5
Measurement of Human Chorionic Gonadotrophin (hCG) in the management of Trophoblastic disease

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5.1 INTRODUCTION

Human Chorionic Gonadotrophin (hCG) is mainly a product of the syncytiotropoblastic cells of the placenta although it is also secreted, in lesser quantities, by cytotrophoblastic cells and several non-placental tissues. It is produced in gestational trophoblastic neoplasms (GTN) and maybe seen in non-trophoblastic neoplasms (Stenman, 2006). hCG is essential in pregnancy as it promotes transformation of cyclic ovary corpus luteum to gravid corpus luteum allowing continued ovarian production of progesterone and oestrogens in the first 6-7 weeks of pregnancy until the luteo-placental shift in progesterone production occurs (Tulchinsky, 1973). The hormone was discovered in 1920 by observing the stimulatory effect of placental extracts on corpus luteal formation and progesterone production in rabbits (Hirose, 1920). The first assay for hCG was developed several years later and was based on the ability of hCG to activate receptors in the gonads of mice (Ascheim and Zondek, 1927). Despite their many drawbacks such animal based bioassays remained the only pregnancy tests until the '60s. It was a quantitative variant of a mouse bioassay that enabled Li *et al* to discover the sensitivity of trophoblastic neoplasms to treatment with methotrexate (MTX) when they observed a decline in urine hCG potency following its administration (Li, 1956). However, it was not until the advent of radioimmunoassays in the early '60s that the first sensitive and truly quantitative tests for determination of hCG became available (Berson & Yallow, 1960). Presently, effective management of trophoblastic disease is heavily reliant on immunoassay determinations of serum hCG which are used at all stages of trophoblastic disease management including diagnosis, treatment planning, monitoring response to therapy and relapse detection (Stenman, 2004, 2006; Muller & Cole 2009; Seckl, 2013; Mangili, 2014). Due partly to clinical reliance and partly the nature of hCG immunoassays, false or inaccurate determinations of hCG have had serious adverse consequences (Rotmensch & Cole, 2000; Stenman 2006, 2013). Hence, mitigating these risks has been a topic of debate and interest to clinicians responsible for the treatment of trophoblastic neoplasms.

In this chapter the structure and biochemistry of hCG, its variant forms, their metabolism and excretion, together with the fundamentals of immunoassay technology will be discussed with the aim of highlighting the pitfalls associated with their use and hence the best practice for their application in trophoblastic disease management.

5.2 STRUCTURE AND BIOCHEMISTRY

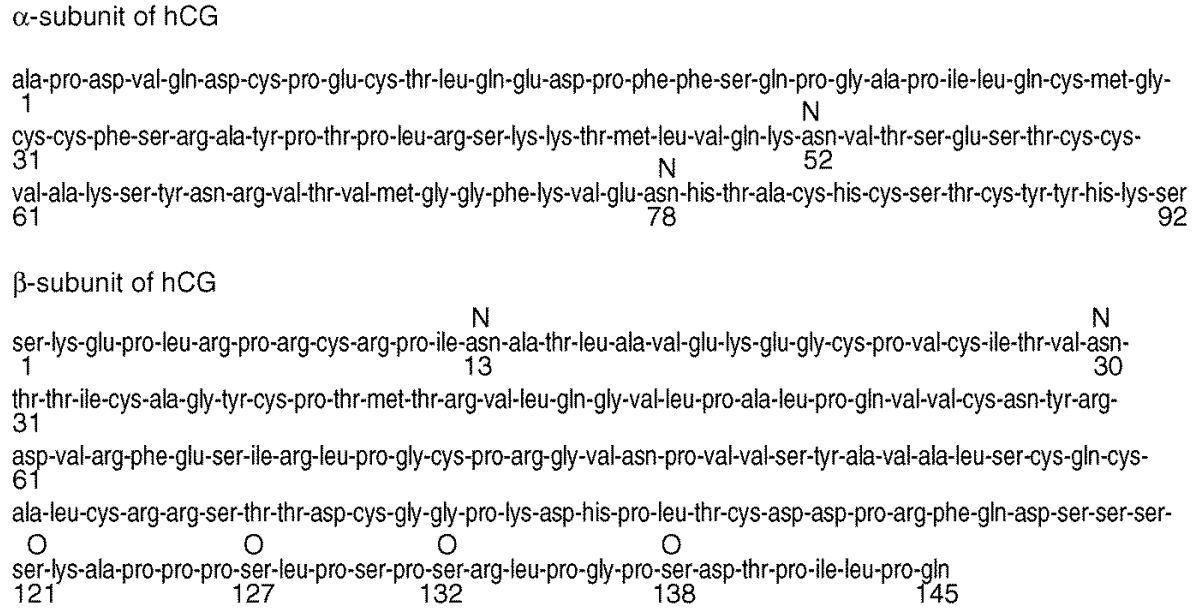
hCG, together with LH, FSH and TSH form the glycoprotein hormone (GPH) family, all of which are heterodimers that share a common non-covalently linked 92 amino acid α-subunit, GPHα and varying degrees of homology in their β-subunits. The first 1-120 amino-acid (aa) sequence of the β-subunit chain of hCG (hCGβ) is highly homologous to LH with which it shares an 85% sequence identity. However, the final C-terminal peptide of hCG (hCGβCTP), comprising aa 121-145, is unique to hCG, LH being only 121 aa in length (Carlsen, 1973; Stenman, 2006). The 4 genes and 2 alleles (CGβ6/7, CGβ3/9, CGβ5, and CGβ8) that encode hCGβ are thought to have developed from a singular ancestral LHβ gene (CG β54, on chromosome 19q13.3) by gene duplications and mutations (Talmadge, 1983). hCGβCTP became incorporated due to loss of the stop codon in the gene encoding for hCGβ (Fiddes, 1980). The hCGβ subunit gene cluster can be subdivided into type I (CGβ6/7) and type II (CGβ3/9, CGβ5, and CGβ8) genes which differ at 2 sites, one of which is a silent mutation; the second results in an amino acid difference at position 1: type I contains Alanine while type II contains Asparagine (Bellet, 1997). Type II genes appear dominant; the CGβ5 gene produces approximately 65% of the transcripts in normal

placentas and the CGβ8 is the second most transcribed (Miller-Lindholm, 1997). The common α-subunit is encoded by a single gene on chromosome 12q21.1-23. The 2 proteins also share biological activity and both are able, when present as their intact α,β –heterodimer forms, to stimulate sex steroid production via the G-protein-coupled LH/hCG receptor, LH/hCG-R (Macfarland, 1989).

Three-dimensional X-ray crystallographic studies demonstrate that GPH subunits share structural homology with a wider grouping of glycoproteins, the cysteine knot superfamily, a group that includes nerve growth factor (NGF), platelet derived growth facter (PDGF) and transforming growth factor β (TGF-β) among others (Sturgeon & McAllister, 1998). The common structural motif that determines the three-dimensional structure shared by both the α and β-subunits of GPH subunits comprises a 2 disulfide bridge that covalently links adjacent folded strands of the peptide chain forming a ring that is axially permeated by a further disulphide bond. The 2 peptide loops (loops 1 and 3) extend to one side of the central knot while a single larger loop, loop 2 extends on the opposite side. In the holodimeric form of hCG the subunits are noncovalently linked 'head-to-toe' such that loops 1 and 3 are adjacent to loop 2 of the other subunit (Lapthorne, 1994).

The average molecular weights (MW) of hCGα, hCGβ and intact hCG isolated from commercial hCG preparations determined by mass spectrometry are 14 000 and 23 500 and 37 500 daltons, respectively (Birken, 2003). However, the average MW of pregnancy derived hCG calculated on the basis of the sum of the peptide and carbohydrate moieties is greater at 38 931 daltons as commercially purified hCG has suffered partial degradation of its carbohydrate antennae (Valmu, 2006). The wide spectrum of MW values determined in mass spectrometry studies is due to variation in glycosylation.

Figure 5.1. Amino acid sequence of hCG ß-subunit and sites of attachment of N- and O-linked oligosaccharides.



5.3 BIOSYNTHESIS AND SECRETION

After transcription of the α and β-subunit genes intracellular synthesis of hCG occurs by a similar process to that of other glycoproteins. Following migration of α and β-subunit mRNA from the nucleus, translation into peptides occurs on the membrane bound ribosomes of the Rough Endoplasmic Reticulum (RER), followed by microsomal peptidase cleavage of signal peptide extensions and release into RER channels for transport to the Golgi where dimer assembly is completed. Post-translational glycosylation occurs by sequential addition of carbohydrate side chains by enzymes located in both the RER and Golgi and is completed shortly before secretion of the assembled dimer (Kornfeld and Kornfeld 1985; Ruddon, 1987; Hanover, 1982). The precise composition of the oligosaccharides added is

dependent on the enzymes present and therefore varies according to the hCG producing secretory cell type.

hCG secretion is regulated by a number of factors including corticoids, progesterone, GnRH, PPARγ agonists, oxygen saturation and hCG (Fournier, 2015). Cell surface receptors that couple to adenylate cyclase can activate hCGα and hCGβ-subunit transcription via phosphorylation of cAMP dependent kinases (for a review see Medeiros and Norman 2009). hCG itself stimulates further release from trophoblasts in an autocrine fashion (Shi, 1993). Other stimulatory mediators including TGFα, epidermal growth factor and leukemia inhibitory factor appear to exert their effects via further promotion of hCG expression suggesting a central role of the hormone in trophoblast development. The α-subunit is usually synthesised in excess and therefore the synthetic rate of dimer production is primarily regulated by β-subunit transcription. In sera the ratio increases throughout pregnancy from an α:β ratio of 1.7 to 1 in first trimester to 12 to 1 by term (Boothby, 1983).

5.4 HETEROGENEIGHTY OF HCG AND DISTRIBUTION OF VARIANT FORMS IN BIOLOGICAL FLUIDS

In addition to the minor variation in protein sequence consequent to gene type or possible mutational polymorphisms, circulating hCG exists in a range of variant forms that can arise from further post-translational and metabolic modifications including dissociation, nicking, truncation, homodimerization and oxidation (Cole, 1982; Ruddon, 1996; Stenman, 2006). hCG occurring in body fluids and tissues is therefore heterogeneous with respect to both protein structure and carbohydrate content and should be considered as a complex family of hCG variants (Berger, 2013). In early pregnancy serum, hCG immunoreactivity consists of intact hCG (>95%) together with minor amounts of hCGβ. The serum concentration of free hCGβ subunit, nicked or other forms parallels the concentration of intact hCG which reaches a peak at around 10 weeks (Stenman, 2006).

Free hCGβ-subunit

The free hCGβ-subunit form is around 1% of total hCG declining to 0.5% at term but in urine it is a major form constituting between 9-40% of the total immunoreactivity (McChesney, 2005). Most of the free hCGβ found in the urine is nicked (Birkin, 1991). Monoclonal antibodies with high specificity for hCGβ (cross-reactivity with hCG <0.3%) have been developed and used in specific free hCGβ assays to study the variation in serum levels in a number of clinical conditions. Higher levels of maternal serum hCGβ appear to be associated with a greater risk of pre-eclampsia (Valliant, 1996; Roiz-Hernandez, 2006), while lower levels have been shown to be predictive for ectopic pregnancy (Okamoto et al., 1987). Maternal serum levels of free hCGβ also vary with fetal ploidy status, being higher in triplody of paternal origin (diandric) with multiples of median (MoM) value of approximately 8.0, and lower in triploidy of maternal origin (digynic) with MoM value of 0.2 (Spencer *et al.,* 2002). In Down's syndrome (trisomy 21) hCGβ levels are between 1.4-3.5 higher than normal pregnancies until the end of the second trimester (Hallahan, 1998; Hsu, 1999).

