoblast hyperplasie) en waarvan er geen enkele specifiek is. Het DNA-triploide patroon maakt een goed onderscheid tussen PM en CM mogelijk, maar niet tussen PM en hydropische abortus. Als trofoblast hyperplasie niet als een *conditio sine qua non* voor de diagnose PM wordt beschouwd, dan zijn er mogelijk in het verleden veel gevallen van PM gemist. Bovendien kunnen er dan in de toekomst gevallen van (DNA-diploide) hydropische abortus worden overgediagnostiseerd als PM, indien DNA analyse niet routinematig wordt toegepast. 3) PGTZ moet als een zeldzame complicatie na een correct gediagnostiseerde casus van PM worden beschouwd. 4) DNA analyse is belangrijk voor de correcte classificatie van de mola hydatidosa en hydropische abortus. Voor het ontstaan van PGTZ heeft DNA analyse geen directe voorspellende waarde, maar het heeft wel een indirecte waarde door een ondersteunende rol bij het stellen van de correcte diagnose.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 4 oktober 1961 te Utrecht. Na de middelbare schoolopleiding (Gymnasium β) aan het Reformatorisch College Blaucapel te Utrecht, begon zij in 1980 met de studie Geneeskunde aan de Rijksuniversiteit Utrecht. In 1983 en 1986 behaalde zij respectievelijk het kandidaatsexamen en het doctoraalexamen cum laude. In de jaren 1982 tot 1984 was zij tevens werkzaam als student-assistent bij de vakgroepen Anatomie (snijzaal), Neuro-anatomie en Pathologie, en in 1984 en 1985 als student-onderzoeker bij de vakgroepen Veterinaire Pathologie en Pathologie. Hier verrichtte zij onderzoek naar de immunotypering van amyloid, onder leiding van Prof.dr. E. Gruys en in samenwerking met Prof.drs. J. Huber. Dit onderzoek resulteerde in een publikatie en een academische beloning (art. 60 W.W.O.). Op 28 augustus 1987 werd de studie Geneeskunde afgerond met het artsexamen.

Van 1 september 1987 tot 1 november 1992 was zij in opleiding tot patholoog bij de vakgroep Pathologie van het Academisch Ziekenhuis Nijmegen / Katholieke Universiteit Nijmegen (opleiders: Prof.dr. G.P. Vooijs en Prof.dr. D.J. Ruiter). In 1989 werkte zij tijdens een wetenschapsstage mee aan de ontwikkeling van een protocol voor interfase cytogenetica op paraffine coupes, onder leiding van dr. A.H.N. Hopman. In 1990 werd een aanvang gemaakt met het in dit proefschrift beschreven onderzoek. In het kader van dit onderzoek is zij lid van de Nederlandse Werkgroep Trofoblasttumoren en een van de referentie pathologen (tezamen met Prof.dr. G.P. Vooijs en dr. A.G.J.M. Hanselaar) van de Centrale Mola Registratie Nederland.

Vanaf 1 september 1992 is zij werkzaam als staflid patholoog aan het Instituut voor Pathologie van het Academisch Ziekenhuis Nijmegen / Katholieke Universiteit Nijmegen, als consulent voor de urologische pathologie, de kinderoncologische pathologie en de kinderobducties.

Op 26 augustus 1994 trouwde zij met Frank J.F. Hulsbergen. Op 1 juni 1995 werd hun zoon Alexander geboren.

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