

FORMS OF HUMAN CHORIONIC GONADOTROPIN IN SERUM OF TESTICULAR CANCER PATIENTS

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Academic dissertation

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*Live as if you were to die tomorrow.
Learn as if you were to live forever.*

– Mahatma Gandhi –

Contents

LIST OF ORIGINAL PUBLICATIONS	6
ABBREVIATIONS	7
ABSTRACT	9
1 REVIEW OF THE LITERATURE	10
1.1 hCG, hCG β AND hCG-h.....	10
1.1.1 Introduction	10
1.1.2 Structure	10
1.1.3 Function	11
1.1.4 hCG expression in healthy subjects	12
1.1.5 hCG expression in malignant disease	13
1.1.6 Stability of hCG in serum and in urine	15
1.1.7 Principles of hCG assays	15
1.1.8 Endogenous interferences in immunoassays	17
1.1.9 Clinical applications.....	19
1.2 TESTICULAR CANCER	20
1.2.1 Introduction	20
1.2.2 Epidemiology.....	21
1.2.3 Origin and predisposing factors	21
1.2.4 Genetic characteristics	22
1.2.5 Histology	23
1.2.6 Serum tumor markers.....	24
1.2.7 Diagnosis	27
1.2.8 Staging and prognosis.....	27
1.2.9 Treatment.....	29
1.2.9.1 ITGCN.....	30
1.2.9.2 Seminoma.....	30
1.2.9.3 NSGCT	31
1.2.9.4 Follow-up after curative therapy and long-term sequelae	32
2 AIMS OF THE STUDY	33
3 MATERIALS AND METHODS	34
3.1 PATIENTS (II, IV, V).....	34
3.2 SAMPLES	34
3.3 DETERMINATION OF DIFFERENT FORMS OF hCG	36

3.4 OTHER ASSAYS	38
3.5 STUDY DESIGNS	38
3.5.1 Stability of hCG in serum and in urine (I, II, IV).....	38
3.5.2 Development of an assay specific for hCG-h (III)	39
3.5.3 hCG, hCG β and hCG-h in serum of testicular cancer patients (II, IV, V).....	39
3.6 STATISTICAL METHODS	39
3.7 ETHICAL ASPECTS	39
4 RESULTS AND DISCUSSION	40
4.1 STABILITY OF hCG AND hCG β IN SERUM	40
4.2 STABILITY OF hCG IN URINE.....	41
4.2.1 Stability in urine during long-term storage	41
4.2.2 Stability in urine during short-term storage	42
4.2.3 Effect of urea and protective additives on the loss of hCG in urine	43
4.3 DEVELOPMENT OF IMMUNOFLUOROMETRIC ASSAY FOR hCG-h	45
4.3.1 Assay design and performance	45
4.3.2 Complement interference	46
4.3.3 Preparation of provisional standard	47
4.4 hCG, hCG β AND hCG-h IN SERUM OF TESTICULAR CANCER PATIENTS.....	48
4.4.1 Preoperative serum concentrations of hCG, hCG β and hCG-h in seminoma patients.....	48
4.4.2 Preoperative serum concentrations of hCG, hCG β and hCG-h in NSGCT patients.....	49
4.4.3 Detection of relapse	51
4.4.4 Prognostic value.....	52
4.4.5 Follow-up samples and false-positive results.....	54
4.4.6 hCG expression is not always caused by tumor tissue	55
5 SUMMARY AND CONCLUSIONS	57
6 ACKNOWLEDGEMENTS	58
7 REFERENCES	60
8 ORIGINAL PUBLICATIONS	76

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. The original publications have been reprinted with the kind permission of the copyright holders.

I - **Lempiäinen, A.**, Hotakainen, K., Alfthan, H. and Stenman, U.-H., Loss of human chorionic gonadotropin in urine during storage at -20 °C. *Clinica Chimica Acta* 2012, 413 (1-2): 232-236

II - **Lempiäinen, A.**, Stenman, U.-H., Blomqvist, C. and Hotakainen, K. The Free β -subunit of Human Chorionic Gonadotropin in Serum is a Diagnostically Sensitive Marker of Seminomatous Testicular Cancer. *Clinical Chemistry* 2008, 54: 1840-1843

III - Stenman, U.-H., Birken, S., **Lempiäinen, A.**, Hotakainen, K. and Alfthan, H. Elimination of Complement Interference in Immunoassay of Hyperglycosylated Human Chorionic Gonadotropin [Letter]. *Clinical Chemistry* 2011, 57:1075-1077

IV - **Lempiäinen, A.**, Hotakainen, K., Blomqvist, C., Alfthan, H. and Stenman, U.-H. Hyperglycosylated Human Chorionic Gonadotropin in Serum of Testicular Cancer Patients. *Clinical Chemistry* 2012, 58:1123-1129.

V - **Lempiäinen, A.**, Hotakainen, K., Blomqvist, C., Alfthan, H. and Stenman, U.-H. Increased Human Chorionic Gonadotropin Due to Hypogonadism after Treatment of a Testicular Seminoma [Letter]. *Clinical Chemistry* 2007, 53: 1560-1561.

ABBREVIATIONS

Ab	Antibody
AFP	Alfa-fetoprotein
BSA	Bovine serum albumin
CI	Confidence interval
CTP	C-terminal peptide
CV	Coefficient of variation
EDA	Ethylene diamine
EDTA	Ethylene diamine tetra-acetic acid
FSH	Follicle stimulating hormone
GCT	Germ cell tumor
GI	Gastrointestinal
HAMA	Human antibodies against mouse antibodies
hCG	Human chorionic gonadotropin
hCG α	Free alfa-subunit of hCG
hCG β	Free beta-subunit of hCG
hCG β cf	Core fragment of hCG β
hCG β -h	Hyperglycosylated hCG β
hCG-h	Hyperglycosylated hCG
%hCG-h	Proportion of hCG consisting of hCG-h
HUCH	Helsinki University Central Hospital
IFMA	Time-resolved immunofluorometric assay
IGCCCG	International Germ Cell Cancer Collaborative Group
IRR	International Reference Reagent
IS	International standard
ITGCN	Intratubular germ-cell neoplasia
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
MAb	Monoclonal antibody

NSE	Neuron specific enolase
NSGCT	Nonseminomatous germ cell tumor of the testis
PLAP	Placental alkaline phosphatase
RPLND	Retroperitoneal lymph node dissection
RT	Radiotherapy
ST	Stage
TGCT	Testicular germ cell tumor
TNM	Tumor-node-metastases
TSH	Thyroid stimulating hormone
UICC	International Union against Cancer
URL	Upper reference limit
WADA	World antidoping agency
WHO	World health organization

ABSTRACT

Testicular cancer is the most common malignant disease in young men. In order to avoid long-term toxicity of adjuvant therapy, treatment of testicular cancer is often limited to surgery, which leads to higher relapse rates. Thus, there is a need for sensitive and specific markers that enable early detection of relapse, and ideally, identify high risk patients needing adjuvant therapy. Human chorionic gonadotropin (hCG) is an extremely sensitive marker of testicular cancer. However, hCG is a heterogeneous protein with several different isoforms, of which the free β -subunit (hCG β) has prognostic value in many nontrophoblastic cancers and hyperglycosylated form of hCG (hCG-h) has been suggested to be the very key to malignant transformation of testicular cancer. Our aim was to study the clinical and prognostic utility of hCG, hCG β and hCG-h as serum markers for testicular germ cell tumors.