Several studies have suggested that separate measurement of serum hCGβ and determination of the percentage proportion of total hCG immunoreactivity (%hCGβ) in the management of GTN may provide additional information to measurement of total hCG alone. The exact proportion is dependent on the assays used, but, based on molar concentrations, a proportion approximating 3-5% is typical of low risk post-molar invasive GTN whilst patients with post-gestational histologically confirmed choriocarcinoma have a higher proportion at around 10% (Stenman, 2006). The potentially most aggressive form of GTN, placental site trophoblastic tumour (PSTT) shows the greatest proportion of hCGβ (Cole, 2006; Harvey, 2008). Due to the rarity of these more aggressive disease forms the number of patients reported in these studies is low but updated figures from the author's laboratory, comprising 19 PSTT, the largest collated to date, confirm these findings (see table 5.1 for summary results). Receiver operator characteristics (ROC) analysis of these data demonstrates that %hCGβ distinguishes choriocarcinoma patients from invasive post-molar disease with an accuracy of 78% ($p < 0.0001$). Due to the greater proportion of free hCGβ-subunit in PSTT the accuracy for distinguishing PSTT from lower

risk invasive post-molar disease was greater than for choriocarcinoma at 82% ($p<0.000001$). At the optimal cut-off of ≥16.5% hCGβ-subunit, a 58% detection and 1.7% false-positive rate was achieved indicating that the approach is best suited for helping to rule out PSTT (only 2/116 patients without a final diagnosis of PSTT had %hCGβ above the cut-off). However, due to the limited accuracy of the test as well as the inability to distinguish PSTTs from patients with choriocarcinoma the approach has limited clinical utility. Due to variation in hCG measurement between assays the precise proportions and hence optimal cut-offs for clinical use will vary according to the combination of immunoassays used. Some reports indicate that a proportion based on molar concentrations greater than approximately 5% are specifically associated with malignant post-gestational trophoblastic neoplasms including the commonest form, post-molar trophoblastic neoplasia (Stenman, 1985; Berkowitz, 1989; Khazaeli, 1989). However a more recent study using a larger number of patients, including 35 invasive post-molar neoplasias found no significant difference between this group and those with benign post-molar disease (Van Trommel, 2005). Our recently updated studies confirm that serum samples from benign and invasive post-molar patients have similar average %hCGβ values of around 5% and the proportion is variable (See table 5.2).

Table 5.1. Median values with Interquartile Ranges (IQR) for total, free hCGβ, and the calculated %hCGβ for low-risk invasive molar disease, choriocarcinoma and PSTT patient groups. Mann-Whitney *U* test *p*-values for comparison of PSTT and choriocarcinoma with invasive molar disease groups are included. The analysis is updated from our previous study (Harvey *et al,* 2008).

Median (IQR)	**Invasive Molar n=116**	**Choriocarcinoma (n=17)**	**PSTT (n=19)**	**Mann-Whitney *U* *p*-value**
Total hCG, pmol/L	**36400 (12100-80600)**	**154000* (4150-331000)**	**1300** (300-21500)**	**0.044* 0.0002****
Free hCGβ, pmol/L	**1470 (640-3890)**	**4890* (590-17800)**	**630** (56-1170)**	**0.0007* 0.01****
%hCGβ	**4.66 (3.08-6.83)**	**8.73* (5.92-20.2)**	**16.7** (6.71-47.3)**	**0.0002* <0.000001****

The %hCGβ has been reported to increase during development of therapy resistance (Vaitukaitis & Ebersole 1976; Vartiainen, 1998). In our recently updated studies determinations of %hCGβ were similar in pre-treatment sera from low-risk MTX cured (median= 5.02%, IQR= 3.25; n= 83) and MTX resistant patients (median= 3.82%, IQR= 3.23; n= 70, p= 0.255) suggesting that the test has no predictive value for resistance to first line therapy with MTX (See table 5.2).

Ectopic production of hCGβ has been identified in nearly all common epithelial tumours studied to date but the incidence is highly variable (Iles, 2010). Consistent with an apparent correlation with tumour type and its aggressiveness across the spectrum of trophoblastic neoplasms, many reports demonstrate that either tissue expression or elevation of serum hCGβ is also associated with adverse prognosis in most cancers (Iles, 2010; Stenman, 2004). hCG targeted delivery of hCG antibody conjugated cytotoxic agents to tumour sites using "Antibody-Directed Enzyme Prodrug Therapy (ADEPT)" has been demonstrated by a significant delay of tumour growth in mice bearing choriocarcinoma xenografts (Bagshawe, 1988; Springer, 1991). Ectopic production of intact hCG has also been observed in nontrophoblastic neoplasms, most often by lung cancer and hepatoblastoma (Stenman, 2004).

hCGβ does not activate LH/hCG-R receptor but several lines of evidence indicate that it has a growth promoting activity in cancer (Stenman, 2006). Addition of anti-hCGβ antiserum to culture media of bladder cancer cell lines inhibits the growth promoting action of secreted hCGβ (Gillot, 1996) and antisense oligonucleotides directed to hCGβ also have an anti-proliferative effect in prostate and cervical tumour cell lines (Devi, 2002; Jankowska, 2008). Induction of tumour host hCG antibodies using a vaccine composed of the CTP of hCGβ conjugated to diphtheria toxoid significantly improved survival in

patients with metastatic colorectal cancer in patients who had higher than or equal to median anti-hCG levels in a phase II clinical trial of 77 patients (Moulton, 2002). It has been speculated that the mechanism involves antagonism of inhibitory growth effects of TGF-β, PDGF and NGF or other forms of such 'cross-talk' among other CKGF mediators such as VEGF based on structural similarities between these molecules and their endogenous ligand receptors (Iles, 2010).

Most commercially available hCGβ-subunit assays are intended for maternal screening of Down's syndrome, and being optimized for the high serum concentrations occurring in pregnancy, they are not well suited for the determination of the low levels of hCGβ that typically occur in the serum of cancer patients (Stenman, 2004). Specific and sensitive assays for hCGβ have been developed by some laboratories (Ozturk, 1987; Alfthan, 1988; Marcillac, 1992; de Medeiros, 1992b) and have been used to estimate reference values for hCGβ (See table 5.4). Serum free hCGβ concentrations are lower than those of intact hCG in men and women, and, unlike intact hCG do not increase with age. In women, postmenopausal levels of hCG up to around 5 IU/l are 5–10 times higher than those of hCGβ (Alfthan, 1992; Stenman, 2004).

Table 5.2. Median values with Interquartile Ranges (IQR) for total, free hCGβ, and the calculated %hCGβ for benign molar and low-risk invasive molar (IM) disease; the latter is further subdivided into MTX sensitive (MTX-S) and MTX resistant (MTX-R) patient groups. Mann-Whitney *U* test *p*-values for comparison of treatment resistance to MTX are included.

Median (IQR)	**Benign Molar n=28**	**Low-risk IM n=116**	**MTX-S n=62**	**MTX-R N=54**	**Mann-Whitney *U* *p*-value**
Total hCG, pmol/L	**4990 (2180-11300)**	**36400 (12100-80600)**	**26100 (7030-67600)**	**43050 (18500-115200)**	**0.048**
hCGβ, pmol/L	**192 (90-500)**	**1470 (640-3890)**	**1160 (435-3670)**	**2120 (840-3980)**	**0.10**
%hCGβ	**4.34 (2.09-6.22)**	**4.66 (3.08-6.83)**	**4.79 (3.44-7.12)**	**4.50 (2.38-6.58)**	**0.27**

Free α-subunit

Free α-subunit, in relation to total circulating hCG, is less than 10% in the first trimester and increases throughout pregnancy reaching 30-60% at term (Benveniste & Scommegna, 1981). In the sera of non-pregnant women and men free α-subunit levels of around 0.1-0.3 μg/L reflect the total pooled production consequent to synthesis of all the pituitary GPH glycoproteins (Norman, 1987). Free α-subunit displays wide variation in glycosylation as well as slight compositional variation in the initial amino acids of the peptide sequence. Levels increase in post-menopause and high levels can be found in some tumors of the pituitary, lung, testes, gastric or pancreatic carcinoid neoplasms and insulinomas (Braunstein et al.,1979). Although assays for hCGα are available the relatively low predictive value of free α-subunit measurements compared with hCG or hCGβ limits their clinical utility in trophoblastic disease (van Trommel, 2005; Medeiros & Norman, 2009).

Nicked forms, hCGn, hCGβn and hCG β-core fragment (hCGβcf)

The nicked forms of hCG, hCGn and hCGβn arise by nicking of either the intact holodimer or the free hCGβ-subunit by proteases secreted by the placenta or by proteinases released by activated leukocytes (Kardana & Cole, 1994). Cleavage occurs between amino acids 47-48 and to a lesser extent between amino acids 42-43, 43-44 or 45 (Kardana, 1991; Birkin, 1991; Elliott, 1997). Nicking results in a more rapid dissociation of the dimer and immunonreactivity for some immunoassays is lost (Cole, 1993; Cole, 2004). Although most of the commercially available total hCG assays are able to detect both hCGn and hCGβn, relative detection is variable and a source of measurement discrepancy between assays (Birkin, 2003; Cole, 2004; Sturgeon, 2009; Harvey, 2010).

Specialist assays employing specific monoclonal antibodies for hCGn have been developed by some laboratories and used to study the distribution of hCGn and hCGβn in health and disease (Kovalevskaya, 1999). hCGn is more abundant in urine, the molar ratio hCGn:hCG increases 8-31% during pregnancy (Cole, 1991), compared with serum at 9-10% (Birkin, 1991) and its proportion is increased in pre-eclampsia, Down's syndrome, testicular and bladder cancer as well as trophoblastic disease. It is a major form in the urine following molar evacuation or during treatment of trophoblastic neoplasias (Cole & Sutton 2003; Kohorn & Cole, 2000; Hoermann, 1994).

hCGβcf is composed of two hCGβ fragments of amino acid residues 6-40 and 55-92 linked by 5 disulfide bonds (Birkin, 1988). It is the terminal product of hCG degradation and the predominant form of hCG present in the urine in both pregnancy (Matthies & Diczfalusy, 1971) and cancer (Stenman, 1993). The availability of highly purified preparations of hCGβcf enabled early development of monoclonal antibodies for use in immunohistochemical studies and for the construction of hCGβcf specific assays (de Medeiros 1992; Krichevsky, 1994; Khan, 2000). Prior to the introduction of the new standards and IFCC nomenclature, because some of the early urine assays that were developed using purified hCGβcf measured several forms of degraded fragments, such assays were collectively termed urinary gonadotrophin fragments or peptides (UGP) assays (Schwartz, 1996). The new recently purified WHO standard for hCGβcf (Birkin, 2005) has been valuable for characterising the epitopes of monoclonal antibodies employed in commercial immunoassays and for ascertaining assay specificity, particularly for urine assays (Berger, 2013).

hCGβcf has been identified in the normal placenta, the pituitary, hydatidiform mole, choriocarcinoma tumour and tissue from various epithelial carcinomas (de Medeiros 1992a; Okamoto, 2001). During pregnancy, hCGβcf can be detected in plasma (Hoermann, 1995; Udagawa, 1998, Nisula, 1989; de Medeiros, 1992a; Khan, 2000) but the concentrations are only ~0.01% of those of hCG (Alfthan & Stenman, 1990). Intact, nicked and free hCGβ can cross the glomerular membrane and are internalised by the proximal renal tubule cells where they are further degraded to hCGβn and hCGβcf (Markkanen & Rejaniemi, 1979; Cole, 1997). In pregnancy hCGβcf emerges as the predominant form of hCG in the urine from 5 weeks following conception and remains in excess of 1.6-9.6 fold relative to intact hCG (de Medeiros, 1992a). Studies on the metabolic clearance rate of hCGβcf show that >99% is formed in the kidneys during renal excretion (Wehmann, 1989). After injection of hCG, hCGβ or recombinant hCG (rhCG), hCGβcf is detected in the urine (Nisula, 1989), but peak concentrations occur ~6 h after the peak in urinary concentration of hCG (Norman, 2000).

