

Gestational Trophoblastic Diseases: 2. Hyperglycosylated hCG as a Reliable Marker of Active Neoplasia.

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Abbreviated title: hCG-H in cancer invasion

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ABSTRACT

Objectives:

To determine whether circulating hyperglycosylated human chorionic gonadotropin (hCG-H), a promoter of choriocarcinoma growth and tumorigenesis, is a reliable marker of active gestational trophoblastic neoplasia (GTN) or choriocarcinoma, and whether hCG-H can consistently discriminate quiescent gestational trophoblastic disease (GTD) from neoplasia.

Methods:

Patients were those referred to the USA hCG Reference Service for consultation. These included a total of 82 women with GTN, including 30 with histologic choriocarcinoma. They were compared with 26 patients with resolving hydatidiform mole and 69 with quiescent GTD (persistent positive low value of real hCG but no clinical evidence of disease). All were tested for total hCG and hCG-H. hCG-H was calculated as the percentage of total hCG (hCG-H(%))

Results:

We compared the utility of total hCG and hCG-H(%) in detecting active GTN and quiescent GTD. There was no significant difference when measuring total hCG (includes regular and hyperglycosylated hCG), between women with quiescent GTD or self-resolving hydatidiform mole compared to choriocarcinoma/GTN cases ($P > 0.05$ and $P > 0.05$). In contrast, hCG-H(%) was significantly higher in choriocarcinoma/GTN cases ($P < 0.000001$, and $P < 0.000001$).

The usefulness of hCG and hCG-H(%) testing was assessed for discriminating between the 69 quiescent GTD cases, which required no therapy, and choriocarcinoma/GTN which need treatment. While hCG would detect 62% and 24% of malignancies at a 5% false positive rate, hCG-H(%) would detect 100% and 84% of malignancies at this same false positive rate. Follow-up data was received and repeat consultations were performed in 23 cases in which active disease was subsequently demonstrated. In 12 of 23 cases, hCG-H(%) results were able to first identify active disease 0.5 to 11 months prior to rapidly rising hCG or detection of clinically active neoplasia. In the remaining 11 cases hCG-H(%) active disease showed at the same time as rising hCG or demonstrable clinical tumor.

Discussion and Conclusion:

hCG-H(%) appears to reliably identify active trophoblastic malignancy. It is a 100% sensitive marker for discriminating quiescent GTD from active GTN/choriocarcinoma. It is also a marker for the early detection of new or recurrent GTN/choriocarcinoma. The data presented appears sufficient to encourage the adoption of hCG-H as a tumor marker in trophoblastic disease. Further studies are now urgently required to confirm and extend our findings.

INTRODUCTION

At the time when it appeared that the problems associated with the diagnosis and treatment of gestational trophoblastic disease (GTD) had been solved by early ultrasound diagnosis of hydatidiform mole, educated clinical management and effective chemotherapy, totally new problems have arisen. To a great extent these are associated with the ability to detect hCG accurately at much lower concentrations by use of modern automated tests, and with significantly greater knowledge about hCG structure and its degradation process [1]. Patients have presented with low levels of hCG persisting for months or years without clinical or imaging evidence of overt trophoblastic disease. These are patients with a history of a hydatidiform mole, gestational trophoblastic neoplasm (GTN) or choriocarcinoma, or spontaneously aborting pregnancy. In these cases, low levels of hCG appear at the completion of or in the months that follow therapy [2-7]. Alternatively, patients have been discovered by incidental pregnancy tests and then shown to have persistent low levels of hCG. This condition is named quiescent GTD [2-7]. By definition the patient has had persistent low levels of hCG present for 3 months or longer with no clear increasing or decreasing hCG trend. In all cases no disease is detectable clinically or by sophisticated imaging. Yet, in most cases investigated, the patient has been treated with single agent chemotherapy and when the hCG fails to decrease, combination chemotherapy and/or hysterectomy or other surgery. We know of no case that appropriately responded to the chemotherapy or surgery [2-7]. Initial studies indicate that these patients have residual syncytiotrophoblast cells with no or very minimal invasive cytotrophoblast cells and therefore have active disease [3,4]. Depending on the report, in 10-25% of these quiescent GTD cases the persistent hCG concentration changes and starts to elevate rapidly 5 months to 10 years after the finding of the persistent elevated hCG. In most of these cases a tumor is subsequently identified, with pathology indicating choriocarcinoma or other GTN [4-7]. This indicates that quiescent GTD is a pre-malignant syndrome with malignant transformation occurring in a proportion of cases [4-7].

Hyperglycosylated hCG (hCG-H) may aid in the identification of quiescent GTD and the early detection of onset of active disease. It is a carbohydrate variant of hCG with double-size sugar side chains, accounting for as much as 40% of the molecular weight of hCG. Interestingly, hCG-H is produced predominantly by invasive cytotrophoblast cells [8,9] making it a marker of invasive cells. The first publication in this series [10] and previous publications [11-12] have demonstrated that choriocarcinoma cell lines in culture produce mainly hCG-H

and that hCG-H is the factor that induces invasion by choriocarcinoma and pregnancy cytotrophoblast cells *in vitro*. When human choriocarcinoma cells are transplanted into athymic nude mice, a xenograph model of human choriocarcinoma, tumor growth and progression occurs. Injection of antibody against hCG-H blocks initial tumorigenesis and disease progression [10]. Independently it has been shown that when all forms of hCG are blocked in choriocarcinoma cells by an antisense RNA and transplanted into athymic nude mice an analogous blockage of initial tumorigenesis and disease progression occurs [13]. hCG-H therefore promotes tumorigenesis and choriocarcinoma progression *in vitro* and *in vivo*. Since hCG-H is secreted and present in the circulation in GTN and choriocarcinoma it may be a useful marker of invasive trophoblast behavior and help discriminate between GTN/choriocarcinoma and quiescent GTD. Here we investigate this exciting possibility.