We first confirmed the validity of our archival samples by re-determining hCG after storage at -20 °C in 152 serum (II, IV) and 74 urine (I) samples. We developed an immunofluorometric assay for hCG-h, since no commercial ones are available (III). We determined hCG, hCG β (II, IV, V) in 3802 and hCG-h (IV) in 176 serum samples from testicular cancer patients and analyzed the association between serum concentrations and known prognostic factors, progression free survival time and disease course.

We found that serum hCG is stable for years at -20 °C (II, IV), but in most urine samples hCG immunoreactivity is lost during storage at -20 °C (I). Urea probably plays a role in the degradation of hCG, but other mechanisms are likely to participate in the process (I).

Complement causes interference in the determination of serum hCG-h when assay uses the monoclonal antibody B152. The interference was eliminated by using EDTA plasma rather than serum, or by inactivating complement in serum with EDTA before the assay (III).

hCG β is a sensitive marker for all types of testicular cancer and in seminomas it is superior to hCG. Separate determination of hCG β provides clinically valuable information, since approximately one third of marker-positive seminomas and of relapses would have been missed by an assay measuring hCG and hCG β together (II). Most of the hCG in testicular cancer patients was shown to be hyperglycosylated. Thus assays used for diagnosis and monitoring of this disease should recognize hCG and hCG-h equally. However, separate measurement of hCG-h does not seem to provide additional information as compared to assays of hCG and hCG β (IV). We also described a case with increasing hCG levels due to hypogonadism causing suspicion of a relapse. Treatment of testicular cancer can be initiated on the basis of marker elevation alone, and therefore it is important to understand the behaviour of tumor markers under various physiological conditions (V).

1 REVIEW OF THE LITERATURE

1.1 HCG, HCG β AND HCG-H

1.1.1 INTRODUCTION

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone that consists of an α - (hCG α) and a β - subunit (hCG β). The α -subunit is common to the pituitary glycoprotein hormones, thyroid stimulating hormone (TSH), luteinizing hormone (LH) and follicle stimulating hormone (FSH), while the β -subunit is hormone-specific and determines their biological function. hCG is expressed by placental trophoblasts during pregnancy and by several trophoblastic tumors, including male germ cell tumors, for which hCG is a valuable marker. hCG β can be produced by several nontrophoblastic cancers and this is usually a sign of adverse prognosis (Alfthan, et al. 1992a; Alfthan, et al. 1992b; Stenman, et al. 2004). Cancer-derived hCG has been found to contain more complex carbohydrate moieties than late pregnancy hCG (Elliott, et al. 1997; Kobata and Takeuchi 1999; Valmu, et al. 2006) and this so called hyperglycosylated hCG (hCG-h) has been suggested to play a central role in cancer invasion (Cole, et al. 2006b).

1.1.2 STRUCTURE

hCG α is encoded by a single gene on chromosome 12 (12q21.1-23) (Boothby, et al. 1981; Fiddes and Goodman 1981) and its peptide backbone contains 92 amino acids (Mise and Bahl 1980). Two carbohydrate moieties are bound to Asn52 and Asn78. It has a molecular weight of 14.5 kDa (Birken 1984). hCG β is encoded by at least six genes arranged in a cluster on chromosome 19 (19q13.3) (Policastro, et al. 1983; Talmadge, et al. 1984). The peptide backbone of hCG β contains 145 amino acids (Morgan, et al. 1975). There is extensive homology (\approx 80%) between LH β and hCG β , the main difference being an extension in hCG β (amino acids 115-145) called the C-terminal peptide (CTP) (Morgan, et al. 1975), which determines the biological function of hCG. Two N-linked carbohydrate moieties are bound to Asn13 and Asn30, respectively, and four O-linked glycans to serines 121, 127, 132 and 138 (Kessler, et al. 1979; Morgan, et al. 1975). Its molecular weight is 22.2 kDa (Birken 1984).

In intact hCG, the subunits are noncovalently held together by a loop of hCG β embracing hCG α like a seat belt (Laphorn, et al. 1994). hCG has a mean molecular weight of 36.7 kDa (Birken 1984), but because of differences in carbohydrate composition (Elliott, et al. 1997; Kobata and Takeuchi 1999; Valmu, et al. 2006), the molecular weight varies considerably. The carbohydrate chains contain variable amounts of terminal sialic acids and therefore hCG also displays extensive charge heterogeneity (Graesslin, et al. 1972; Sutton 2004).

When excreted into urine, much of hCG is proteolytically degraded to the beta-core fragment (hCG β cf) which consist of amino acids 6-40 and 55-92 linked by disulfide bridges (Amr, et al. 1984; Birken, et al. 1988). In addition, the glycans on Asn 13 and 30 are smaller in hCG β cf than in intact hCG (Birken, et al. 1988).

In cancer-derived hCG-h, the carbohydrates are more complex in structure than in hCG derived from late pregnancy urine. The N-linked carbohydrates are more complex containing more triantennary and monoantennary Asn-linked carbohydrates (Elliott, et al. 1997; Valmu, et al. 2006). Also, triantennary glycans are often linked to Asn-30 and fucosylation of the Asn-13-bound glycan is increased (Valmu, et al. 2006) and there is an abnormal N-glycan with two antennae attached to the same mannose residue (Kobata and Takeuchi 1999). In addition to complex oligosaccharides, the content of sialic acid in cancer-derived hCG has been shown to be either reduced (Imamura, et al. 1987; Nishimura, et al. 1981) or increased (Yazaki, et al. 1985). Thus, there is considerable variation in the glycosylation of cancer-derived hCG. hCG and hCG β expressed by testicular cancer have also been found to be hyperglycosylated (Valmu, et al. 2006).