The concentration of hCGβ in the urine is usually much lower than in the serum as it is mostly degraded by the kidneys to hCGβcf during excretion to urine (Alfthan, 1992). In non-trophoblastic cancers hCGβcf concentrations reflect those of hCGβ in the serum, and when measured by highly sensitive assays, serum hCGβ is reportedly the better marker (Alfthan, 1992).

Separate measurement of urine hCGβcf has been suggested as a possible alternative marker for follow-up of trophoblastic disease and some cancers (Norman, 1993; de Medeiros, 1992a). As PSTT produces lower levels of hCG, urine hCGβcf has also been suggested as an alternative marker for this condition (Seki, 2004). However, the use of these assays for monitoring of cancer is hampered by large day-to-day variation in the urine concentrations of hCGβcf, which is not eliminated by normalization against urinary

creatinine (Ngan, 1995). Because of these problems, commercial assays for hCGβcf are presently not widely available.

Part of the heterodimeric hCG in urine is nicked (hCGn), i.e. the peptide chain is cleaved at various positions between amino acids 44 and 48. This nicked hCG may also occur in the serum of cancer patients (Cole, 1991; Jacoby, 2000). Part of the hCGβ isolated from urine is also in the hCGβn form. hCG missing the carboxyl terminal extension (hCG-CTP) may also occur in the serum and urine of some cancer patients (Cole, 1982, 2004; Harvey, 2010). In this situation, assays requiring epitopes harboured on the CTP can give misleading results (Cole, 2004; Harvey, 2010). Many commercially available total hCG assays do not recognise hCGβcf or other less well defined degradation products including truncated hCG and hCGβ missing the CTP. Assays that do not measure hCGβcf are not suitable for urine assays based on measurement of total hCG as a disease biomarker (Cole, 1993, 2004; Hoermann, 1994; Sturgeon, 2009; Harvey, 2010).

Pituitary hCG

A pituitary chorionic gonadotrophin form isolated from human pituitary glands has been characterized that contains sulphate and has, on a molar basis, about half of the sialic acid content of hCG purified from urine (Odell & Griffin, 1987; Birken, 1996). Purified sulfated pituitary hCG has only 50–65% of the biological activity of hCG purified from urine (Birken, 1996). Pituitary secreted hCG increases in perimenopausal and older women (Odell & Griffin, 1987) and serum levels ranging up to 25-32 IU/L may be observed although levels around 7-15 IU/L are more common (Braunstein, 2002; Cole, 2007). Lack of GnRH suppression when sex steroid levels fall leads to raised LH and FSH which is occasionally accompanied by the secretion of low levels of intact hCG. Furthermore, postmenopausal hCG can be suppressed with oestradiol and these findings taken together indicate that such hCG is derived from the pituitary (Stenman, 1987, 2006).

5.5 WHO PROTEIN ISOFORM STANDARDS

The best characterised protein variants; intact biologically active heterodimeric hCG (hCG), nicked intact hCG (hCGn), free α-subunit (hCGα), free β-subunit (hCGβ), nicked hCG β-subunit (hCGβn) and hCG β-core fragment (hCGβcf) are available as defined International Reference Reagent (IRR) standard preparations, as established by the International Federation of Clinical Chemistry (IFCC) and endorsed by the WHO for calibration and characterisation of immunoassays (Birkin, 2003; Bristow, 2005). The newly assigned values for latest standards were based on amino acid analysis and use molar concentration which is more appropriate for immunoassay measurements as they reflect molar concentrations and not bioactivity. When the new reference reagents are used to calibrate immunoassays for hCG, hCGβ, hCGα and hCGβcf the concentrations should be expressed in pmol/L. Presently however commercially available hCG assays are still calibrated using the previous international standard preparations comprising standards for hCG (75/537 and 75/589), hCGβ (75/551) and hCGα (75/569) which are impure preparations that have been calibrated in IU against preceding sets by bioassay (Canfield & Ross 1976; Storring, 1980). Total or intact hCG assays are calibrated with reference to the WHO 4th International standard, 75/589 and 0.11μg = 1 IU = 2.93 pmol. Since hCGβ subunits lack hCG activity, they were assigned values based on a mass with 1 μg corresponding to 1 IU which equates to 42.5 pmol. Thus, the units are not comparable with those of hCG. Estimation of ratiometric proportions such as %hCGβ must be performed using the molar concentrations (Stenman, 2006). The issue is further complicated by the fact that total hCG assays vary in their sensitivity to free hCGβ and rarely show equimolarity with intact hCG. The IFCC has provided a nomenclature (Table 5.3) for describing the forms and recommended its use to help improve scientific communications and understanding (Stenman, 2006).

Table 5.3. The WHO 1st International Reference Reagents (IRR) for hCG and related variants.

WHO code	Symbol	Molecular Structure and approximate weight (kDa)
99/688	hCG	Intact αβ-heterodimer, bioactive hCG (36.7 kDa)
99/720	hCGα	Free α-subunit, 92 aa, glycosylated (14.5 kDa)
99/863	hCGβ	Free β-subunit, 145 aa, glycosylated (22.2 kDa)
99/708	hCGβcf	Core fragment of hCGβ; hCGβ6-40 linked to hCGβ 55-92 (9.8 KDa)
99/642	hCGn	Nicked hCG, missing linkages in the region hCGβ 44-48 (36.7 KDa)
99/692	hCGβn	Nicked hCGβ, missing linkages in the region hCGβ44-48 (22.2 KDa)

5.6 GLYCOSYLATION OF HCG

About one-third of the molecular weight of hCG consists of carbohydrates and glycosylation is of structural and functional importance affecting both the half-life in circulation and activity at the LH/hCGR receptor (Lustbader, 1998). Both subunits are glycosylated; hCGα has two N-linked glycoside moieties attached at Asn-52 and Asn-78, hCGβ has N-linked carbohydrate antennae at Asn-30 and 13 and 4 putative O-linked glycosylation sites at Ser-121, Ser-127, Ser-132 and Ser-138. Our understanding of the glycosylation structure of hCG in pregnancy is based upon several independent Mass Spectrometry (MS) studies of pharmaceutical hCG preparations derived from urine pooled from pregnant women and a single analysis of a pregnant individual (Valmu, 2006). According to these studies the two N-linked glycans on hCGβ at Asn-13 and Asn-30 have been shown to consist of bi-antennary and to a lesser extent tri-antennary, complex-type N-glycans with terminal sialic acids and a variable content of fucose (Carlsen, 1973, Kessler, Reddy, 1979; Weisshaar, 1991; Valmu, 2006). On the CTP portion of hCGβ are four putative serine-linked oligosaccharides (O-glycans) attached to serine residues 121, 127, 132, and 138 that have been shown to consist of monoantennary, so-called core-1 O-glycan structures with two sialic acids attached to each glycan (Kessler, Mise, 1979). However in later studies, unoccupied glycosylation sites and the presence of core -2 type bi-antennary glycans at Ser-121 and other sites in early pregnancy has been demonstrated (Lui & Bowers, 1997, Valmu, 2006). Early pregnancy hCG urine samples collected from a single woman at 5 and 7 weeks compared with 2 pregnancy samples collected at 35 weeks from 2 women were found to have an increased content of tri-antennary complex-type N-linked carbohydrates attached to hCGβ Asn-13 (Valmu, 2006). Asn 30 linked N-glycans in several reports are nearly entirely fucosylated in pregnancy hCG (Kessler, Reddy, 1979; Weisshaar, 1991; Valmu, 2006). In the later study by Valmu *et al* 2006 Asn-13 linked N-glycans are more highly fucosylated in early pregnancy (Valmu, 2006).

Tumor-derived hCG has been reported to contain increased amounts of triantennary N-glycans (Elliott, Kardana, 1997), 'abnormal' biantennary N-glycans (Kobata & Takeuchi, 1999), and biantennary core-2 type O-glycans (Birken, 2003; Elliott, 1997). hCG produced by trophoblastic cancers occasionally displays reduced content of sialic acid (Nishimura, 1981; Imamura, 1987), but variants with unusually low pI values indicating increased content of sialic acid have also been described (Yazaki, 1987). The degree of fucosylation of N-glycans is enriched in malignancies including choriocarcinoma (Mizuochi, 1983). Site specific analyses showed that Asn-30 linked glycans were consistently nearly totally fucosylated, alike pregnancy hCG (Kessler, Reddy, 1979; Weisshaar, 1991) whereas the degree of Asn-13linked glycan fucosylation varied and was higher in malignancies alike the early pregnancy urine sample in the Valmu *et al* 2006 study. The previously reported increased content of mono-antennary N-glycans attached to Asn-13 and Asn-30 in choriocarcinoma (Elliot, 1997) was confirmed by Valmu *et al* 2006 but the earlier observed increase in tri-antennary N-glycans was seen only in those attached to Asn-30.

Regarding O-type glycans, aside from the differences at Ser-121 highlighted above, the Valmu *et al* study found that Ser-138 had an increased proportional occupancy by core-2 type in choriocarcinoma, testicular

GCT and the early pregnancy urine sample (Valmu, 2006). Although site specificity of glycan analysis between sites at Ser-127 and Ser-132 was not possible as both moieties reside on the same peptide fragment, it was also observed that in invasive post-molar, choriocarcinoma, testicular GCT and in the early pregnancy urine sample these sites were mostly occupied by core-2 type oligoscharides (Valmu 2006, See figure 2).