METHODS

Patients

Serum samples were collected from patients referred to the USA hCG Reference Service, first at Yale University and then at the University of New Mexico, USA. Between 1998 and 2005, 151 patients were referred with active or suspected trophoblastic neoplasia and genuine hCG (excludes those with false positive hCG). These 151 patients do not include those with placental site trophoblastic tumors or non-trophoblastic malignancies who are being reported in a separate article in this series [14]. In addition, the Charing Cross Gestational Trophoblastic Disease Center (Imperial College, London, UK) provided serum samples from 26 patients with hydatidiform mole. These 177 cases are categorized in Table 1. With an obvious exception, no case from our Reference Service or from Charing Cross meeting these criteria were in any way selected or excluded for this evaluation. The exception is 10 cases initially suspected of having choriocarcinoma/GTN that were later demonstrated by histology to have a placental site trophoblastic tumor or non-trophoblastic malignancy.

To protect patient confidentiality all spreadsheets generated from accrued data were coded, without any identifiable personal information. Unfortunately, due to the nature of study, an analysis of accrued patient data, obtaining patient consent, or review board approval from referring institutions was not possible. This study and the evaluations of patient records were fully approved by University of New Mexico and Yale University Internal Review Boards (protocols 99-349 and 02-548), with the understanding that no request could be made to referring institutions for further medical records.

The USA hCG Reference Service is a consulting service that evaluates parallel serum and urine samples from women with gestational trophoblastic diseases or from those with unusual or questionable hCG results. Patient records are evaluated and a comprehensive array of tests for hCG and hCG-related molecules are performed [4]. The basic tests are a total hCG test (all forms of hCG-related molecule), a repeated total hCG test at multiple serum dilutions (to confirm the correctness of results), a total hCG test after treatment with heterophilic antibody blocking agent (HBR, Scantibodies Inc, San Diego CA) to consider false positive results, an hCG free β -subunit only test, and an hCG-H-only test (also repeated at multiple dilutions, where possible, to confirm results). The percentage of hCG-H in relation to total hCG (hCG-H(%)) is calculated. Additional tests are performed as indicated. These are an intact hCG only test, a nicked hCG only test, a nicked free β -subunit only test, and a β -subunit core fragment only test [3, 4]. After the tests are completed, the Reference Service prepares a

formal report for patient records, discussing the case in light of the results. The same basic tests were performed with the 26 patients providing serum samples from Charing Cross.

In this publication we have followed the recommendations of the Society of Gynecologic Oncologists that the term gestational trophoblastic disease is a collective noun that includes all types of trophoblastic disease particularly hydatidiform mole both benign and partial, invasive mole, histologic choriocarcinoma and other trophoblastic tumors. The term trophoblastic neoplasia implies an invasive process requiring chemotherapy and/or surgery. Terms such as malignant trophoblastic disease, invasive mole, persistent trophoblastic disease are being studiously avoided. Both SGO and FIGO require that placental site trophoblastic tumors and non-trophoblastic malignancies be reported separately from other gestational tumors. As such, these are both excluded here and are discussed in the following article in this series [14]. The staging and risk factor staging nomenclature implemented by FIGO in 2000 is used throughout this report.

Laboratory Tests

The USA hCG Reference Service laboratory is certified by the Department of Health and Human Services for performing clinical tests for patient records (CLIA certification 32D0972561). The consistency of laboratory tests is monitored by the College of American Pathologists (CAP certification 7176750-01).

Serum samples were received frozen and thawed and tested immediately upon arrival. All basic testing involved automated assays using pre-formulated reagent packs. Serum total hCG was measured using the robotic chemiluminescence DPC Immulite hCG test (DPC Inc., Las Angeles CA). This assay detects hCG, hCG-H and free β -subunit on an equal molar basis. When the concentrations of pure hCG, hCG-H and free β -subunit were determined in molar units (nmol) by absorbance at 278 nm, near-identical results were observed (in mIU/ml) in the DPC Immulite hCG test for these 3 molecules (hCG-H result 99% and free β -subunit result 100% of hCG standard concentration) [15].

Serum samples were tested for hCG-H using the Nichols Institute Diagnostics robotic chemiluminescence hCG-H assay (Nichols Institute Diagnostics, San Clemente CA). This assay has <0.1% cross-reactivity with hCG [4, 15], and is calibrated in ng/ml using choriocarcinoma hCG-H JEG-3 standard. This standard was purified from JEG-3 choriocarcinoma cell culture medium and shown by carbohydrate structure studies to be

representative to choriocarcinoma patient hCG-H [1, 11]. hCG-H mass values can be converted to hCG equivalents (in mIU/ml) by multiplying by 11 [11, 15].

It should be noted, that while the total hCG assay and the hCG-H assay are both FDA-approved tests, they are only approved for pregnancy applications. While gestational trophoblastic diseases are gestation-related applications the use of these and all other hCG assay for GTD is considered by some manufacturers as “off-label” applications. We have carefully evaluated both the total hCG and the hCG-H tests and demonstrated their particular suitability and accuracy, compared with other commercial hCG tests, for gestational trophoblastic disease applications [4, 16]; this does not however, necessarily surmount FDA regulations.

Protection of patient confidentiality

Clinical records were requested in each case from the referring physician along with the serum and urine samples. Follow-up information was also requested. The completeness of clinical records and follow-up information varied greatly. We have wanted to complete records and follow-up information, however, guidance rules governing Internal Review Boards have precluded us from requesting or obtaining further information from patient records from other institutions.

Data Analysis

Recently, all accrued test results by the USA hCG Reference Service from 1998-2005, dates, ages, diagnoses, antecedent gestation data, and pertinent treatment histories, were digitized by entry into a Microsoft Excel 2003 spreadsheet (Microsoft Inc., Redmond WA). Basic mean, range and standard deviation statistics and t statistics were determined in the Excel 2003 spreadsheet. Data groups were ranked, non-parametric 95th centiles were determined, and detection rates were calculated. Receiver operating characteristics (ROC) curves were plotted and areas under curves, asymptotic standard errors (SE) and multiple ROC curve structural components were calculated and predictive values compared using AccuROC software, version 2.4 (Accumetric Corp., Motreal, QC).