1.1.3 FUNCTION

In pregnancy, hCG is strongly expressed by the placental trophoblasts and increased serum concentrations can be detected as soon as 5-7 days after conception. hCG mediates its action through the LH/hCG receptor (McFarland, et al. 1989). The major function of hCG is to support steroid production of the corpus luteum (Yoshimi, et al. 1969), thus ensuring adequate progesterone production during early pregnancy when LH concentrations decrease after the luteal phase of the cycle. During pregnancy, hCG also stimulates steroid production in the fetal ovaries and testosterone production in the testes (Huhtaniemi, et al. 1977). Recently, hCG has been found to exert functions that are not mediated by the LH/hCG receptor. Recombinant hCG has been shown to promote IL-8 production by monocytes, which do not express LH/hCG receptors (Kosaka, et al. 2002). Furthermore, D-mannose interferes with the binding of hCG to the monocyte cell surface suppressing hCG-induced IL-8 production. However, mannose receptor mRNA was not detected in these monocytes (Kosaka, et al. 2002). The mannose receptor has been shown to bind glycoproteins with N-linked carbohydrate side chains (East and Isacke 2002). In another recent study, hCG was shown to induce proliferation of uterine natural killer (NK) –cells. When hCG was deglycosylated or D-mannose was added to the culture, proliferation was not induced. Mannose receptor was present on the NK cell surface, and hCG and the mannose receptor co-localized to the cell surface. This indicates that hCG-carbohydrate side chains were recognized by the mannose receptor, which might be the mediator behind the growth promoting effect of hCG on NK cells (Kane, et al. 2009).

hCG β does not bind to LH/hCG –receptor and lacks hCG activity. However, hCG β has been shown to prevent apoptosis of tumor cells in culture (Butler, et al. 2000). The mechanism mediating this activity is not known, but it has been suggested that hCG β interferes with the growth-inhibiting effect of transforming growth factor β , platelet-derived growth factor -B and nerve growth factor (Butler and Iles 2004). However, the receptor mediating this effect remains unclear.

In early pregnancy, hCG-h is produced by immature cytotrophoblasts (Kovalevskaya, et al. 2002b) and it has been suggested to stimulate invasion of the developing placenta into uterine wall and also of cancer to adjacent tissues (Cole 2007). However, so far no other receptor than the LH/hCG –receptor has been found and the physiological function of hCG-h

remains unclear. Because the mannose receptor is thought to recognize glycan structures on hCG and because it mediates immunological responses (Kane, et al. 2009) that are of importance during implantation, it is tempting to speculate that hCG-h might also act through the mannose-receptor. However, this remains to be shown.

1.1.4 hCG EXPRESSION IN HEALTHY SUBJECTS

During pregnancy, hCG is expressed by placental trophoblasts beginning around the time of fetal implantation, which starts approximately 5-7 days after fertilization (Lenton, et al. 1982; Marshall, et al. 1968). Thereafter, hCG production increases exponentially and serum hCG concentrations double approximately every 1.5 days during the first six weeks (Lenton, et al. 1982; Pittaway, et al. 1985). The concentration peaks at around 300 000 pmol/l or 100 000 IU/l at pregnancy weeks 8 - 10. After week 12, the concentrations decrease until they plateau at around 90 000 pmol/l at the 20th week (Braunstein and Hershman 1976). The serum concentrations of hCG β correlate with those of hCG: In early pregnancy the proportion of hCG β has been reported to be as high as 16% but decreases to <5 % later in pregnancy (Cole, et al. 1984; Hay 1985). Urine hCG concentrations correlate with those in serum. In early pregnancy, the urine concentrations are on average 50 % of those in serum but the concentrations vary depending on urinary flow rate. In early pregnancy, intact hCG is the main form of hCG in urine but after week 5, hCG β cf becomes the dominant form (Kato and Braunstein 1988; Wehmann, et al. 1990). In pregnancy, the proportion of hCG β in urine is small (Norman, et al. 1987).

In early pregnancy, most of the hCG in circulation is hyperglycosylated (Kovalevskaya, et al. 1999). Thus, hCG-h is the predominant form of hCG both in serum and urine during the first weeks of pregnancy with a hCG-h to hCG ratio of 1.3 - 6.2. The ratio diminishes rapidly to about 0.2 - 1.0 at 10 weeks of pregnancy and to 0.06 at 29 weeks (Kovalevskaya, et al. 1999). Tissue expression of hCG-h has been detected in first trimester placentas but not in the second or third trimester (Kovalevskaya, et al. 2002b). During early pregnancy, placental cytotrophoblasts mainly produce hCG-h, while the more mature syncytiotrophoblasts secrete hCG (Kovalevskaya, et al. 2002b).

Low concentrations of hCG are produced by the pituitary (Odell and Griffin 1987; Stenman, et al. 1987) giving rise to measurable hCG concentrations in serum of non-pregnant healthy individuals (Alfthan, et al. 1992a). The serum concentrations of hCG correlate with those of LH, and increase with age especially in women and less in men (Stenman, et al. 1987). The age- and gender-specific upper reference limits are given in Table 1 (Table 1). Serum hCG β is measurable in the majority of healthy individuals, but the concentration increases only slightly with age (Alfthan, et al. 1992b).

hCG immunoreactivity has been detected in the male reproductive organs. In the normal testis tissue, hCG β immunoreactivity is found in peritubular cells, presumably Leydig cells (Berger, et al. 2007). Seminal fluid contains high concentrations of free subunits, especially hCG α , but only minimal concentrations of intact hCG (Berger, et al. 2007). Seminal vesicles themselves do not seem to contain hCG α -immunoreactive cells (Berger, et al. 2007), and the origin of hCG α in seminal fluid is not clear. It has been suggested to originate from the

Table 1. Age- and gender specific upper reference limits (pmol/l), based on the 97.5 percentile, of hCG, hCG β and of hCG β cf in serum and urine (Alfthan, et al. 1992a).

Gender	Women, pmol/l		Men, pmol/l	
	<50	>50	<50	>50
Age, years	<50	>50	<50	>50
Serum hCG	8.6	15.5	2.1	6.1
Serum hCG β	1.6	2.0	1.9	2.1
Serum hCG β cf	1.1	1.1	1.1	1.1
Urine hCG	8.8	11.5	2.9	8.4
Urine hCG β	1.7	4.3	1.3	3.6
Urine hCG β cf	8.1	9.5	6.7	8.5

prostate, where there are two cell types that secrete hCG α : prostate fibroblasts and neuroendocrine epithelial cells (Berger, et al. 2007; Rumpold, et al. 2002). The physiological function of the hCG subunits in the male reproductive system is not known, and no receptors for them have been found. Normal bladder epithelium has also been found to express hCG β (Hotakainen, et al. 2002).