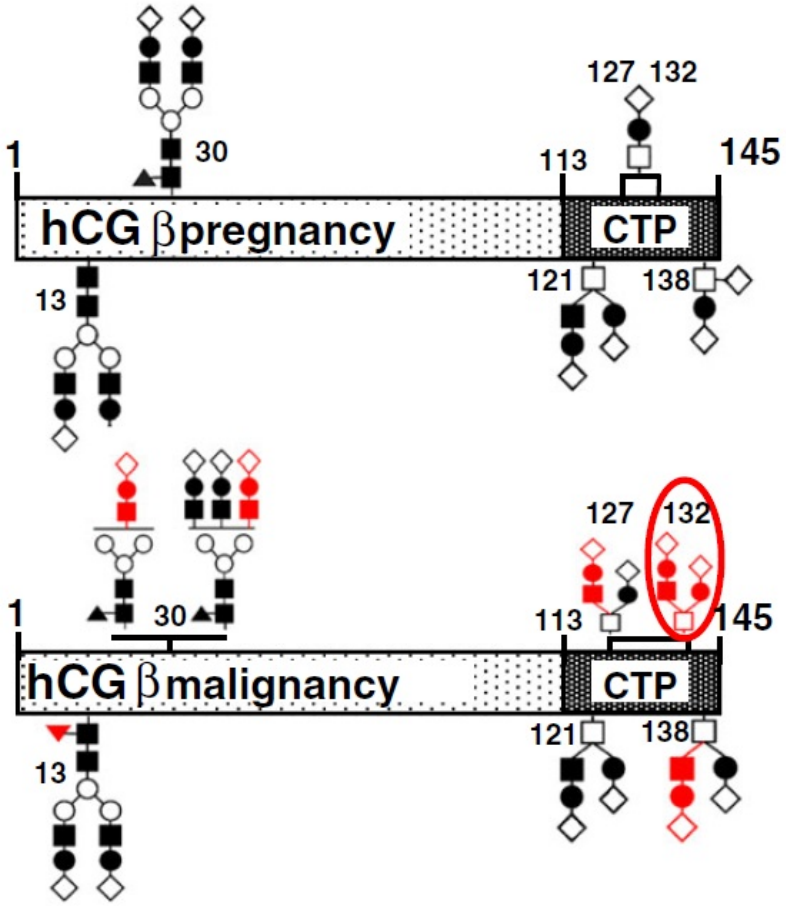


Figure 5.2. Glycosylation variants of hCGβ (according to Valmu *et al* 2006). Immunoassays for hCG-h based on mAb B152 recognize the encircled glycan at Ser-132 and surrounding peptide structure. The major differences in carbohydrate antennae composition between early and mid-to-late pregnancy- and malignancy-derived hCG are depicted in red. Filled square GlcNAc, filled diamond Fuc, empty square GalNAc, empty circle Man, filled circle Gal, empty diamond NeuAc. Reproduced from Berger *et al* 2013 open access article at Springerlink.com.

5.7 HYPERGLYCOSYLATED HCG

The expression 'hyperglycosylated hCG' was initially used to describe hCG containing increased complex carbohydrates (Elliott, 1997) but, following use of the B152 antibody in a number of clinical studies, has come to mean hCG detected by assays employing antibody B152. This is misleading as B152 detects only a single glycosylated epitope variant of hCG and hence belies the complexity and heterogeneity of hCG glycosylation evident from MS studies (Stenman, 2006). Hyperglycosylated hCG (hCG-h) recognised by assays employing the B152 monoclonal antibody is very likely to contain a multitude of molecular variants. hCG-h has also been named invasive trophoblastic antigen, ITA (Cole, 1999).

Using B152 based assays it has been demonstrated that hCG-h is the predominant form of hCG in early pregnancy serum and urine, representing 90-100% of total hCG in the first 3-4 weeks of gestation and decreasing to less than 2% at 20 weeks (Kovalevskaya, 2002; Stenman, 2006). Low levels have been associated with pregnancy loss (Kovalevskaya, 2002; Sutton-Riley, 2006; Sasaki, 2008) and pre-eclampsia (Bahado-Singh, 2002; Keikkala, 2013) while high levels have been associated with Down´s syndrome

(Cole, 1999). hCG-h is also reported as a major form in a variety of malignant conditions including cervical, colon, bladder, lung, germ cell tumour and trophoblastic disease (Kelly, 2007; Kovaleskaya, 2002). Cole *et al* have reported that hCG-h can discriminate benign from malignant GTN, where it accounts for 30-100% of total hCG immunoreactivity, with 100% sensitivity and specificity but these findings have not been validated by other groups (Cole, 2006).

The shift in the glycosylation pattern observed from early to mid-pregnancy may result from hCG production initially by cytotrophoblastic cells, which dominate in the early placenta, changing to syncyciotrophoblasts which predominate later in pregnancy and produce 'normally glycosylated' hCG (Kovalevskaya, 2002). As it is the cytotrophoblastic population of cells that form the invasive extravillous cytotrophoblasts, strong expression of hCG-h by these cells together with the association of low serum hCG-h levels in early miscarriages and pre-eclampsia (Zhou, 1997; Burton, 2009; Meekins, 2004; Keikkala, 2013) suggest that hCG-h could be a biomarker for the presence of 'normally' functioning invasive extravillous cytotrophoblast (Zhou, 1997; Cole, 2009; Keikkala, 2013). *In vitro* experiments have shown that supernatant from the cultures of invasive cytotrophoblast contains 4-6 times more hCG-h than villous trophoblast (Guibourdenche, 2010) and that addition of purified hCG-h to choriocarcinoma cell lines promotes growth and invasion in an autocrine fashion (Badaho-Singh, 2002; Cole & Khanlian 2006, Handshuh, 2007). Furthermore the B152 antibody has been used to inhibit tumour growth in mice injected with a choriocarcinoma cell line suggesting a role for hCG-h in promoting growth of transformed trophoblasts (Cole*,* 2006). A molecular mechanism for the ascribed invasive function of hCG-h has not been identified although it has been speculated that the observed effects may be caused by hCG-h specific inhibition of TGFβ or antagonism at the TGFβ-RII receptor in a similar fashion as has been proposed for free hCGβ (Iles, 2010, Cole, 2009). Evidence for potent angiogenic actions of hCG and more recently hCG-h has emerged (Zygmund, 2002; Berndt, 2006, 2013). While hCG appears to mediate angiogenic effects via activation of LH/hCGR competitive binding, immunoblotting studies and TGFβ gene reporter assays in vitro suggest that hCG-h, like its purported stimulation of trophoblast invasion, is mediated via TGFβ-RII receptors. Furthermore hCG-h abililty to stimulate endothelial cell outgrowth and pericyte migration in aortic ring preparations persists in aortas from LH/hCGR knockout mice providing further evidence that these effects are not mediated via the LH/hCG receptor (Berndt 2013). Doppler US studies have shown that during normal placentation uterine artery blood flow increases by up to 3.5-fold as extravillous syncitiotrophoblast invade the endometrial spiral arteries to establish a high-flow, low resistance vasculature (Thaler*,* 1990; Kliman, 2000). Impaired implantation of the placenta in early onset pre-eclampsia has been associated clinically with abnormally high impedance to uterine artery blood flow as detected by Doppler ultrasound at 11-13 weeks pregnancy (Harrington, 1997; Martin, 2001 Plasencia, 2007; Poon, 2009; Onwudiwe, 2008). These observations, together with evidence from morphological studies, suggest that the underlying mechanism for development of pre-eclampsia is poor trophoblast invasion of maternal spiral arteries and their conversion from narrow muscular vessels to wide non-muscular channels independent of maternal vasomotor control (Poon, 2009).

We have shown that impedance to uterine blood flow in low-risk MTX resistant GTN is significantly lower at 9-13 weeks gestation compared with MTX cured patients suggesting that, opposite to the observation in PE, trophoblast invasion is deeper and spiral artery conversion more advanced (Agarwal, 2002). However, follow-up studies by our group have shown that the %age of serum hCG-h was equivalent with a median of 33-34% in both MTX sensitive and resistant invasive post-molar disease groups and highly variable (in press). The average proportions and variability of %hCG-h in our studies were comparable with the mean value of 50% previously reported for 'active disease'; i.e. a combined invasive post-molar and choriocarcinoma patient set (Cole*,* 2006). In addition to these purported invasive effects hCG-h has also been reported to exert immune modulatory effects via the mannose receptor (Kane, 2009). Hence the relative importance or specific regulatory role, if any, that hCG-h may play in normal placentation or tumour invasion is unclear.

Assays for the measurement of hCG-h are not available to most laboratories. A commercial assay has been marketed by Nichols Laboratories but is no longer available. Two in-house high sensitivity immunoassays utilising the B152 antibody have been described (Stenman, 2011; Strom, 2012). Stenman *et al.,* have reported the isotype of B152 to be an IgGa type antibody and demonstrated susceptibility of the antibody to interference by complement which can produce falsely low results unless eliminated

(Stenman, 2011). Due to uncertainty regarding the glycosylation of hCG and the variety and distribution of glycosylated variants in normal pregnancy and trophoblastic or non-trophoblastic neoplasms an international standard for hCG-h has not been formulated. As the carbohydrate composition of hCG produced by tumours may vary considerably determining an appropriate source for such a standard is problematic (Birken, 2005, Stenman, 2006). As with other glycoproteins large variations of glycoforms in both intra-individual and inter individual variation of hCG glycoforms in pregnancy and tumours appear to exist but no study has yet quantified the extent of this variation. It remains to be seen whether earlier promising results regarding the proposed clinical application of assays for hCG-h can be verified (Stenman, 2011).

5.8 METABOLISM AND CLEARANCE OF CIRCULATING HCG

Degradation by enzymic nicking as previously described is a source of variant forms and a process that affects clearance of hCG from the blood. The rate of clearance is important as it affects both serum concentration and rates of change in biomarker determinations and hence their clinical interpretation. The clearance of hCG after an abortion or a term pregnancy is best described by a triphasic model with median half-lives of 3.6, 18 and 53 h (Korhonen, 1997). Clearance after injection of recombinant hCG is slower and biphasic with half-lives of 5–6 h and 24–33 h (Rizkallah, 1969; Wehmann & Nisula, 1981). The slower clearance may reflect differences in glycosylation of the purified recombinant form or partial degradation due to the purification process itself (Stenman, 2006: Medeiros, 2008). Most of the hCG in circulation is metabolized by the liver, whereas about 20% is excreted by the kidneys (Nisula, 1989). During excretion, a major part of hCG is degraded to subunits, nicked forms and especially hCGβcf (Wehmann & Nisula, 1980; Nisula, 1989) which is the predominant form of hCG present in urine from the 5th week of pregnancy onwards (de Medeiros, 1992a; Norman, 1987).

The free hCGβ subunit isoform disappears more slowly than intact hCG with half-lives of 1, 23 and 194 hours. Hence following pregnancy the proportion of hCGβ relative to the total hCG immunoreactivity increases from 0.8% at term to around 27% after 3 weeks. Similarly, following successful therapy as hCGβ is cleared from the circulation more slowly than intact hCG, the proportion will also increase while the total hCG levels decrease (Korhornen, 1997). It is important therefore that when evaluating the value of %hCGβ that sampling occurs before commencement of treatment (Stenman, 2006).

The half-life of hCGα is shorter than that of hCGβ, and after term pregnancy half-lives of 0.6, 6 and 22 h have been observed (Korhonen, 1997).