RESULTS

Of the 177 women, 82 had active disease (together called “choriocarcinoma/GTN,” based on the history and presence/absence of pathology). This includes 30 cases with histology proven choriocarcinoma and 52 with no histology or GTN (Table 1). We also evaluated 69 women diagnosed with quiescent GTD and 26 with self-resolving hydatidiform mole. This is 95 cases with “absence of clinically active disease. The histories of these groups are outlined in Table 1.

The “Absence of clinically active disease” patients include 69 described as quiescent gestational trophoblastic disease (quiescent GTD). Quiescent GTD was diagnosed in these cases by the observation of persistent low levels of hCG, with no increasing trend, continuing over a period of 3 months or longer (table 2). It also was indicated by the absence of tumor by clinical evaluation or sophisticated imaging techniques. In all the cases of quiescent GTD (table 2), persistent hCG results ranged from 1 mIU/ml to 212 mIU/ml, with a mean level (\pm standard deviation, SD) of 34 ± 38 mIU/ml and a 95th centile of 101 mIU/ml. As shown in Table 2, in all cases neither surgery nor chemotherapy eliminated the low level of hCG. In 67 of these 69 cases the presence of quiescent GTD was associated with the complete absence hCG-H. The exceptions were 2 of 69 (2.9%) cases with very low proportions of hCG-H (5% and 10%), in whom active disease appeared unlikely because of greater than 6 months of persistent very low levels of hCG (1.9 and 7.2 mIU/ml) with minimal variation. It is therefore inferred that a diagnosis of quiescent gestational GTD requires persistent low levels of hCG below 101 mIU/ml (the 95th centile of cases) persisting for 3 month or greater, with no detectable hCG-H, or extremely low proportions of hCG-H (in cases with longer term persistent low hCG results). There must be no clinical or imaging evidence of tumor. Ineffectiveness of chemotherapy and surgery, in eliminating persistent low levels of hCG is also characteristic of quiescent GTD.

The experience presented here shows that most cases of quiescent disease occur in those with clear history of gestational trophoblastic disease, 48 of 69 (70%) cases, whether choriocarcinoma (6 cases), GTN (9 cases), complete hydatidiform mole (28 cases), or partial hydatidiform mole (5 cases) (Table 1 and 2). While no cases were recorded following parturition, the remaining 21 cases followed a history of miscarriage or ectopic pregnancy with no demonstrated pathology. Of the 69 cases, 41 received ineffective single agent or multi-agent chemotherapy. In all 41 cases, this failed to significantly reduce the serum hCG

concentration. Hysterectomy or other major surgery was performed in 9 cases, and this also failed or only partially reduced hCG results. We have not recorded a single case of defined quiescent GTD in which chemotherapy or surgery successfully abated the condition.

The Reference Service encourages feedback from physicians to supplement its learning experience. In over 20 cases of quiescent gestational trophoblastic disease we have had personal interaction with physicians at conferences, by e-mails, and by phone about eventual outcomes (note entered into spreadsheet). This told us that within a 6 month period, persistent low levels of hCG had spontaneously resolved. We encourage courtesy follow-up evaluations for total hCG and the proportion of hCG-H or total hCG (hCG-H(%)). In 3 further patients we were asked to confirm the self resolution by a follow up consultation. In one case self-resolution occurred 4.5 years after initial consultation, and in 2 others, 3 month and 9 months following diagnosis of quiescent gestational trophoblastic disease. In contrast, we have had follow-up consultations with 13 patients with a history of quiescent gestational trophoblastic disease in whom persistent hCG results were followed by rising hCG showing active neoplasia (GTN or histology-confirmed choriocarcinoma) (Table 2). Two notes in our spreadsheet concerning patients not followed-up by the Service describe later development of active disease (no follow-up consultation). Therefore 15 of 69 (22%) patients with quiescent disease developed active disease. This occurred between 6 months and 4 years after the initial identification of quiescent GTD. Table 3 summarizes the histories of the 13 patients who provided follow-up serum samples to the Service (Table 3, cases 1 to 13).

We compared the usefulness of hCG and hCG-H(%) testing in detecting and differentiating active choriocarcinoma/GTN (Table 4). When examining hCG results alone, no significant difference was observed between results for quiescent gestational GTD cases or self-resolving hydatidiform mole cases with choriocarcinoma/GTN cases ($P>0.05$ and $P>0.05$). In contrast, a very significant difference was observed measuring hCG-H(%) ($P<0.0000001$ and $P<0.0000001$). As shown (Table 5), independent of an arbitrary cut-off value (ROC area under the curve analyses) hCG-H(%) would be $100 \pm 0.2\%$ accurate in discriminating active disease or quiescent GTD from choriocarcinoma/GTN, and 96% accurate in discriminating self resolving hydatidiform mole from choriocarcinoma/GTN. Considering the 95th centiles of the quiescent GTD and self resolving hydatidiform mole control groups as cut-off, hCG would detect 62% and 29% of malignancies at a 5% false positive rate, and hCG-H(%) would detect 100% and 89% of malignancies at this false positive rate (Table 5). These cut-off values ($>0\%$ and $>13\%$ hCG-H, for quiescent GTD and self resolving hydatidiform mole, respectively)

should be considered for clinical applications. Using ROC area under the curve analysis, hCG-H is $100 \pm 0.2\%$ accurate in differentiating active disease from quiescent GTD, and $96 \pm 1.8\%$ accurate in differentiating active disease from self resolving mole.