1.1.5 HCG EXPRESSION IN MALIGNANT DISEASE

Ectopic expression of hCG in cancer was first detected because of symptoms caused by gonadotropin secretion, i.e., precocious puberty and gynecomastia, in patients with lung and liver cancers (Becker, et al. 1965; Fusco and Rosen 1966; Hung, et al. 1963; Reeves, et al. 1959). Later, hCG immunoreactivity in serum was detected in a variety of malignancies (Braunstein, et al. 1973; Hattori, et al. 1978), including testicular cancer (Braunstein, et al. 1973). It was also soon recognized that samples from cancer patients could contain free subunits with (Vaitukaitis 1973) or without concomitant increase in intact hCG (Rosen and Weintraub 1974; Weintraub and Rosen 1973).

hCG is expressed by trophoblastic disease and by cancers with trophoblastic components. Virtually all cases of female trophoblastic disease, i.e., molar disease, choriocarcinoma and placental site trophoblastic tumors, have elevated serum concentrations of hCG and hCG β . The extremely rare ovarian cancers containing trophoblastic components often produce hCG (Kurman and Norris 1976; Roger, et al. 1984). In testicular cancer, elevated serum hCG concentrations occur in roughly half of the patients with nonseminomatous testicular cancer (NSGCT), but only in 10–15% of those with seminomatous testicular cancer (Braunstein, et al. 1973; Lange, et al. 1977; Norgaard-Pedersen, et al. 1984; Scardino, et al. 1977; Scardino and Skinner 1979; Schultz, et al. 1978). However, a variable proportion, 10–80%, of seminomas have been found to secrete hCG β (Hoshi, et al. 2000; Madersbacher, et al. 1992; Mann, et al. 1993; Mann and Siddle 1988; Marcillac, et al. 1992).

hCG-h is a major form of hCG produced by trophoblastic tumors and has been suggested discriminate between malignant and pre-malignant gestational trophoblastic disease with 100% sensitivity and specificity (Cole, et al. 2006a). hCG-h is thought to be the major form of

hCG also in testicular cancer. It has been suggested to mediate invasiveness of cancer cells and the malignant transformation of testicular germ cell tumors (Cole, et al. 2006b; Cole, et al. 2006c). However, studies on hCG-h in testicular cancer are very limited comprising only a few patient samples or hCG produced by tumor cells in culture. So far, the prognostic value or follow-up of the disease has not been studied (Cole, et al. 2006c; Kelly, et al. 2007; Valmu, et al. 2006). hCG-h has also been found in serum and urine of patients with cervical, colon, bladder and lung cancers (Kelly, et al. 2007).

Expression of hCG β is common in nontrophoblastic cancers, the serum concentrations being slightly or moderately elevated in 20–50 % of various non-trophoblastic cancers (Marcillac, et al. 1992; Papapetrou, et al. 1980; Stenman, et al. 2004). Expression of hCG β is especially common in transitional cell carcinoma of the bladder and urinary tract. Depending on the assay and cut-off level used, 10 to 75% of patients have elevated serum concentrations (Crawford, et al. 1998; Hotakainen, et al. 2002; Marcillac, et al. 1992). Increased urine concentrations of “total hCG β immunoreactivity”, which has later been shown to consist mainly of hCG β , correlate with adverse prognosis (Iles, et al. 1996). In renal cell cancer, 23% of the patients have elevated serum concentrations of hCG β and hCG β is an independent prognostic factor (Hotakainen, et al. 2003). Increased hCG immunoreactivity has also been observed in urine of prostate cancer patients (Fukutani, et al. 1983; Papapetrou, et al. 1980; Shah, et al. 1987).

Expression of hCG-immunoreactivity occurs in most types of gastrointestinal (GI) cancers, the overall incidence being around 20% (Stenman, et al. 2004). This immunoreactivity consists mainly of hCG β , but occasionally hCG is concomitantly expressed (Alfthan, et al. 1992b; Marcillac, et al. 1992). The frequency of hCG immunoreactivity is highest in biliary (60%), pancreatic (46%) and gastric (40%) cancers (Alfthan, et al. 1992b; Louhimo, et al. 2002b; Marcillac, et al. 1992; Stenman, et al. 2004). Tissue expression and elevated serum concentration of hCG β in colorectal cancer are associated with adverse prognosis (Carpelan-Holmström, et al. 1996; Louhimo, et al. 2002a; Lundin, et al. 2001). Elevated serum concentrations of hCG α have been observed in patients with neuroendocrine tumors of the GI-tract (Kahn, et al. 1977; Öberg and Wide 1981).

In lung cancer, serum concentrations of hCG β above a relatively high cut-off level, 5 IU/l (15 pmol/l), have been observed in 12–14% of small cell lung cancers, and this was associated with short survival (Szturmowicz, et al. 1999; Szturmowicz, et al. 1995). Tissue expression of hCG has been observed in 30–80% of lung cancers (Kimura and Ghandur-Mnaymen 1985; Kuida, et al. 1988; Skrabanek, et al. 1979; Slodkowska, et al. 1998; Wilson, et al. 1981).

In breast cancer, tissue expression of hCG β has been detected in 10–60 % of the cases (Agnantis, et al. 1992; Kuida, et al. 1988; Lee, et al. 1985) and serum hCG β is elevated in roughly half of the patients with advanced disease, but serum concentrations do not reliably reflect the course of the disease (Sjöström, et al. 2001).

In ovarian, endometrial, cervical and vulvovaginal cancers, 20–75 % of the patients have elevated urine concentrations of hCG β cf (Carter, et al. 1994; Cole, et al. 1988; Nam, et al. 1990), which is derived from hCG β produced by the tumor (Grossmann, et al. 1995; Nishimura, et al. 1998). In ovarian cancer, hCG β is a strong independent prognostic factor (Vartiainen, et al. 2001).

In oral cancer, tissue expression of hCG is found in 64% of the tumors (Bhalang, et al. 1999) and in 14% of head and neck cancer patients serum concentrations of hCG β are elevated (Hedström, et al. 1999). Increased serum hCG-immunoreactivity in serum has also been observed in lymphoma patients (Braunstein, et al. 1973; Moller 1996).

1.1.6 STABILITY OF HCG IN SERUM AND IN URINE

Repeated freezing at -20 °C and thawing have only a minimal effect on the serum concentrations of hCG and hCG β (Cowans, et al. 2010; Spencer, et al. 1993). However, at room temperature, hCG subunits dissociate, and the concentration of intact hCG decreases while that of hCG β increases being 3-fold after four weeks. The concentrations of the so-called nicked forms of hCG and of hCG β , increase even more (Kardana and Cole 1997). Antimicrobials diminish the nicking of hCG and hCG β suggesting that microbial protease activity is its primary cause (Kardana and Cole 1997). Nicking and dissociation is slower at +4 °C, and there are only minor, statistically nonsignificant changes in the recoveries of serum hCG and hCG β at +4 °C after four weeks (Kardana and Cole 1997). If serum samples are heated to 56 °C, in order to deplete serum complement, hCG will dissociate into subunits increasing their concentrations (Bidart, et al. 1991; Gau, et al. 1984; Spencer, et al. 1993) and decreasing that of intact hCG.