5.9 METHODOLOGY OF HCG MEASUREMENT USING IMMUNOASSAYS

Since the development by Wide and Gemzell of a more rapid and convenient means of detecting hCG based on haemagglutination inhibition, assays utilising antibodies, 'immunoassays' have remained the predominant method for determining hCG concentration (Wide & Gemzell, 1960; Ekins, 1998). However the first truly specific immunoassay for accurate quantitation of serum hCG concentration was a radioimmunoassay developed over a decade later (Vaitukaitis, 1972). The rabbit antiserum that it used, 'SB6' was raised by immunization with the hCGβ subunit and due to its clinical utility was widely distributed. On distribution the antisera was labelled 'beta subunit assay,' which confused the recipients into thinking that only hCCβ form was detected and explains the origin of the misleading expressions 'β-hCG assay' or hCG-beta-assay' commonly used to describe total hCG immunoassays to this day. Radioimmunoassays are classified as competitive format immunoassays as the principle of measurement is based on competition between a fixed and known amount of radiolabelled hCG and any sample hCG for a fixed, limited number of antibody binding sites (Stenman & Alfthan, 2013). Several trophoblastic centres are still using radioimmunoassays of this type, including that of the UK Trophoblastic Screening and Treatment Centre in London, which has used the same rabbit polyclonal antisera since the early '80s. Some of these assays, including that of the UK trophoblastic service, have the advantage of recognizing all of the major protein isoforms of relevance in both serum and urine (Harvey, 2010; Sturgeon, 2009).

Radioimmunoassays for hCG are no longer commercially available and presently all commercial immunoassays for quantitation of serum hCG are of the non-competitive so called sandwich type. Sandwich format, 'immunometric' immunoassays employ 2 antibodies, a solid phase antibody to capture hCG molecules from the sample and a second antibody labeled with an enzyme, fluorophor or luminescent marker to enable detection of the captured hCG. Since the antibodies can be used in relative excess they have higher sensitivity than their competitive format counterparts. Most of these assays use 2 monoclonal antibodies each of which recognizes a single site on the target molecule known as an epitope, both of which must be present on the hCG molecule for detection. The sandwich principle is utilized by both qualitative and quantitative type assays. Qualitative pregnancy test type assays are most convenient when all that is required is a simple yes/no answer whereas quantitative immunometric assays are used for serological measurements requiring greater test accuracy or estimation of trophoblastic tissue burden and are required for all other clinical applications. Most quantitative immunometric assays are 'heterogeneous' with one antibody immobilised to a solid phase (capture) antibody and a second (detection) antibody solution added after sample incubation (for reviews see Sturgeon & McAllister 1998, Stenman & Alfthan 2013). The type of label chosen to enable detection (i.e. whether enzyme, fluorophore or a luminescent marker) also has a considerable influence on assay performance. Time-resolved immunofluorometric assays (TR-IFMA) based on the use of fluorophores with a long decay time increase sensitivity and assay range in comparison with conventional enzyme linked or fluorometric immunassays (Lövgren and Petterson, 1990). TR-IFMA type assays are the most sensitive immunoassays for serum measurement of hCG in clinical use (Alfthan, 1988).

5.10 HCG EPITOPES AND RECOMMENDED ANTIBODY COMBINATIONS FOR IMMUNOASSAYS

One of the most important factors determining the performance and clinical utility of an immunoassay is that of the precise epitope specificity of the antibodies used in its formulation. The antigenic regions or 'epitopes' recognised by antibodies that have been raised to hCG have been characterised by the hCG epitope mapping workshop held under the auspices of the International Society of Oncodevelopmental Biology and Medicine (ISOBM) (Berger, 2013). These collaborative studies comprised mapping of 27 mAbs directed against hCG and hCG variants provided by several major diagnostic companies and defined the molecular location of a total of 24 epitopes (See figure 5.3). On the intact hCG molecule, hCGα displays five epitopes (α1-α5), while hCGβ has seven (β1-β5 and β7-β9) of which β8-β9 are present on the C-terminal peptide region, hCGβCTP. In addition, there are four conformational epitopes (c1-c4) that are present on the intact hCG molecule, with only c3 and c4 being retained on hCGn. Free hCGα displays an additional two epitopes (α_6 and α_7), as does free hCGβ (β_6 and β_7). Finally, epitopes β10-β13 are only present on, and hence highly specific for, hCGβcf. Most epitopes are determined by the protein backbone (Berger, 1993; Lottersberger, 2003) but a few rare mAbs targeting the hCGβCTP portion that are influenced by O-linked carbohydrates attached to Ser132 or Ser138 have been discovered (Berger, 2002). Knowledge of the epitope distribution on variant forms and the epitope specificity of available monoclonal antibodies facilitates design of assays specific for each form of hCG (Bidart, 1985; Alfthan, 1992). This knowledge, combined with an understanding of the structures of clinically relevant hCG variants and their distribution in biological fluids, has enabled formulation of practical recommendations regarding the most appropriate mAb specificities and immunoassay designs for hCG methods for clinical use. For oncology purposes, broad assay specificity is preferable in order to detect both intact hCG and molecules derived from hCGβ. To construct a sandwich type assay to fulfil these requirements the ISOBM recommends that a monoclonal antibody against the β_1 epitope (which recognises the cystine knot region) should be used as a capture antibody together with a detector antibody that recognises the β_2 or β_4 epitopes at the top of loops 1 and 3 of the hCGβ (Berger, 2002; Berger, 2013). These recommendations should influence manufacturer's choice of antibodies for the next generation of hCG immunoassays and thereby lead to improved between-laboratory comparability of assays for hCG and hCG variants (Berger & Sturgeon, 2008).

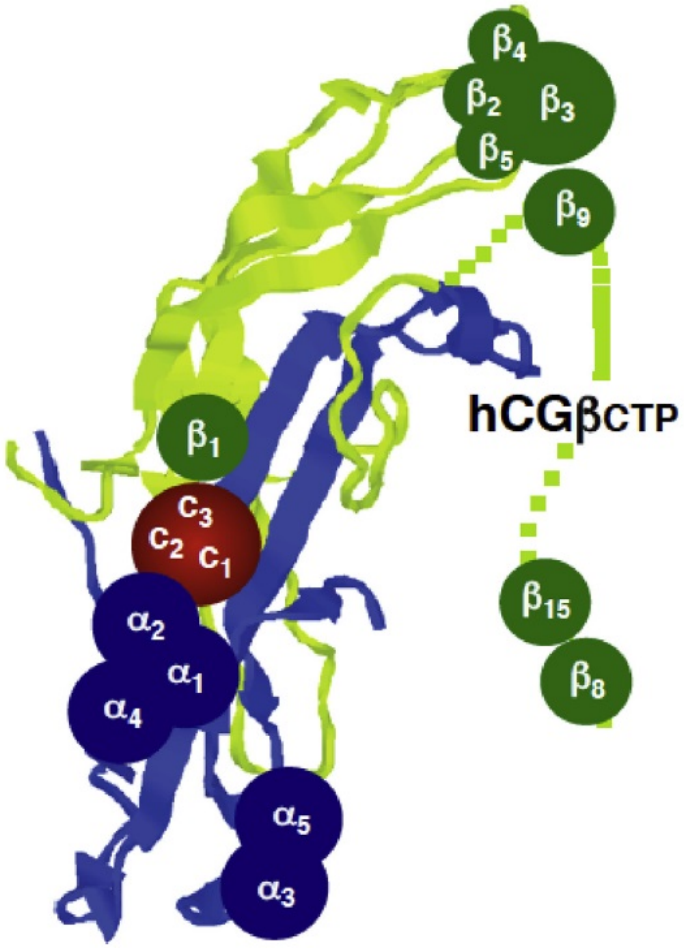


Figure 5.3. Epitope map showing the major epitopes discerned by monoclonal antibodies on hCGα (in blue) and hCGβ (in green) and intact hCG (in red) on a 3-D model of intact hCG. Modified from Berger *et al.,* 2013 open access article at Springerlink.com and reproduced with permission from Springer Verlag.

5.11 SELECTION AND VALIDATION OF HCG IMMUNOASSAYS

Between method variation in total hCG assay results due to analyte heterogeneity and variation in assay design is a long standing and widely recognised problem (Bock, 1990; Cole, 1992; Cole, 2004; Mitchell, 2007). This is especially true for samples from patients with gestational trophoblastic disease, germ cell tumours, and other neoplasias, conditions in which higher proportions of free subunits, partially degraded fragments and glycosylated variants of hCG may be present (Marcillac, 1992; Cole, 1997, 2004; Mitchell, 2007; Valmu, 2006; Lottersberger, 2003; Harvey, 2010). Discrepancies between results from different assays are unsurprising given the multiple different forms of hCG, hCG subunits and degradation products that are present in serum, the multiple different epitopes present on these molecules, as well as the continued use of assay calibrants aligned with the first IRP that was purified from pregnancy urine and is known to be contaminated with nicked hCG and hCGβcf (Canfield & Ross, 1976; Birken, 2003).

Variability of detection between assays among the variant hCG isoforms appears greatest for hCGβ and hCGβcf (Harvey, 2010; Sturgeon, 2009). While polyclonal competitive immunoassays such as that used by the UK Trophoblastic Disease Service generally recognise most hCG variants, some two-site monoclonal assays do not detect all forms. For example, among 5 of the most popular commercially available sandwich assays presently in use, only the Siemens Immulite 2500 and Roche Elecsys assays detected the hCGβcf form measuring 73% and 31% of the target value, respectively (see figure 5.4; Sturgeon, 2009; Harvey, 2010). The problem of poor between method comparability of hCG results in the management of trophoblastic disease has been minimised in countries such as the UK and

Netherlands by establishment of centralised trophoblast reference centres that manage all patients using a single assay.

Despite the epitope recommendations of the ISOBM workshop and the availability of the new WHO defined standards, manufacturers do not currently describe the epitope specificity of their assays in their provided instructions for use (IFU). Therefore it is necessary to confirm assay specificity by using the latest WHO standards (figure 4 shows the results of such an experiment). In order to minimise false negative results and inaccuracy due to fluctuations in the proportions of isoforms present it is recommended that assays used for management of trophoblastic disease recognise each of the 5 IRRs for the clinically relevant protein isoforms. Since equimolarity for these isoforms has not been observed for any assays evaluated to date and, it has been argued, is theoretically impossible (Ekins, 1991), reasonable thresholds for defining an assays acceptability within the current state-of-the-art of available immunoassays should be; no less than 50% or greater than twice that of the intact hCG IRR (99/680) measurement for each of the latest WHO standards

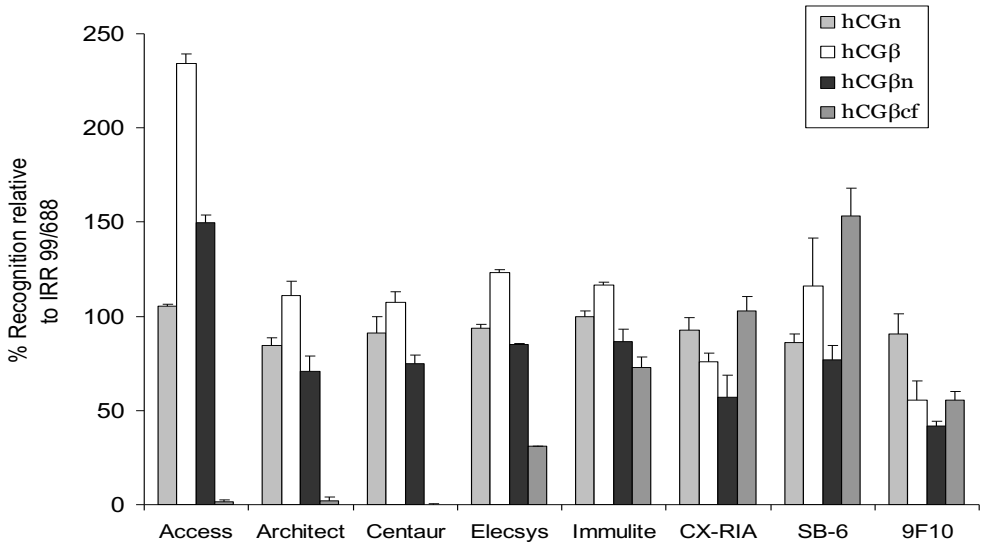


Figure 5.4. Percentage recognition of first WHO IRR isoform preparations for hCGn (99/642), hCGβ (99/863), hCGβn (99/692) and hCGβcf (99/708) relative to intact hCG IRR (99/688) in the various assays included in the study. Data are presented as percentage recovery, that is, 100 × (isoform result divided by 99/688 result). The ratio plotted is the mean of 2 results for each of the IRR preparations measured at 75pM and 300pM ± SD. Reproduced from Harvey *et al* 2010.