At present, rapidly rising hCG results (i.e. low levels doubling) are used as the principal test for identification or recurrence of disease or transformation of a quiescent gestational trophoblastic disease. Follow-up information was received or repeat consultations were performed in 23 cases in the months after initial consultation with the Service, in whom onset of active disease was demonstrated, in 13 cases with quiescent GTD (Table 3, cases 1-13) and 10 other cases (Table 3, cases 14-23). In 12 of 23 (52%) patients, hCG-H(%) results were able to first identify active disease, prior to rapidly rising hCG or demonstration of a tumor. In cases 1, 2, 3, 4, 5, 6, 7, 19, 20, 21, 22, 23 (Table 3) active disease was indicated by hCG-H(%) at 3, 2, 0.5, 1, 3, 11, 0.75, 2, 2, 2, 0.5 and 1 months, respectively, prior to rising hCG results or imageable or demonstrated tumor. In all these cases, diagnosis may have been made earlier and therapy initiated earlier if the physician had accepted hCG-H(%) as signifying active disease. In the remaining 11 cases the presence of hCG-H(%) confirmed the otherwise demonstrated presence of tumor (Table 3). These 23 cases comprised 13 patients with the diagnosis of quiescent GTD who developed choriocarcinoma/GTN, and 10 who developed a new or recurrent of GTN/choriocarcinoma with no proven quiescent GTN. The recurrence in 10 of 10 of these patients was initially shown by the presence of hCG-H.

DISCUSSION

The evidence presented here shows that hCG-H is a very high sensitivity marker of active GTN or choriocarcinoma, and for discriminating quiescent GTD. With close to absolute statistics it differentiates active disease requiring chemotherapy from quiescent GTD not requiring therapy. Currently, multiple measurements of rising hCG results are required to identify new, recurrent or active choriocarcinoma/GTN. As indicated here, a single measurement showing the presence of hCG-H is sufficient to conclude the presence of active disease. This allows therapy to be initiated at the earliest time or shows the need to delay treatment until hCG-H is present. The data presented appears sufficient to encourage the adoption of hCG-H as a tumor marker in trophoblastic disease.

As indicated, the 95% centile of quiescent GTD and self resolving hydatidiform mole cases, >0% of >13% hCG-H, should be considered as a cut off for clinical utility. Using this test 100% accuracy is achieved in discrimination active disease (choriocarcinoma/GTN) from quiescent GTD and 96% accuracy in differentiating active disease from self-resolving mole.

As shown in the preceding article [10] and confirmed in other studies [13] hCG-H is an essential promoter of human trophoblastic invasion, both *in vitro* and *in vivo*. Here we report the translation of this basic science information to clinical practice and demonstrate that, indeed, the presence of hCG-H as a proportion of hCG is a reliable marker of active trophoblastic neoplasia. This study by the USA hCG Reference Service with 69 quiescent GTD and 82 neoplastic cases, including 23 serial studies, is uniquely comprehensive, representing the largest study of its kind. This is in part due to the specialized role of the Reference Service in consulting in a significant proportion of gestational trophoblastic disease cases in the USA. They have for 1 year, since the FDA approval of the hCG-H test, been using hCG-H(%) results to state whether active disease is present or absent (46 cases), without identified problem.

It is a serious problem that in spite of the absence of detectable tumor both on clinical examination and by using sophisticated imaging 41 of 69 (59%) quiescent GTD patients were treated with single agent or combination chemotherapy and had surgery, usually hysterectomy, without appropriate decline in the level of hCG (chemotherapy had no clear effect, hysterectomy, in some cases, partially suppressed hCG results). Fortunately, so far only one patient has died of bleomycin pulmonary toxicity after two years of several single agent and combination chemotherapy in the absence of hCG-H and without any detectable active disease. Physicians presently appear reluctant to initiate chemotherapy in patients until hCG levels are significantly high or metastases, usually to lung or brain, have developed, in spite of

the presence of elevated hCG-H(%). In 12 cases, therapy was not started until 0.5 - 11 months after active disease was indicated by the increased proportion of hCG-H. Thus, there are two problems, failure to stop therapy when hCG-H indicates quiescent GTD, backed-up by no physical or imageable presence of disease, and second, failure to react to an elevated hCG-H(%) finding, indicating the presence of active disease. Physicians wait until hCG steeply rises or disease is confirmed by the demonstration of a tumor. The purpose of this paper is to encourage physicians treating GTD to avoid unnecessary treatments, and to initiate treatment of neoplastic disease at the earliest time. Cytotrophoblast cells are the emergent or invasive trophoblast cells [17]. Since hCG is produced by syncytiotrophoblast and hCG-H by cytotrophoblast we conclude that the absence of detectable hCG-H in patients with quiescent GTD must be associated with rapid conversion of cytotrophoblast to syncytiotrophoblast or by the loss of cytotrophoblast cells in patients treated with chemotherapy. Since patients with quiescent GTD have no clinically detectable disease the number of cells present must be less than 10^9 the number of cells necessary to be imaged [17], and more than 10^7 , a level below which hCG becomes undetectable [17]. It is therefore inferred that the number of cytotrophoblast cells present in quiescent GTN are insufficient to generate detectable hCG-H but the derived syncytiotrophoblast is sufficient to make the low levels of detectable hCG. Clearly, when quiescent GTD becomes active the number of cytotrophoblast cells increases sufficiently to produce detectable hCG-H. This increase in the number of cytotrophoblast cells explains the delay in the detection of clinical disease by rising hCG levels and in detecting a clinically evident tumor. It is also inferred that the percentage of hCG-H is representative of the ratio of cytotrophoblast to syncytiotrophoblast cells.

The FDA has approved the automated hCG-H test (Nichols Advantage hCG-H Test). Because hCG-H is not effective in an hCG corpus luteal cell progesterone promoting biological assay it cannot be calibrated in mIU/ml like hCG. This test is calibrated in ng/ml using a choriocarcinoma hCG-H standard. The hCG-H mass values can be converted to hCG equivalents in mIU/ml by multiplying by 11 [11, 15]. Once this is known hCG-H(%) can be determined by dividing this value by the hCG value. While the data presented here is sufficiently persuasive to encourage the adoption of hCG-H(%) as a tumor marker for predicting malignant behavior at the 95th centile (>0%), using the FDA approved test, repeat studies are urgently needed to confirm and expand upon the findings.

Table 1. Clinical characteristics of gestational trophoblastic disease patients referred to the USA hCG Reference Service (excludes PSTT).