In urine, the various forms of hCG have been considered to be relatively stable. hCG is stable at +4 °C for at least for 3 to 4 weeks (de Medeiros, et al. 1991; McChesney, et al. 2005). Recently, Robinson et al. reported that recovery of urinary hCG is inconsistent after storage at -20 °C for up to three months, but the magnitude or rate of the change was not described (Robinson, et. al. 2010). In other studies, loss of hCG in urine has not been observed: Wilcox et al. found that hCG concentrations increase slightly during storage at -20 °C, probably due to evaporation (Wilcox, et al. 1985). McCready et al. studied the stability of hCG in pregnancy urine, which was either frozen at -20 °C after one week of storage at room temperature or +4 °C, or without delay. They found that hCG concentrations decreased in samples maintained at +4 °C as compared to samples frozen at sample arrival, but hCG was considered stable at freezing conditions (McCready, et al. 1978). Based on their unpublished data, Spencer et al. have considered urine hCG β stable at +4 °C for at least 2 weeks and at -20 °C for at least three months (Spencer, et al. 1996). At +37 °C, urine hCG dissociates into subunits (Cole 1997) and thus hCG β increases. Urine hCG-h has been found to be stable at -20 °C for at least 3 years (Cole, et al. 1999b).

1.1.7 PRINCIPLES OF HCG ASSAYS

Quantitative hCG determinations are usually performed on serum while urine is mainly used for point-of-care pregnancy tests, which are the most commonly used hCG determinations. Quantitative urine measurements are used in doping control.

Virtually all presently used quantitative hCG assays are sandwich immunoassays based on monoclonal antibodies (MAb): a capture antibody (Ab) on a solid phase binds hCG in the sample and the amount of bound hCG is measured using a detection Ab that binds to hCG captured on the solid phase. The detection Ab is labeled with a fluorochrome, an enzyme or

a chemiluminescent substance, the quantity of which correlates to the content of the analyte in the sample (Figure 1).

Most commercial serum hCG assays detect hCG and hCG β together. In these assays, both Abs are directed against the β -subunit. In many assays one of the antibodies is directed against an epitope on CTP eliminating the crossreaction with LH. Some of these assays detect hCG and hCG β fairly equally but there are still considerable differences in calibration between assays (Harvey, et al. 2010; Sturgeon, et al. 2009). A few assays detect hCG, hCG β and hCG β cf together, which may be an advantage when analyzing urine. Assays using a pair of Abs, of which one is directed against the α - and the other against β -subunit, detect only intact hCG. Another possibility is to use an Ab directed to a conformational epitope comprising both subunits. Assays specific for free α - or β -subunits utilize antibodies directed against epitopes hidden in the heterodimer.

A monoclonal Ab, B152, recognizes a biantennary core 2 o-glycan at Ser 132 present on hyperglycosylated hCG (Birken 2005; Birken, et al. 2003b). Another glycan specific Ab, CTP104, detects a sialylated glycan on Ser138 (Birken, et al. 2003b). Of these, MAb B152 has been used in a previous commercial assay specific for hCG-h (Pandian, et al. 2003).

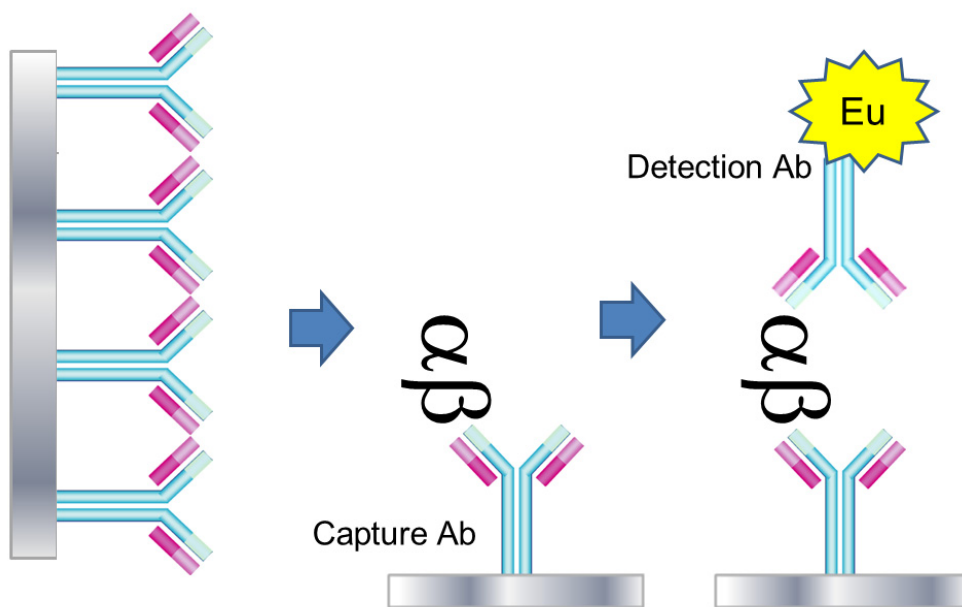


Figure 1. Principle of sandwich assays employing two analyte-specific antibodies. The capture antibodies are bound to solid surface, bind to analyte in the sample and then detection antibody bind to the analyte forming a “sandwich”. The detection antibodies contain a label, the quantity of which is measured.

1.1.8 ENDOGENOUS INTERFERENCES IN IMMUNOASSAYS

Immunoassays are vulnerable to rare, but possible sporadic errors caused by endogenous, interfering substances within the specimen. These substances can be anti-reagent or anti-analyte Abs, complement factors or cross-reacting substances. Endogenous interferences are usually difficult to detect. The detection relies on clinical suspicion, *i.e.* the clinician realizing the possibility of a false result, which does not fit to clinical picture. Routine laboratory screening of each specimen for endogenous interferences is unlikely to be cost-effective (Sturgeon and Viljoen 2011) or even possible.

Serum from some patients contain Abs against animal immunoglobulins used in the assay. Such Abs may form a bridge between capture and the detector Ab causing a signal in the absence of analyte and falsely elevated concentrations, or they can block the binding sites of assay Abs, thus preventing sandwich formation and causing falsely low results (Figure 2). The most relevant interfering Abs are human anti-mouse antibodies (HAMA), heterophilic antibodies and rheumatoid factor (Table 2). Usually, interferences caused by HAMA's are more pronounced than those of heterophilic antibodies and of rheumatoid factor. The frequency of endogenous interfering Abs has varied in different studies between 0.5 and 12% (Hawkins, et al. 1980; Ismail, et al. 2002a; Ismail, et al. 2002b; Warren, et al. 2005). In a recent, large study of 11 000 serum samples, the frequency of interference in an immunoassay for carcinoembryonic antigen was approximately 4%, but could be reduced to 0.1% by removing the constant Fc portion of the capture antibody (Bjerner, et al. 2002).