5.12 SEPARATE MEASUREMENT OF ISOFORMS

It is also possible for laboratories to formulate their own sandwich type assays by choosing monoclonal antibodies of appropriate affinity and epitope recognition to use in pairs for either broad spectrum total hCG measurement or selected isoform determination (Berger, 2002). One antibody, the 'capture' antibody is used to pre-coat a solid phase substrate, such as a polystyrene 96-well plate, while a second 'detection' antibody is labelled with a suitable tracer (i.e, radioisotope, enzyme or fluorophore) and added in solution after sample incubation and washing. By labelling of the detection antibody with a europium chelate time-resolved immunofluorometric assays for hCGβ, hCGβcf and intact hCG have been formulated that, due to lower background noise:signal ratios possible through the use of fluorophores with a long decay time, achieve sub-picomolar measurement (Pettersson*,* 1990; Aflthan, 1988, 1990). While use of such assays has been helpful in elucidating the biochemistry, physiological distribution and metabolism of hCG and are useful for research into the possible clinical utility of separate measurement of isoforms a clinical requirement for their use in the management of trophoblastic disease has not been established. Furthermore in-house formulation and processing of 96-well plate assays is both labour intensive and subject to greater operator error and poorer precision than current state-of the-art automated immunoassay analysers. The specialist knowledge and skills required to setup, troubleshoot and maintain sufficient rigour for clinical service requirements are unlikely to be available in most non-specialist and/or less well funded general clinical laboratories.

5.13 MASS SPECTROMETRY

Due to rapid developments in mass spectrometry (MS) based technology, particularly concerning introduction of soft ionization techniques that facilitate analysis of large biomolecules, the possibility of using a MS based method for quantitation of hCG isoforms either together or separately is increasingly attractive. Soft ionization techniques that have been used include matrix assisted laser desorption ionisation (MALDI) and electro spray ionization (ESI). While MALDI provides mainly single charged ions comprising a mass range suitable for most serum proteins which simplifies interpretation, the sensitivity decreases rapidly with increasing molecular weight and the method is not quantitative (Hortin, 2006). All quantitative MS methods that have been developed for hCG measurement use ESI coupled with HPLC reverse phase chromatographic columns. Their mass range is limited to approximately 3000 Da and therefore larger proteins need to be fragmented prior to analysis which is usually achieved by enzymatic degradation using trypsin. ESI coupled with hybrid mass spectrometers employing both quadrapoles together with a time-of-flight (TOF) MS unit provides a suitable arrangement for precise structural studies, such as those used for glycoproteomic analysis, but they are less suitable for quantitation (Valmu, 2006; Stenman, 2008). For accurate quantitation of proteins the best results have been achieved using ESI coupled to triple quadrapole mass spectrometers which are widely used by laboratories providing analytical services for doping control and increasingly used by clinical labaoratories. To remove interfering substances and improve sensitivity hCG must firstly be purified by an immunoextraction step. Using this approach a recent method has been developed that could detect hCGβ, nicked hCG forms and hCGβcf in urine or sera with a limit of quantitation of 5 IU/L (Lund 2012). The method could not however distinguish hCG and hCGβ as both isoforms contain the same "βT5" peptide fragment used for quantitation. By use of several monoclonal antibodies with differential selectivity for hCG isoforms a further immunoextraction method for LC ESI-Triple Quadrapole MS has been developed for measuring intact hCG separately from hCGβ and βcf in urine as required for hCG testing in males for sports according to the World Anti- Doping Agency, (Woldermariam and Butch, 2014). This method had a 25-fold lower limit of quantitation (0.2 vs 5 IU/L) and precision of ≤10.4% for each isoform at concentrations of 2-7IU/L which approaches the performance of current immunoassays. While the analytical sensitivity and precision are sufficient for urine testing in trophoblastic disease the method, including its ability to discriminate intact hCG, hCGβ and βcf in urine remains dependent on the use of antibodies with appropriate epitope specificity. Furthermore, due to the overall complexity of the method, particularly the requirement for multiple sample pre-treatment steps, the throughput is limited and estimated to be only 60 samples per week (Woldermariam and Butch, 2014). In summary, despite the principal theoretical advantages of LC-MS/MS based methods of high analytical sensitivity and specificity, due to their greater complexity, slower throughput and lower precision, they are not yet a

viable alternative to immunoassays for measurement of hCG in the management of trophoblastic disease for most clinical laboratories.

5.14 REFERENCE VALUES FOR SERUM AND URINE

Reference limits for the upper limit of 'normal' (ULN) of total serum hCG concentration in non-pregnant women are used to define pathologically raised hCG and are particularly important in providing clinical decision thresholds for cessation of chemotherapy and for relapse detection in the management of GTN. Because of differences in calibration and specificity between various methods, it is desirable that reference values should be determined separately for each assay. Manufacturers provide upper reference limits with their assays and these differ for total hCG assays. They range from 3-4 to over 5 IU/L for premenopausal (non-pregnant) women, reflecting variation in assay calibration due to the impure standard IS 579 and variant assay specificity for different isoforms. The ULN is variably defined by assay manufacturers and represents the concentration that comprises either 95, 97.5 or 99% of the hCG measurements of a 'normal' non-pregnant healthy population. For most hCG assays the ULN for total hCG in men and women is close to the analytical sensitivity of the assay. According to the Siemens Immulite assays' instructions for use (IFU) supplied with the reagent pack, in a study of 369 non-pregnant females, 95% had serum values below 2.7 IU/L, and the highest value was 5.2 IU/L. For the Abbott Architect method the IFU recommends a ULN of <5 IU/L and cites Tietz NW, Clinical Guide to Laboratory Tests, 3rd Ed. 1995 as the source.

More accurate reference ranges for intact hCG, free hCGβ-subunit and hCGβcf in serum and urine for men and pre- and post-menopausal women have been determined using highly sensitive immunofluorometric assays. The values are shown in Table 5.4 and, strictly are valid only for these assays (Alfthan, 1992). These studies confirm that hCG levels increase with age, most noticeably for intact hCG in post-menopausal women due to pituitary secretion of hCG. Serum levels of hCGβ-subunit are lower and occasionally undetectable even using high sensitivity assays. The of hCGβ levels do not increase with age. The urine concentrations of intact and free hCGβ-subunit are similar to those of the serum. Urinary hCGβcf concentration is also similar to that of serological intact hCG and therefore the total hCG levels are higher in urine than in serum. Urine total hCG concentrations, though strongly correlated to that of serum, vary considerably due to greater heterogeneity of isoforms present and hydration status. As urinary determination of hCG is not as accurate a reflection of disease status as serological measurement and commercial assays aren't validated for urine quantitation, accurate urine reference ranges for total hCG assays have not been determined. Hence, the cut-off used for determining the positive threshold for qualitative pregnancy testing by most manufacturers is cautiously high and usually stated as 25 IU/L (Stenman 2006, 2008).

Table 5.4. Upper reference limits for serum and urine concentrations of hCG, hCGβ and hCGβcf in non-pregnant women (n=606) and in men (n=423). Reproduced from Alfthan *et al.*, 1992.

Fluid	*Women, 97.5 percentile*				*Men, 97.5 percentile*			
	<50 years		≥50 years		<50 years		≥50 years	
	pmol/l	IU/l	pmol/l	IU/l	pmol/l	IU/l	pmol/l	IU/l
Serum								
hCG	8.6	3.0	15.5	5.4	2.1	0.7	6.1	2.1
hCGβ	1.6		2.0		1.9		2.1	
Total hCG	9.0		17.0		3.2		7.1	
Urine								
hCG	8.8	3.1	11.5	4.0	2.9	1.0	8.4	2.9
hCGβ	1.7		4.3		1.3		3.6	
hCGβcf	8.1		9.5		6.7		8.5	
Total hCGβ	13.6		20.4		8.0		21.5	

5.15 POST-MOLAR SCREENING AND DIAGNOSIS OF GTN

Post-molar GTN, the commonest form of GTN requiring treatment, is usually diagnosed by repeated hCG measurements following evacuation of a histologically confirmed molar pregnancy. 15-20% of complete moles develop invasive trophoblastic disease characterised by persistently elevated serum hCG after evacuation whereas the frequency of invasive disease following evacuation of partial moles is only 0.1-5% (Seckl, 2010; Lurain, 2010). Criteria for the diagnosis of post-molar GTN requiring chemotherapy were agreed in 2000 by the International Federation of Gynecology and Obstetrics (FIGO) as follows:

- Weekly hCG rising for at least 3 consecutive measurements for a period of at least 2 weeks (days 0, 7, and 14)
- Weekly hCG plateauing for at least 4 consecutive measurements for a period of at least 3 weeks (days 0, 7, 14, and 21)
- Persistence of hCG more than 6 months after evacuation
- Histological diagnosis of choriocarcinoma

Plateaued hCG is defined as four or more equivalent values of hCG over at least 3 weeks (days 1, 7, 14 and 21) and rising hCG as two consecutive rises in hCG of 10% or greater over at least 2 weeks (days 1, 7 and 14) (Seckl, 2013). The precise hCG surveillance protocol varies by country. Definitive follow-up after evacuation of CHM or PHM requires serial serum and/or urine hCG measurements every 1 to 2 weeks until at least 2 consecutive tests show normal serum levels, after which, hCG levels should be determined monthly for up to 6 months in patients with CHM but can be stopped in those with PHM because the risk for subsequent GTN is less than 1:3000 (Mangili, 2014). An elevated but falling serum hCG 6 months after molar evacuation is no longer an absolute requirement for commencing chemotherapy because patients undergoing continued surveillance all subsequently normalise (Agarwal, 2012). The overall risk of recurrence is low, 0.6%–2% after one molar pregnancy although much increased after consecutive molar pregnancies (Seckl, 2013). Because GTN rarely occurs after hCG levels have spontaneously fallen to normal contraception for only 6 months rather than 1 year is now recommended (Sebire, 2007; Lurain, 2010).