Description	cases
1. Choriocarcinoma/GTN	
Choriocarcinoma, proven by histology	30
GTN, no pathology	52
Total	82
1a. "Advanced" choriocarcinoma/GTN (FIGO stage III and IV)	
Lung metastases only	17
Lung and brain metastases	9
Lung and liver metastases	4
Liver and uterus metastases	1
Liver metastases only	1
Brain metastases only	1
Total	33
1b. "Early" choriocarcinoma/GTN	
Pelvic, vaginal or uterine lesion only (FIGO stages I and II)	4
Post hydatidiform mole rising hCG, no disease imageable	20
Persistent elevated hCG, imaging confirmed active disease up to 8 weeks later	25
Total	49
2. Absence of clinically active disease (controls)	
Quiescent gestational trophoblastic disease, prior or following therapy	69
Hydatidiform mole, self resolving, 1-5 weeks post D&C	26
Total	95
2a. Quiescent gestational trophoblastic disease	
After complete hydatidiform mole	28
After partial hydatidiform mole	5
After choriocarcinoma	6
After GTN	9
After spontaneously aborted pregnancy, no pathology	16
After ectopic pregnancy	5
Total	69
2b. Hydatidiform mole, self resolving	
Complete hydatidiform mole	17
Partial hydatidiform mole	9
Total	26

Table 2. Summary of 69 cases with quiescent gestational trophoblastic disease (Quiescent GTD) referred to the USA hCG Reference Service. In all patients with a history of choriocarcinoma, diagnosis was demonstrated by histology. "Therapy received" is that given during time when persistent low levels of hCG were present, and not that with the initial disease. Therapies are Mtx or methotrexate, ActD or actinomycin D, EMA-CO or etoposide-Mtx-ActD (EMA) alternating with cyclophosphamide-vincristine (CO) combination therapy, EMA-EP or EMA alternating with etoposide-cisplatin (EP), Ifo or ifosfamide, Hys or hysterectomy, and BSO or bilateral salpingo-oophorectomy. Therapy effect summarize the results of therapy received, where none means no obvious change in persistent low hCG levels and partial means a partial reduction in hCG. In no cases did therapy completely suppress hCG. HCG-H(%) is hCG-H as a proportion of total hCG.

Case	hCG (mIU/ml)	hCG-H (ng/ml)	HCG-H(%)	Antecedent gestation	Therapy received	Therapy Effect
1	1	<0.2	0	Choriocarcinoma	None	
2	29	<0.2	0	Choriocarcinoma	None	
3	8	<0.2	0 ^a	Choriocarcinoma	Mtx, EMA-CO, Hys	None
4	48	<0.2	0 ^a	Choriocarcinoma	EMA-CO, EMA-EP	None
5	16	<0.2	0 ^a	Choriocarcinoma	Taxol, Ifo	None
6	37	<0.2	0 ^a	Choriocarcinoma	None	
7	3	<0.2	0	Complete Mole	Mtx, ActD	None
8	3	<0.2	0	Complete Mole	Mtx	None
9	5	<0.2	0	Complete Mole	Mtx, ActD	None
10	8	<0.2	0	Complete Mole	Mtx, ActD	None
11	8	<0.2	0	Complete Mole	Mtx	None
12	12	<0.2	0	Complete Mole	Mtx	None
13	12	<0.2	0	Complete Mole	Mtx	None
14	14	<0.2	0	Complete Mole	None	
15	24	<0.2	0	Complete Mole	Mtx, EMA-CO, Hys	None
16	25	<0.2	0	Complete Mole	None	
17	27	<0.2	0	Complete Mole	Mtx	None
18	28	<0.2	0	Complete Mole	None	
19	29	<0.2	0	Complete Mole	Mtx, ActD	None
20	34	<0.2	0	Complete Mole	Mtx, ActD	None
21	35	<0.2	0	Complete Mole	Mtx, ActD, EMA-CO	None
22	38	<0.2	0	Complete Mole	None	
23	48	<0.2	0	Complete Mole	Mtx, ActD, EMA-CO	None
24	48	<0.2	0	Complete Mole	EP	None
25	70	<0.2	0	Complete Mole	Mtx, ActD, Hys, BSO	Partial
26	80	<0.2	0	Complete Mole	Mtx	None
27	90	<0.2	0	Complete Mole	Mtx	None
28	110	<0.2	0	Complete Mole	Hys, BSO, ActD	Partial
29	11	<0.2	0 ^a	Complete Mole	Mtx, ActD	None
30	12	<0.2	0 ^a	Complete Mole	Mtx	None
31	13	<0.2	0 ^a	Complete Mole	None	
32	24	<0.2	0 ^a	Complete Mole	None	
33	57	<0.2	0 ^a	Complete mole	EMA-CO	None
34	161	0.72	5% ^b	Complete Mole	Mtx	None
35	10	<0.2	0	Ectopic Pregnancy	None	
36	2	<0.2	0	Ectopic Pregnancy	Mtx	None
37	43	<0.2	0	Ectopic Pregnancy	None	

38	59	<0.2	0 ^a	Ectopic Pregnancy	Mtx	None
39	212	1.9	10% ^b	Ectopic pregnancy	Mtx, BSO	None
40	35	<0.2	0	GTN	Mtx, ActD, Hys, BSO	Partial
41	51	<0.2	0 ^a	GTN	none	
42	144	<0.2	0	GTN	Mtx, ActD	None
43	6	<0.2	0	GTN	Mtx, ActD	None
44	44	<0.2	0	GTN	Hys, Mtx, EMA-CO	Partial
45	6	<0.2	0	GTN	Mtx	None
46	10	<0.2	0	GTN	Hys	Partial
47	15	<0.2	0	GTN	EMA-CO	None
48	44	<0.2	0	GTN	EMA-CO	None
49	13	<0.2	0	Partial Mole	None	
50	18	<0.2	0	Partial Mole	None	
51	50	<0.2	0	Partial Mole	EMA-CO	None
52	62	<0.2	0 ^a	Partial mole	Mtx	None
53	11	<0.2	0	Partial Mole	Mtx	None
54	14	<0.2	0	Miscarriage	None	
55	19	<0.2	0	Miscarriage	Mtx	None
56	90	<0.2	0	Miscarriage	Mtx, ActD, EMA-CO	None
57	7	<0.2	0	Miscarriage	None	
58	8	<0.2	0	Miscarriage	Mtx	None
59	10	<0.2	0	Miscarriage	None	
60	10	<0.2	0 ^a	Miscarriage	None	
61	13	<0.2	0	Miscarriage	None	
62	16	<0.2	0	Miscarriage	None	
63	21	<0.2	0	Miscarriage	None	
64	28	<0.2	0	Miscarriage	None	
65	29	<0.2	0	Miscarriage	None	
66	38	<0.2	0	Miscarriage	None	
67	8	<0.2	0	Miscarriage	None	
68	22	<0.2	0 ^a	Miscarriage	None	
69	27	<0.2	0	Miscarriage	Hys, BSO	None