Table 2. Characteristics of the most important endogenous antireagent antibodies. Modified from Sturgeon and Viljoen, 2011.

Type of antibody	Etiology	Characteristics
Human anti-mouse antibody	Immunization in response to direct antibody stimulus, most frequently following treatment or imaging with mouse MAb	Most readily identified antireagent antibodies. High-affinity Abs, which may be present in high concentrations and may persist long-term.
Heterophilic antibody	Poorly defined Abs, immunogen unclear	Variable analytical relevance. Two types reported, of which only the other binds to mouse Abs
Rheumatoid factor	Autoantibodies often present in serum of patients with autoimmune disease, especially rheumatoid disease, but can also follow infections.	May interfere with two-site immunoassays. Bind to multiple sites in Fc region of IgG.

To avoid possible endogenous interference, most assays include reagents that aim to block the interference. These usually consist of nonspecific animal immunoglobulins, their fragments or mixture of MAbs from the same species used to raise the assay Ab (Kricka 1999). Blocking Abs aim to bind the endogenous anti-animal Abs and thus act as a surrogate for assay Abs. There are also commercially available heterophilic blocking

reagents, e.g. HBR (Scantibodies Laboratory) which claims to actively block interference by inactivating the variable part of heterophilic antibodies (Scantibodies Laboratory Inc, 2012). Another approach is to fragment the capture Ab and use only the variable, Fab region of the Ab. Since approximately 80% of the interfering Abs bind to the constant, Fc region of the Ab (Lind, et al. 1991) removing or modifying the Fc fragment decreases the likelihood of interference (Csako, et al. 1988; Vaidya and Beatty 1992). Also, using humanized Abs may reduce interference (Sturgeon and Viljoen 2011). There are also several simple methods to detect, although not prevent, assay interference: the test can be repeated with another assay, which should give roughly equivalent results. The sample can be serially diluted after which the analyte recovery should follow a linear pattern. If not, assay interference is possible (Sturgeon and Viljoen 2011). Analysing urine specimen can also be of help, since Abs are not secreted into urine, but many analytes are.

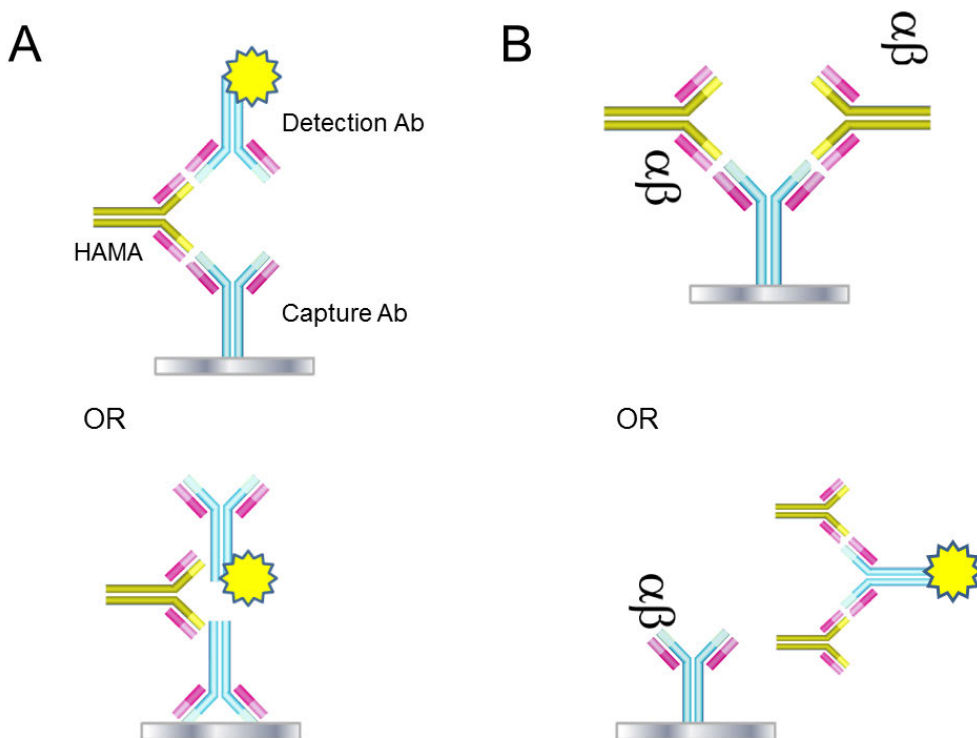


Figure 2. Human anti-mouse antibodies (HAMA) may interfere with sandwich assays A) by forming a bridge between assay antibodies regardless of the analytes presence, and thus causing an increased signal and falsely high result or B) by interfering with the antibody binding of either capture or detection antibody, and thus reducing sandwich formation, decreasing signal and causing falsely low results.

In vivo, formation of multimolecular immune complexes activates the complement system. *In vitro*, antibody-coated plastic surfaces, *i.e.* assay well with capture Abs, acts like aggregated immunoglobulin (Baatrup, et al. 1986). Complement factors C1q and C3 bind in large excess

to capture Abs, which in turn reduces the antibody-antigen binding and leads to falsely low assay results (Børmer 1989; Käpyaho, et al. 1989). Complement has been found to interfere some immunoassays, especially those employing IgG2 subclass Abs (Børmer 1989). Formation of C1q complex requires Ca²⁺ ions, and therefore complement activation can be inhibited by EDTA, which binds Ca²⁺ ions (Børmer 1989; Käpyaho, et al. 1989). Also, heat inactivates complement. Complement interference requires fairly large concentration of complement factors and dilution of serum samples reduces or removes interference (Børmer 1989; Käpyaho, et al. 1989). Also, removing or modifying the Fc portion of the capture Ab should prevent complement interference, since C1q binds to Fc region (Käpyaho, et al. 1989).

Patients with autoimmune disease or patients treated with analyte-like substances may have auto-antibodies against measured analytes, *e.g.* insulin Abs in insulin-treated patients, and these can cause assay interference. The prevalence of anti-analyte Abs is difficult to assess, but they may cause clinically relevant false results (Sturgeon and Viljoen 2011; Zouwail, et al. 2008). Cross-reacting substances are substances detected in the assay for some other analyte, *e.g.* hCG β is detected by most commercial hCG assays and LH still cross-reacts in some qualitative hCG assays. To avoid the risk of misinterpretation, it is important to know the clinically relevant cross-reacting substances in each assay, *i.e.* to understand what the immunoassay actually measures.