Although the incidence of GTN following a non-molar pregnancy is much lower and estimated to be 1-9 per 40,000 pregnancies, GTN should also be considered in the differential diagnosis of patients with unusual presentations and serum hCG should be performed as part of the workup of such patients

(Ngan, 2015). For postpartum GTN, chemotherapy is indicated either when a histological diagnosis of choriocarcinoma is established or when there are clinical features such as unexplained widespread metastasis in a woman of childbearing age with elevated hCG levels (Mangili, 2014).

Placental site trophoblastic tumour (PSTT) is a rare neoplastic variant of trophoblastic disease that arises from implantation site intermediate trophoblastic tissue. Epithelioid trophoblastic tumor is a rare variant of PSTT that simulates carcinoma (Mangili, 2014). Most PSTTs follow non-molar gestations and appears to be the most aggressive form of GTN (Schmid, 2009). The FIGO prognostic scoring system is not used for determining therapy in these patients. PSTT often presents with low and slowly changing concentrations of hCG and can be difficult to differentiate from the more common conditions of choriocarcinoma and invasive trophoblastic disease. The differentiation of PSTT from early stage choriocarcinoma is particularly problematic as the accuracy of histological diagnosis is often limited by the amount of tissue obtained at endometrial curettage. It has been suggested that a percentage free hCGβ-subunit of total hCG immunoreactivity exceeding 35% distinguishes PSTT from choriocarcinoma and so called quiescent GTD with a 100% detection and zero false-positive rate (Cole*,* 2006). A later study by our group confirmed that PSTT showed the greatest proportion of free hCGβ at approximately 45% but due to wide variability within the disease groups was not an absolute test and could not reliably differentiate PSTT from choriocarcinoma (Harvey, 2008). Due to the rarity of these more aggressive disease forms the number of patients reported in these studies is low but updated figures from the author's laboratory, comprising 19 PSTT, the largest collated to date, confirmed that the test could not distinguish PSTTs from patients with chorocarcinoma (AUC ROC curve =0.60; p =0.2, see table 5.1) and hence the approach is of limited clinical utility.

5.16 TREATMENT MONITORING

The treatment of GTN is generally by chemotherapy. Determination of the recommended regimen to commence treatment is guided by risk scoring according to the 2000 FIGO staging and classification system. A risk score of 6 and below is classified as low-risk and above 6 is considered high-risk (Ngan, 2015). The FIGO prognostic scoring system is not valid for determining therapy in patients with rarer PSTT and ETT disease forms, but FIGO staging is used to help adapt treatment intensity (Seckl 2013). Patients with low-risk GTN should be treated with either MTX or actinomycin D whereas multiple agent chemotherapy regimens are used to treat high-risk GTN, most commonly with EMA-CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine). The FIGO risk scoring system is based on hCG measurement, imaging and other clinical findings. As points are assigned based on hCG thresholds at 1000 IU/L (which receives one point), 10 000 IU/L (two points) and 100 000 IU/L (four points) variant measurement of hCG by different assays can affect the risk stratification, particularly at high concentrations.

Over 80% of patients are classified as low-risk and the 8-day methotrexate (MTX) protocol modified by Bagshawe et al, 1989 is the most commonly used treatment in Europe for this group of patients (Savage, 2008; Chalouhi, 2009; Kerkmeijer, 2009). MTX treatment is continued until either normalisation of serum hCG concentration or resistance to MTX therapy is detected (Seckl, 2010). In cases of MTX resistance, observed in nearly one-half of low-risk cases (Sita-Lumsden, 2012), patients are changed to either actinomycin D (ACT-D) or to combination therapy with EMA-CO (Powles, 2007; Patel & Desai, 2010). Tumour chemoresistance is frequently defined as an increase or stagnation of hCG levels over a 2- to 3-week period, but no consensus guideline has been defined (Foulmann, 2006). Ambiguity concerning the definition of MTX resistance based on changing hCG levels increases the likelihood that patients will receive repetitive ineffective cycles of MTX chemotherapy. Recent studies indicate that a kinetic parameter derived by population based modelling of declining hCG levels has significantly greater prognostic power for 1st line treatment resistance to MTX in low risk GTN than FIGO score (You & Harvey*,* 2013). This is likely because (i) weighting of points for FIGO scoring have not been mathematically derived and (ii) reduction of serial measurements to discontinuous categories for FIGO scoring reduces the inherent predictive value of serial hCG measurements.

5.17 RELAPSE DETECTION

Chemotherapy should be continued until the serum hCG is normal and then for a further 3 treatment courses over 6 weeks. This helps to eliminate any residual tumour cells and to minimise the chances of relapse (Seckl, 2013). A retrospective comparative analysis from 2 treatment centres suggests that increasing the consolidation cycles from 2 to 3 reduce the risk of relapse from 8 to 3% in low-risk GTN (Lybol, 2012).

After treatment, hCG surveillance in serum and/or urine is necessary to monitor for relapse. The frequency and duration of hCG tests are not standardized. Some centres measure hCG monthly for up to 18 months while others test more frequently initially and slowly taper to 6 monthly urine samples for life (Seckl, 2013). Due to the poorer accuracy of urine total hCG measurements, raised urine measurement should be confirmed by further urine and serum samples. Because most relapses occur in the first 12 months, avoiding pregnancy during this period seems sensible unless other reasons for an earlier pregnancy exist, such as advancing age (Seckl, 2013). During early pregnancy hCG concentrations increase exponentially with a doubling rate of 1.5-2 days to a peak of 20-100,000 IU/L at 7-10 weeks which is faster than for most relapses (Alfthan, 1988).

5.18 PITFALLS OF HCG IMMUNOASSAYS; CAUSES OF MEASUREMENT ERROR

False or inaccurate determinations of hCG have had serious adverse consequences and the clinical risk of analytical errors using hCG immunoassays in the management of GTN is a significant concern (Rotmensch & Cole, 2000; Stenman 2006, 2013). Broadly, analytical errors can be categorised as being of two main types; exogenous or endogenous. Exogenous errors are independent of the intrinsic properties of individual patient specimens and due to problems in processing of samples including mishandling at venepuncture, mislabelling of specimens or instrument failures including pipetting errors and carryover. Their risk can be minimised by adherence to good quality control and clinical governance procedures. As all medical laboratory laboratories should have procedures to deal with these errors a detailed discussion is beyond the scope of these guidelines and they shall not be considered further. Endogenous errors on the other hand are patient sample dependent and due to the presence of infrequently occurring cross-reacting substances interacting with either the assay's antibodies themselves or binding to the target analyte and, being random and sporadic in nature, are hard to detect. Such assay interferences may be caused by a number of possible disturbant binding or signal generating interactions which maybe either analyte dependent or analyte independent. Analyte independent interferences are those due to the presence of abnormally high concentrations of normal serum constituents commonly haemolysis, icterus and lipaemia. Immunoassays are much less affected by haemolysis and icterus than analytes measured by spectral means. Lipaemia can interfere in immunoassays, especially in those using nephelometry or turbidimetry but most current commercially hCG assays do not use these methods. Turbidity in lipeamic samples and haemolysis can be visually detected. Automated detection of haemolysis icterus and lycopenaemia is available for some automated analytical instruments and the advantages of this approach have been reported (Young, 2007; Guder, 2009; Vermeer, 2005). The effects of excess concentrations of normal serum constituents on immunoassay has been thoroughly investigated and the thresholds below which such common cross-reactants would not be expected to interfere are stated in the manufacturers instructions provided with assay kits.

Analyte-dependent interferences refer to those measurement errors caused by interactions between sample constituents with one or more of the reagent antibodies and, due to their greater prevalence represent the greatest problem. The simplest form of such cross-reacting interference is that due to structural similarity with other proteins, the best example of such regarding hCG immunoassays is that of the susceptibility of early assays to cross reaction with LH as previously described. The causative agents of endogenous interferents of greatest concern in the use of modern hCG immunoassays include (i) heterophile antibodies and autoantibodies including rheumatoid factor, (ii) human anti-animal antibodies (HAAA), (iii) auto-analyte antibodies, (iv) complement or (v) some other infrequently expressed miscellaneous endogenous proteins.

Heterophilic antibodies

Heterophilic antibodies are naturally occurring antibodies with low affinities for many antigens, including self-antigens, and the variable region of other antibodies (anti-idiotypic antibodies). They maybe of either IgG or IgM subtype (Covinsky, 2000). Heterophilic antibodies such as those that occur in infectious mononucleosis are inherently produced from B cells prior to antigen exposure and result from random combinations of the immunoglobulin genes of the heavy and light chain variable regions. It has been proposed that they have roles in immune system regulation and removing foreign antigens from the intestinal tract (Levinson, 2002). They may cause either positive or negative interference as they can interfere with reagent Ab-Ag interaction in a number of ways including directly binding the antigen and altering the 'free' analyte concentration, by mimicking the structure of the antigen and blocking reagent Ab sites or by the cross-linking the capture and detection antibodies of 2-site sandwich (Ismail, 2002; Tate & Ward 2004). Direct cross-linking of capture and detection antibodies in the absence of the target analyte results in the accumulation of labelled detection antibody in the reaction vessel causing a false positive signal. False-positive results due to cross-linking in sandwich assays is estimated to be the most frequently occurring type of immunoassay interference. Single site competitive type immunoassays are not susceptible to this form of interference (Sturgeon, 2011; Halsall, 2013).

Heterophile antibody interference due to the presence of auto-antibodies that are often present in the sera of patients with autoimmune rheumatoid disease antibodies and hence commonly referred to as 'Rheumatoid factor' cause assay interference by binding to the Fc region of assay antibodies leading to aggregation. Such interference may cause either positive or negative interference in sandwich and competitive type immunoassays. Sandwich assays are more susceptible to interference from weak heterophilic antibodies as there is less competition for binding in antibody reagent excess reactions unlike competitive format immunoassays where the primary antibody has high analyte affinity and whose concentration is limited in the assay reaction vessel. Falsely increased results may be generated in competitive assays by antibodies which are able to sequester or block assay Ab binding sites. Falsely low results in competitive assays due to heterophilic interference are also possible but rare, as in this case the interfering antibody must prevent displacement of the tracer from the primary antibody (Halsall, 2013). It has been reported that up to 30% of IgA deficient individuals are found to give false positive hCG results due to heterophilic antibodies (Knight, 2005). As prevalence is relatively high in European populations varying from approximately 1/220 to 1/1000, measurement of IgA is recommended in individuals with proven heterophilic interference (Burks, 1986; Hammarstrom, 2000).

Human anti-animal antibodies (HAAA)

Species specific human anti-animal antibodies (HAAA) are an important type of interfering antibody that are distinguished from weak poly specific heterophilic antibodies as they are of high affinity for a single chemical motif and commonly species specific (Kricka, 1999). HAAA are most commonly human anti-mouse antibodies (HAMA), but also include antibodies to other animals and are reported to occur in 30-40% of patient samples (Selby, 1999). HAMA type antibodies have been reported for numerous analytes and are responsible for the highest observed false positive interferences. They can be generated as a consequence of iatrogenic exposure to mouse Abs which are increasingly used both in modern diagnostic imaging procedures and in recombinant therapies. Accordingly, in patient's known to have been exposed to mouse monoclonals the index of suspicion for such false positives should be raised (Sturgeon, 2011). Sandwich type methods that use only one mouse monoclonal antibody are less prone to interference from HAMA than those using mouse mAbs for both detection and capture antibodies (Selby, 1999).