^a In 13 cases, follow-up serum samples were received by the USA hCG Reference Service, and transformation to active disease was confirmed after a varying period of 6 months to 4 years with quiescent GTD. The histories of transformed cases are outlined in Table 4. It is noteworthy that 4 of 6 cases with a history of choriocarcinoma transformed. We are informed of transformation in 2 further cases (no follow up confirmation). This indicates transformation in 15 of 69 (22%) of quiescent GTD cases.

^b In two cases diagnosed as quiescent GTD, low proportions of hCG-H were detected (5% and 10%). Active disease appeared unlikely because of greater than 6 month of persistent low levels of hCG (1.9 and 7.2 mIU/ml) with minimal variation.

Table 3. Twenty three case histories are presented to demonstrate the usefulness of hCG-H measurement to diagnose active GTN. Cases 1-13 involve quiescent GTD, and cases 14-23 the use of hCG-H(%) to detect onset of active disease in other GTN/choriocarcinoma cases. This study is limited to cases developing active disease. No cases were excluded. The study was also limited to serial clinical data or outcome data on just the 23 cases in which information was provided at time of consultation or in a follow up consultation. Internal Review Boards prohibited us from seeking further patient records for this study. Only pertinent case histories are shown. Significant rising hCG is defined as that doubling over the course of one month. Clinical status is clinical examination and CT/MRI imageable. NED is no evidence of disease, Lu is lung and Br is brain metastases; Mtx is methotrexate and AcD is actinomycin D. HCG-H(%) is hCG-H as a proportion of total hCG.

Dates	hCG (mIU/ml)	HCG-H(%)	Clinical status
<i>Case 1. History of uterine choriocarcinoma confirmed by histology and treated by chemotherapy. Two years later, persistent low hCG (20-170 mIU/ml) detected over a 2 year period, while NED. Received during this time 4 different combination chemotherapy regimens, none diminished hCG. Referred Dec 03 to consider quiescent GTD. Absence of hCG-H(%) indicated quiescent GTD and the likely presence of quiescent GTD for the preceding 2 years. Jan 04 significant hCG-H(%) observed, and confirmed Feb 04. Significantly rising hCG observed Feb 04.</i>			
<i>Active recurrence of disease first identified by hCG-H(%), later confirmed by rising hCG, therapy delayed to this time. Chemotherapy failed to treat quiescent GTD.</i>			
Dec-03	48	0%	NED
Jan-04	53	20% a,c	NED
Feb-04	3669	23%	NED
<i>Case 2. After evacuation of complete mole, persistent hCG levels detected for six months (8-12 mIU/ml); remained NED.</i>			
<i>Mtx and AcD ineffective in abating persistent hCG. Quiescent GTD indicated by absence of hCG-H(%) (Feb 02). 16 months later, slight rise of hCG to 16 mIU/ml, patient re-referred. Active disease indicated by 19% hCG-H. Over next 3 weeks hCG rose to 22 mIU/ml, and lung metastases identified; therapy started.</i>			
<i>Quiescent disease transformed to active disease. Active recurrence of disease first identified by hCG-H(%), later confirmed by rising hCG, therapy delayed to this time. Chemotherapy failed to treat quiescent GTD.</i>			
Feb-02	11	0%	NED
Jul-03	16	19% a,c	NED
<i>Case 3. Ectopic pregnancy treated with methotrexate. hCG levels persisted at 40-65 mIU/ml for 3 years while patient was NED. Referred in March 02, when hCG-H was absent thus confirming quiescent GTD. In April 02 rising hCG detected and 33% hCG-H detected. Active disease identified by both hCG-H(%) and rising hCG, yet patient remained NED. Presence of hCG-H(%) confirmed May 02. With the appearance of lung metastases in June 02 therapy was started.</i>			
<i>Thus active disease first identified by hCG-H(%) and rising hCG, therapy delayed until tumor identified. Quiescent disease transformed to active disease. Quiescent disease transformed to active disease.</i>			
Mar-02	59	0%	NED
Apr-02	194	33% b,c	NED
May-02	1969	34%	NED
Jun-02	6000	81%	Lu
<i>Case 4. After evacuation of complete mole persistent low hCG levels detected for 5 months (10-15 mIU/ml) while patient was NED. Upon referral quiescent GTD indicated by absence of hCG-H (Dec 04). One month later, with rising hCG referred again, hCG-H detected (15%).</i>			
<i>Quiescent disease transformed to active disease. Active disease identified by both rising hCG and rising hCG-H(%).</i>			
Dec-04	12	0%	NED
Jan-05	74	15% b,c	NED

Case 5. Persistent low hCG level detected (15-30 mIU/ml) for 6 months after a miscarriage, while patient was NED. Quiescent disease was indicated by absence of hCG-H (January 02). A major rise in hCG was detected 3 months later. Referred again and active disease confirmed by 57% hCG-H. Lung metastases were discovered one week later.

Quiescent disease transformed to active disease. Active disease identified by both rising hCG and normalized hCG-H(%).