1.1.9 CLINICAL APPLICATIONS

A large number of qualitative and quantitative hCG assays intended for diagnosis and monitoring of pregnancy are commercially available. These assays are not approved for cancer diagnostics in the US, but are nevertheless used for diagnosis and monitoring of trophoblastic tumors and testicular cancer. No quantitative hCG assays are approved for urine measurements but are used for that purpose in doping control (Stenman, et al. 2008).

Most commercially available hCG β assays are intended for maternal screening of Down's syndrome and are therefore optimized for the high concentrations occurring in pregnancy, which are much, even more than thousand-fold, higher than those that need to be measured in cancer patients. Therefore such hCG β assays are not well suited for use in cancer diagnostics. There are no commercial assays for hCG-h.

Pregnancy can be detected on the basis of elevated serum or urine concentrations of hCG about one week before the first missed menstrual period. Commercially available pregnancy tests detect concentrations above 30–150 pmol/l (10–50 IU/l), thus some home pregnancy tests are very sensitive (Cervinski, et al. 2009). During pregnancy, hCG concentrations should double every 1.5 days until pregnancy week 8-10 (Lenton, et al. 1982; Pittaway, et al. 1985). Low concentration of hCG in relation to pregnancy week and a slower than normal increase of hCG concentration that starts decreasing (Bignardi, et al. 2008; Korhonen, et al. 1994) is a marker of failing pregnancy, either early pregnancy loss or resolution of an ectopic pregnancy. A single measurement of hCG-h with a threshold of 13 μ g/l has been suggested to discriminate between failing and continuing pregnancies (Sutton-Riley, et al. 2006). When the serum hCG concentration is above 3000 pmol/l, an ectopic pregnancy can be diagnosed on the basis of the absence of an intrauterine gestational sac as detected by

ultrasonography (Cacciatore, et al. 1989). In resolving ectopic pregnancy, serum hCG should diminish expectedly (Silva, et al. 2006).

Determination of serum hCG or hCG β is used for screening of Down's syndrome in combination with other markers. Assay of serum hCG β and pregnancy-associated plasma protein-A in combination with ultrasonographic measurement of fetal nuchal translucency (Wald, et al. 2005) are now routine methods for first trimester screening, whereas measurement of hCG β together with alfa-fetoprotein (AFP), estriol and sometimes inhibin-A are used in the second trimester (Extermann, et al. 1998; Phillips, et al. 1992; Renier, et al. 1998). Determination of hCG-h in urine has been reported to give better discrimination in Down's screening than that of hCG in urine (Cole, et al. 1999b) but when determined on serum, hCG-h did not provide additional clinical information compared to hCG β (Palomaki, et al. 2005).

Virtually all cases of gestational trophoblastic disease, i.e., benign molar disease, choriocarcinoma and placental site trophoblastic tumor, have elevated serum concentrations of hCG and hCG β , and especially hCG is used for diagnosis and follow-up of these diseases. Treatment can be initiated on the basis of increasing serum hCG concentrations in the absence of other evidence of disease. The proportion of hCG β to hCG may also be used in differentiation between benign and malignant gestational trophoblastic disease: the proportion is below 5% in benign disease and above this in choriocarcinoma (Ozturk, et al. 1988; Vartiainen, et al. 1998). Also, the proportion of hCG-h to hCG of >40% has been suggested to indicate aggressive gestational trophoblastic disease (Cole, et al. 2010). Unfortunately the clinical use of hCG β and hCG-h is limited by lack of suitable commercial assays.

Quantitative determination of hCG in urine is used for doping control. Administration of hCG is used to restore gonadal function that has been suppressed by the use of anabolic steroids and testosterone (Stenman, et al. 2008) and hCG is included in the list of prohibited substances of the World Antidoping Agency (WADA) (WADA 2010). There are no regulations as to which type of hCG assay should be used in doping control and the minimum required detection limit of the hCG assay is 5 IU/l (WADA 2012). However, recently WADA has recommended, that positive results obtained with an assay measuring hCG and hCG β together should be verified with an assay measuring only intact hCG.

1.2 TESTICULAR CANCER

1.2.1 INTRODUCTION

Testicular cancer represents only 1% of all malignancies in males but it is the most common malignant tumor in men aged 15–35 years. The incidence of testicular cancer varies considerably between different countries and has more than doubled in the last 40 years (Huyghe, et al. 2003). About 95% of all malignant testicular tumors are of germ cell origin, most of the rest being lymphomas, Leydig cell tumors and mesotheliomas. Germ cell tumors have traditionally been classified as seminomatous and nonseminomatous testicular cancer, because there are major differences in the management of these. Seminomatous testicular

cancer consists solely of seminomatous type of tumor tissue and tumors containing any other germ cell tumor component are classified as nonseminomas.

Currently, the cure rates of testicular cancer are excellent, more than 90%, and treatment of low-stage disease is often limited to surgery in order to avoid long-term toxicity of radio- and chemotherapy (Albers, et al. 2005; Krege, et al. 2008a). However, without adjuvant therapy relapses are fairly common. Thus there is a need for markers enabling early detection of relapse and, ideally, identification of high risk patients requiring adjuvant therapy in addition to surgery. Either AFP or hCG in serum is elevated in serum in about 80% of the patients with NSGCT, but in seminoma, AFP is not useful and hCG is elevated in only 10–15 % of the cases Braunstein, et al. 1973; Lange and Fraley 1977; Lange, et al. 1977; Norgaard-Pedersen, et al. 1984; Germa-Lluch, et al. 2002).

1.2.2 EPIDEMIOLOGY

The incidence of testicular germ cell tumors (TGCT) varies widely between different countries. In Europe, the age-adjusted incidence is lowest in Lithuania (0.9/ 100 000), intermediate in Finland (2.5/ 100,000) and highest in Denmark (9.2/ 100 000) (Huyghe, et al. 2003). Caucasians of Northern European descent have the highest incidence while men of African or Asian descent have the lowest (Chia, et al. 2010). Differences in incidence persist after migration. Interestingly, the incidence also varies between different races being five times more common in white American than in African-American men (McGlynn, et al. 2003).

The incidence in European countries has increased by 2–5% annually (Huyghe, et al. 2003) and in the USA the increase from mid 70ies to mid 90ies has been 52% (McGlynn, et al. 2003). This suggests that changes in environmental factors contribute to the development of testicular cancer. However, the ultimate mechanism behind the increase remains unclear (reviewed in Horwich, et al. 2006).