Anti-hCG antibodies

Anti-analyte antibodies to hCG do not naturally occur as women are immunologically tolerant to their own production of hCG. This tolerance, however, can be overcome by injection of hCGβ conjugated to tetanus toxoid with adjuvant as has been demonstrated in efforts to produce hCG vaccines for fertility control (Talwar, 2013). A recent report of a single case of false positive hCG measurements due to 'macro-hCG' of ~40 IU/L in a 38 year old lady who had had intra uterine insemination suggests that

therapeutic use of hCG injections may also induce anti-hCG antibodies in some individuals (Heijboer, 2011). In this report the presence of anti-hCG antibodies responsible for formation of large molecular weight polymolecular complexes with hCG, 'macro-hCG' was identified by precipitation of the macro-analyte complexes using Polythylene Glycol (PEG) in the same manner as routinely performed in screening raised serum prolactin measurements for macroprolactinaemia, the best known and most prevalent macro-analyte condition. By further analysis using gel filtration though a Superdex 200 10/300 GL column a high molecular mass peak corresponding to the molecular mass of IgM was identified. The anti-hCG antibody was further confirmed to be of IgM subtype by immunoextraction using anti-human IgM bound to agarose.

Other proteins

Other proteins that can interfere with the reagent Ab-ag reaction include complement, lysozyme and paraprotein. Binding of an IgG kappa type paraprotein to a TSH assay antibody causing steric inhibition of binding and a falsely low TSH measurement has been reported (Luzzi, 2003). Complement binds to the Fc fragment of immunoglobulin molecules reducing their binding capacity and therefore can produce falsely-low results in sandwich assays and higher results in competitive assays (Bormer 1989). IgG2 subtype antibodies often react with complement and are therefore infrequently used by assay manufacturers. Lysozyme may bind to immunoglobulins and can also cause false-positives by cross-linking of the solid-phase capture IgG and detection antibody of sandwich immunoassays (Selby 1999).

Blocking reagents

The prevalence of interference is assay dependent and has been reported for the most frequently occurring heterophile or HAMA type interferences as ranging from as low as 0.05% to up to 6% (Tate and Ward, 2004). Commercial manufacturers have made efforts to minimise the risk of antibody interference by the addition of reagents to their assays. HAAAs can be specifically targeted by addition of excess non-immune (or non-specific) polyclonal IgG without affinity for the target analyte as decoy targets for the interfering anti-animal antibodies or by the addition of 'heterophilic blocking' agents. Heterophilic blocking agents often comprise antibody fragments that specifically block the invariant Fc tail region where heterophiles usually bind. While such measures have undoubtedly reduced the frequency of problematic interference since the target region, affinity and quantity of interfering antibodies can vary from patient to patient the amount of blocking afforded by the added substances can be overwhelmed (Marks, 2002). Alternatively the use of Fab fragments in the formulation of immunoassays instead of the entire Ig molecule can similarly protect against heterophile interference. In a large study of over 11,000 serum samples measured using a sandwich assay constructed using mouse monoclonal Abs the frequency of interference was reduced from 4% to 0.10% by removing the Fc fragments from the capture antibody (Bjerner, 2002).

5.19 IDENTIFYING ANTIBODY INTERFERENCE

The first line of defence in minimizing the risk of antibody related interferences should always be to suspect results that do not fit the clinical picture (Sturgeon 2011). The laboratory should carefully check results before reporting and clinicians and nurses should contact the laboratory should they find a suspicious result. Laboratory processes should be in place to enable quick confirmation of interference in suspected samples. There are several simple approaches that can be used to confirm the presence of substances interfering in hCG immunoassays but as they all lack sensitivity a combination of approaches is required. Use of three basic methods; (i) use of an alternate assay, (ii) paired urine and serum analysis and (iii) linearity studies and/or measurement before and after addition of species specific blocking reagent should lead to positive confirmation of interference in most cases.

(i) Analysis using an alternative immunoassay
The simplest means of investigating suspected interference is to repeat the measurement using an alternate immunoassay. It is preferable to use an assay which uses antibodies from a different species to that of the primary assay and ideally one that has a different architecture. For example if the laboratory's primary assay is an immunometric 2-site assay then a competitive assay should preferably be used as the second assay. Heterophilic false-positive interference in a sandwich assay will likely give lower results using an alternate assay but much lower results in a competitive assay. If higher results are determined using the alternate assay this could indicate interference by complement. Complement interference is rare as most assays no longer use IgG2 subclass antibodies. Interference by Rheumatoid factor or other miscellaneous serum proteins can cause positive or negative interference in either assay format. Agreement between assays does not completely exclude interfering antibodies nor do discrepancies, due to poor lack of assay standardisation, confirm their presence (Tate and Ward, 2004).

(ii) Repeat analysis of paired serum and urine samples
As interfering antibodies are too large to be filtered at the glomerular apparatus into the urine, provided the suspected original serum result is >50IU/L, a negative result using a paired urine sample is a reliable sign of a false positive serum measurement. The FP needs to be greater than 50IU/L otherwise the formation of a dilute urine could theoretically also cause a negative urine result (Stenman 2004).

(iii) Linearity, recovery and blocking reagent experiments
Non-linear dilution using the assay diluent buffer provided that the diluent buffer contains blocking reagents or under recovery of a known amount of the analyte (eg using the assay standard) added to the suspected serum sample may indicate heterophile interference. Using the dilution method the presence of heterophilic antibodies often results in a dramatic reduction in apparent concentration (lower than expected result) as previously overwhelmed blocking agents become effective when the heterophile is diluted out. Recovery experiments are performed by adding a known amount of hCG analyte (eg using the assay standard) to the suspected serum sample and re-assaying. The presence of heterophillic type interferents is confirmed by under recovery (lower than expected result); over recovery suggests either macro-hCG or matrix type interference by other serum components which may elude exact identification. Macro-hCG can be confirmed by PEG precipitation as previously described (Heijboer, 2011).

An alternative procedure for identifying a suspected interfering antibody is measurement before and after pre-treatment by adding sample to a heterophile blocking tube (eg Scantibodies) or the addition of a blocking reagent to the sample and the finding of a significantly different result. If the result remains unchanged further addition of more blocking agent and the use of a combination of blockers from different animal sources may help avoid the possibility of the blocking agents being overwhelmed (Ward, 1997) .

More complex investigations including Gel filtration chromatography, Protein A/G/L immunoadsorption chromatography or the use of specialist 'non-sense' interference assays can be used to further clarify the nature of interferences (see Sturgeon and Viljoen 2011 for further details).

5.20 HIGH-DOSE HOOK OR PROZONE EFFECT

The Prozone effect or 'High-dose hooking' occurs in sandwich type immunometric assays when analyte concentrations are so high that saturation of antibody binding sites prevents formation of the Ab-hCG-Ab* sandwich and ultimately favours formation of capture-Ab and free detection Ab-hCG*. The degree to which the result is falsely lowered varies depending on the degree of saturation so that any degree of false lowering could occur including reduction to below detection limit. Failure to recognise extremely high concentrations in gestational trophoblastic neoplasia constitutes a critical clinical error which at best is likely to delay treatment (Sturgeon, 2006). Hence it is vital that all samples from new patients suspected of trophoblastic disease with no previous results must be assayed at multiple dilutions (eg. neat 1 in 100 and 1 in 10,000). The precise requirements for dilution of new patient samples will depend on the assay as high-dose hook thresholds vary. So called one-step sandwich assays, in which there is no wash step between the addition of sample and the detection Ab, have lower high-dose hook thresholds than 2-step assays as washing after sample incubation with the capture Ab washes away excess free antigen (Sturgeon 1998).

5.21 CAUSES OF APPARENTLY FALSE POSITIVE RESULTS; PITUITARY HCG AND HCG INJECTIONS

Menopausal and peri-menopausal women sometimes have raised serum levels of hCG; levels up to 25 IU/L have been reported (Braunstein, 2002) but levels around 7-15 IU/L are more common. Using high sensitivity assays the hCG levels are 3-10% of those of LH and fluctuate in a pattern similar to that of LH (Odell & Griffin, 1987). Lack of GnRH suppression when sex steroid levels fall leads to raised LH and FSH which is accompanied by the secretion of low levels of intact hCG. Furthermore since postmenopausal hCG can be suppressed with oestradiol these findings taken together indicate that the hCG is derived from the pituitary (Stenman, 1987; Stenman, 2006). The mechanism is believed to be due to low level transcription from genes encoding the hCGβ-subunit which are located on the same chromosome and in close proximity to the LH gene (Cole, 2009). Free α-subunit production is not regulated and is produced in relative excess such that the holodimer is always formed. Chemotherapy also induces gonadal suppression and transient post-chemotherapy low level elevation of hCG following successful therapy may not indicate resistant disease or relapse (Catalona, 1979; White, 2010). The problem can be identified by also measuring LH/FSH and estradiol. If necessary, suppression of hCG by estradiol replacement can be used to further confirm such iatrogenic hCG elevation (Stenman, 2006).

Athletes may use hCG injections to stimulate gonadal steroid production and this may also be a cause of a elevated hCG measurement that could easily be misinterpreted (Braunstein, 2002). hCG injections are also used for controlled ovarian hyper-stimulation in assisted reproductive therapy, ART (Stenman, 2006).

5.22 QUIESCENT GESTATIONAL TROPHOBLASTIC DISEASE

Quiescent gestational trophoblastic disease is a purported variant of trophoblastic disease that is associated with persistent low-level hCG (Khanlian, 2005; Cole, 2010; Cole & Muller, 2010). However the existence of quiescent gestational trophoblastic disease as a distinct disease entity has not been widely accepted and, due to its purported characteristics including low reactivity to B152 based immunoassay for 'hyperglycosylated' hCG and small MRI negative disease foci in patients with a previous history of gestational trophoblastic disease, is difficult to verify, as assays for hCG-H are not commercially available. The recommendation to not treat such patients unless their hCG level reaches or exceeds 3000 IU/L (Cole & Muller, 2010) before commencing treatment has been strongly criticised by the medical trophoblastic community (Seckl, 2010).

5.23 FAMILIAL HCG

Familial hCG is a very rare genetic condition with an estimated prevalence of 1:60,000 in which serum and urine concentrations are persistently elevated for several years and probably the lifespan of affected family members. The hCG concentrations are usually low but high enough (10–200 IU/L) to cause suspicion of pregnancy or cancer. Familial hCG, due to rarity, is a diagnosis of exclusion once other causes of false positive hCG have been ruled out and confirmed by the finding of a similarly raised hCG isoform profile in first degree relatives. In all 10 reported families, all individual cases produced a combination of intact hCG, hCGβ or hCGβ-CTP isoforms. It is important to recognize this condition because it may lead to unnecessary treatment (Cole, 2012; Stenman, 2013).

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