Jan-02	22	0%	NED
Apr-02	194	57% b,c	Lu (one week later)

Case 6. Complete mole evacuated. hCG levels diminished and then persisted at 50-70 mIU/ml for 5 months. Patient NED and chemotherapy did not abate hCG. Referred and quiescent GTD indicated by absence of hCG-H (July 02). Next month significantly rising hCG detected along with 58% hCG-H, showing active disease.

Quiescent disease transformed to active disease. Active disease identified by both rising hCG and hCG-H(%). Chemotherapy failed to treat quiescent GTD.

Jul-02	57	0%	NED
Aug-02	174	58% b,c	NED

Case 7. Uterine choriocarcinoma confirmed by histology and successfully treated by chemotherapy. Two years later persistent low levels of hCG detected, 3-12 mIU/ml, while patient was NED. Referred for quiescent GTD; confirmed by absence of hCG-H (December 01). This continued for 2 further years at 6-12 mIU/ml, and did not respond to hysterectomy or chemotherapy. Referred again when significant rising hCG observed, active disease confirmed by hCG-H(%).

Quiescent disease transformed to active disease. Active disease identified by both rising hCG and hCG-H(%). Chemotherapy and hysterectomy failed to treat quiescent GTD.

Dec-01	8	0%	NED
Dec-03	28	55% b,c	NED

Case 8. After evacuation of a complete mole, persistent low hCG detected for a 4 month period at 20-28 mIU/ml; patient is NED. March 02 referred; quiescent GTD indicated by absence of normalized hCG-H. In August 02 a significant rise of hCG detected and patient re-referred. Active disease confirmed by normalized hCG-H. Two weeks later Lu and Br detected.

Quiescent disease transformed to active disease. Active disease identified by both rising hCG and hCG-H(%).

Mar-02	24	0%	NED
Aug-02	433	43% b,c	NED
Aug-02	1135	Not determined	Lu, Br

Case 9. Uterine choriocarcinoma confirmed by histology and successfully treated by chemotherapy. For four months had persistent low hCG, 5-21 mIU/ml. Referred; quiescent GTD indicated by absence of hCG-H. Quiescent GTD continued for 2 years, until significant rise in hCG found and lung metastases identified. Referred again and active disease shown by 15% hCG-H.

Quiescent disease transformed to active disease. Disease identified by hCG-H(%), rising hCG and lung metastases.

Aug-01	16	0%	NED
Oct-03	225	15% b,c	Lu

Case 10. Partial mole evacuated. For 1 year after persistent low levels of hCG detected at 40-74 mIU/ml; patient NED. Mtx and AcD were ineffective in treating persistent hCG. Referred (October 99) and quiescent GTN indicated by absence of hCG-H. Quiescent GTN continued for 17 months, then rising hCG detected. 37% hCG-H confirmed active disease.

Quiescent disease transformed to active disease. Active disease identified by both rising hCG and hCG-H(%). Chemotherapy failed to treat quiescent GTD.

Oct-99	62	0%	NED
May-01	231	37% b,c	NED

Case 11. History of GTN. Low levels hCG, 27 to 55 mIU/ml, for 1 year, patient NED. hCG not effected by combination chemotherapy, or hysterectomy. Referred; quiescent GTD shown by absence of hCG-H. Over 3 years, hCG rose gradually and lung metastases were identified. hCG-H (14%) confirmed active disease. Disease identified by hCG-H(%), rising hCG and presence of lung metastases. Quiescent disease transformed to active disease. Chemotherapy, combination chemotherapy and hysterectomy all failed to treat quiescent GTD.

Aug-00	44	0%	NED
Feb-03	275	14% ^{b,c}	Lu

Case 12. History of uterine choriocarcinoma. One month after completion of therapy persistent low levels of hCG, 25-42 mIU/ml, detected for 6 months; patient NED. Referred July 01; quiescent GTD identified by absence of hCG-H. Two months later, significant rise in hCG detected. hCG-H (37%) confirmed active disease. Disease identified by both normalized hCG-H and rising hCG.

Disease identified by both hCG-H (%), and rising hCG. Quiescent disease transformed to active disease.

Jul-01	37	0%	NED
Sep-01	173	37% ^{b,c}	NED

Case 13. Complete mole evacuated, leading to persistent low levels of hCG, 5-25 mIU/ml over 2 months. Referred December 04 and quiescent GTD indicated by absence of hCG-H. Over the course of 2 months significant rise in hCG (to 113 mIU/ml). 26% hCG-H confirmed active disease.

Disease identified by both hCG-H (%), and rising hCG. Quiescent disease transformed to active disease.

Dec 04	13	0%	NED
Feb 05	113	26% ^{b,c}	NED

Case 14. Uterine choriocarcinoma with lung metastases. hCG declined to 30 mIU/ml following chemotherapy, and persisted around this level for 1 month; patient was NED. Referred November 02 to consider recurrence, and 28% hCG-H detected. Three months later there was a significant rise in hCG and chemotherapy started. With resistance to chemotherapy, Lung metastases reappeared and brain metastases became evident.

Thus active disease first identified by hCG-H, later confirmed by rising hCG, therapy delayed to this time.

Nov-02	40	28% ^a	NED
Jun-03	596.600	88%	Lu, Br

Case 15. Two months after miscarriage, persistent low levels of hCG detected 70-90 mIU/ml over 2 months. Patient is NED, and is referred to consider GTN; 14% hCG-H detected. 8 weeks later significant rising hCG detected and lung metastases detected.

Thus active disease first identified by hCG-H and later confirmed by rising hCG and presence of tumor, therapy delayed to this time.

Jan-04	87	14% ^a	NED
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Case 16. Uterine choriocarcinoma successfully treated by chemotherapy. Three years later, persistent low hCG (10-35 mIU/ml) detected over 3 months while patient NED. Referred to consider false positive hCG. Real hCG identified with a 41% hCG-H component. Lung metastases were discovered 2 weeks later and chemotherapy was started.

Thus active disease first identified by hCG-H, later confirmed by finding tumor, therapy delayed to this time.

Oct-04	32	41% ^a	NED
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Case 17. Complete mole successfully evacuated. Three months later positive hCG detected (5.2 mIU/ml). Patient NED, false positive results suspected. Real hCG identified with a 91% normalized HCG-H component. We were informed that 1 month later that hCG levels rapidly rose and therapy was started.

Thus active disease first shown by hCG-H, later confirmed by rising hCG, therapy delayed to this time.

Nov-03	5.2	91% ^a	NED
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Case 18. Ectopic pregnancy, treated first with Mtx and then with Salpingectomy. Persistent low hCG detected for 6 months (20-50 mIU/ml). Hysterectomy did not completely reduce hCG, patient remained NED. Referred December 98 to consider GTN; 26% hCG-H was detected. Eleven months later, there was significantly rising hCG and therapy was started.

Thus disease first identified by hCG-H(%), later confirmed by rising hCG, therapy delayed to this time.

Dec-99	47	26% ^a	NED
Nov-00	179	28%	NED

Case 19. Patient had history of successfully treated GTN, 10 years prior. Over 13 months, January 04 to February 05, hCG levels increased continuously from 1 mIU/ml to 42 mIU/ml, while patient NED. False positive and quiescent GTD were considered. The presence of significant hCG-H (35%) was shown in February 05 indicating active disease. Epithelial trophoblast tumor found 2 months later after hysterectomy. Active disease initially identified by HCG-H(%), confirmed 2 months later by tumor. Therapy was delayed.

Jan 04	1	not detectable	NED
Feb 05	42	35% ^a	NED

Case 20. History of spontaneous abortion, followed by 15 months of persistent low levels of hCG, 108-167 mIU/ml. Patient was NED; no referral was made so quiescent GTD was not confirmed. Referral February 05 with the finding of 35% hCG-H. GTN indicated, and confirmed 2 month later by imaging tumor in uterus. Active disease first shown by hCG-H (%), confirmed 2 month later by imaging. Therapy was delayed.

Feb 05	305	37% ^a	NED
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Case 21. History of early pregnancy loss, hCG of 4320 mIU/ml discovered 3 month later. hCG levels persisted for 1 month with no clear increasing trend for 6 weeks. Referred May 05 with the finding of 23% hCG-H. GTN indicated and confirmed 2 month later by imaging tumor in lung. Active disease first shown by hCG-H(%), confirmed 2 months later by imaging. Therapy was delayed.

May 05	5290	23% ^a	NED
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Case 22. History of uterine choriocarcinoma. After chemotherapy hCG results plateaued in the range of 9-29 mIU/ml for 1 month, NED. Referred May 05 with the finding of 21% hCG-H.

Active disease first shown by hCG-H(%), confirmed 2 weeks later by imaging. Therapy was delayed.

May 05	9.2	21% ^a	NED
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Case 23..History of GTN, following a hysterectomy and combination chemotherapy hCG plateaued at 7-10 mIU/ml for 2 months while NED. Referred June 05 with finding of 100% hCG-H.

Active disease first shown by hCG-H(%), confirmed 1 month later by tumor. Therapy was delayed

May 05	9.1	100% ^a	NED
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^a In 12 of 23 cases (52%), active disease (GTN or choriocarcinoma) first associated with presence of hCG-H(%), before sharply rising hCG or imageable tumor.

^b In remaining 11 of 23 cases active disease detected by significantly rising hCG or identification of tumor, identified or confirmed at same time by presence of hCG-H(%).

^c Cases of quiescent GTD undergoing transformation to active disease (GTN or choriocarcinoma), n=13.

Table 4. Use of total hCG and hCG-H(%) (hCG-H as a proportion of total hCG) to discriminate gestational trophoblastic diseases. Description of study categories are presented in Table 1. Values are mean \pm standard deviation. Results are compared using Student's t test.

Description	hCG (mIU/ml)	hCG-H(%)
Choriocarcinoma/GTN (n = 82) range	16298 \pm 70590 ^a 5.2 - 597,000	50% \pm 39% ^a 7 - 100%
Quiescent gestational trophoblastic disease (n =69) range	34 \pm 58 1 - 212	0.47% \pm 2.1% 0 - 10%
Hydatidiform mole, self resolving only (n = 26) range	4327 \pm 7795 62 - 30,355	5.9% \pm 4.4% 0.5 - 16%

^a Measuring hCG, no significant difference is observed between quiescent gestational trophoblastic disease or self resolving hydatidiform mole cases (control categories) and the "early" choriocarcinoma/GTN cases. ($P > 0.05$). Measuring hCG-H(%), a significant difference is observed ($P < 0.0000001$ and $P < 0.0000001$).

Table 5. Use of hCG and hCG-H(%) tests for differentiating quiescent gestational trophoblastic disease (Quiescent GTD) from active malignancy, and for differentiating self resolving hydatidiform mole (Mole-SR) from active malignancy. Sample sets and numbers of cases are those described in Table 1. In control diagnoses, individual HCG-H(%) and hCG concentrations are ranked and 95th centiles are determined, and detection calculated at these 95th centile concentrations for those with active disease diagnoses (detection at 5% false positive rate). ROC tests are used to evaluate the accuracy of diagnostic tests, independent of a single cut-off concentration. Areas under the ROC curve \pm standard error (SE) results are presented. This is a direct measure of test accuracy, together with structural component analyses to compare the accuracies between hCG-H(%) and hCG tests (P values).

Serum test	hCG (mIU/ml)	hCG-H(%)	hCG (mIU/ml)	hCG-H(%)
Test used for differentiating				
Control diagnoses	Quiescent GTD		Mole-SR	
Active disease diagnosis	Choriocarcinoma/GTN		Choriocarcinoma/GTN	
Controls, 95 th centile	101	0%	20735	13%
Active disease, choriocarcinoma/GTN	62%	100%	24%	89%
Area under ROC curve \pm SE	84 \pm 3.3%	100 \pm 0.2%	34 \pm 5.3%	96 \pm 1.8%
Comparison, HCG-H : hCG test		P<0.000001		P<0.000001

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