1.2.3 ORIGIN AND PREDISPOSING FACTORS

TGCTs are thought to develop during fetal development from primordial germ cells. TGCTs progress through a non-invasive phase called testicular intratubular germ-cell neoplasia (ITGCN) (Giwerzman, et al. 1991; Høie-Hansen, et al. 2004), also called carcinoma in situ, which precedes most germ cell tumors (GCT) with the exception of spermatocytic seminomas and prepubertal GCTs (Soosay, et al. 1991). All ITGCNs are likely to progress to TGCTs, since the frequencies of ITGCN and TGCT are similar (Giwerzman, et al. 1991). Also, gene products that act on primordial germ cell or early embryonic development, such as transcription factors OCT3/4, AP-2 γ and c-KIT, are also expressed by ITGCN (Høie-Hansen, et al. 2004; Looijenga, et al. 2003; Rajpert-De Meyts and Skakkebaek 1994).

Postpubertal testicular tumors present a distinct histological and genetic pattern compared to prepubertal ones, and the origin and biology of prepubertal and postpubertal GCTs may be different. Testicular tumors in adult patients most often consist of seminoma, embryonal carcinoma or mixed germ cell tumors, but in children, yolk sac tumors and teratomas are most frequent (Weissbach, et al. 1984). Furthermore, there is no association between

ITGCN and prepubertal GCTs. Postpubertal tumors often contain isochromosome i(12p) (Bosl, et al. 1994; Rodriguez, et al. 1992), while prepubertal ones are usually diploid. Therefore, in addition to a division based on histology, TGCT's can be divided according to biology: infantile/prepubertal GCTs, postpubertal GCTs and spermatocytic seminoma of elderly men.

Familial clustering has been observed but the cause is unknown (Dong, et al. 2001; Forman, et al. 1992; Lutke Holzik, et al. 2004). In addition to a family history of testicular tumors among first grade relatives, other known risk factors include hypotrophic (<12 mm) or atrophic testicles, infertility, cryptorchidism or undescended testis, Klinefelter's syndrome, Down's syndrome, and tumor or ITGCN of the contralateral testis (reviewed in Bosl and Motzer 1997).

1.2.4 GENETIC CHARACTERISTICS

Among TGCT patients, 1–3% have affected first-degree relatives (Dong, et al. 2001; Forman, et al. 1992; Lutke Holzik, et al. 2004). So far, genes responsible for this have not been found, but linkage to the Xq27-region has been reported (Rapley, et al. 2000).

Adult TGCTs are invariably aneuploid. A strikingly consistent finding in more than 80% of adult TGCTs is a gain of the chromosome arm 12p, usually in the form of isochromosome i(12)p. In the absence of i(12)p, amplifications in the subregions of 12p, at 12p13 or 12p11.2–p12.1, have been described (Henegariu, et al. 1998; Roelofs, et al. 2000; Suijkerbuijk, et al. 1993; Summersgill, et al. 2001). Genes at 12p13 encode, among others, protein CCND2, which regulates cell cycle, and NANOG and STELLAR that are associated with maintaining pluripotency in stem cells (Clark, et al. 2004; Houldsworth, et al. 1997; Zaehres, et al. 2005).

Several other chromosomal regions are also imbalanced though to a lesser extent than 12p: gain of genetic material has been observed in the regions 4q12, 17q21.3, 22q11.23 and Xq22 and loss of material from 5q33, 11q12.1, 16q22.3 and 22q11 (McIntyre, et al. 2004; Skotheim, et al. 2003; Skotheim, et al. 2002). The *KIT* gene encoding a tyrosine kinase receptor is overexpressed and amplified in some seminomas (McIntyre, et al. 2005). Spermatocytic seminoma is either diploid or aneuploid with loss of chromosome 9 rather than gain of isochromosome 12p (Verdorfer, et al. 2004).

During normal fetal development of primordial germ cells, uniparental genomic imprinting is established (Surani, et al. 1984). However, hypomethylation, in which genomic imprinting has been erased, is seen in both seminomas and nonseminomatous tumors (Costello, et al. 2000; Honorio, et al. 2003; Koul, et al. 2002; Smiraglia, et al. 2002; Smith-Sorensen, et al. 2002), and the unmethylated region at the *XIST*-gene has been suggested to be a diagnostic marker for testicular cancers (Kawakami, et al. 2004). Changes in methylation pattern alter gene expression and have been suggested to affect the phenotype of the tumor (Horwich, et al. 2006). Expression profiling shows that embryonal carcinomas and ITGCN have similar patterns of gene expression as pluripotent embryonic stem cells (Almstrup, et al. 2004; Sperger, et al. 2003), but seminomas are less stem cell -like (Sperger, et al. 2003). These changes are consistent with the hypothesis that testicular cancer arises from primordial germ cells during fetal development.

1.2.5 HISTOLOGY

Seminomas comprise about half of germ cell tumors, the rest being NSGCTs. These may contain one or several histological components such as embryonal carcinoma, choriocarcinoma, yolk sac tumor, seminoma and teratomas. Most are mixed germ cell tumors of the testis. The rare spermatocytic seminoma is a subtype that occurs in elderly men.

Pure seminoma accounts for about half of all GCTs and seminomatous components are present in a large proportion of mixed GCTs (Krag Jacobsen, et al. 1984). The peak incidence of seminoma is between 34 and 45 years. Usually seminoma cells are large and uniform, and having a solid or nested growth pattern separated by lymphocyte-rich trabeculae. Large hCG-expressing cells are seen in approximately 5-10% of seminomas (Hedinger, et al. 1979; Niehans, et al. 1988).

Spermatocytic seminoma accounts only for 1–2% of all testicular tumors (Krag Jacobsen, et al. 1984; Talerman 1980) and is believed to have a different pathogenesis and different cytogenetic characteristics than other GCTs. It is not associated with ITGCN or other germ cell neoplasias, and it is never seen as a component of mixed GCTs (Krag Jacobsen, et al. 1984; Talerman 1980). It typically occurs, but is not restricted to, men older than 50 years (Chung, et al. 2004). Histologically, spermatocytic seminoma is characterized by a diffuse proliferation of polymorphic cells supported by stroma. Immunohistochemically spermatocytic seminomas do not express hCG (Dekker, et al. 1992).

Pure embryonal carcinoma is the second most common single-cell –type GCT after seminoma and embryonal carcinoma is a component in up to 80% of mixed GCTs (Krag Jacobsen, et al. 1984; Mostofi, et al. 1988). Pure embryonal carcinoma occurs most often between 25 to 35 years of age. Histologically, embryonal carcinoma is characterized by marked anaplasia and undifferentiation of the cells. The growth pattern, however, is variable, from solid to acinar, tubular or papillary. Immunohistochemically embryonal carcinoma is commonly hCG positive (Kurman, et al. 1977; Niehans, et al. 1988).

A yolk sac component is found in 40% of mixed GCTs, but pure yolk sac tumors are extremely rare in adults (Talerman 1975). Histologically, yolk sac tumors display a variety of histological patterns, such as reticular-microcystic, macrocystic, endodermal sinus, papillary or hepatoid pattern. A distinctive histological feature is the occurrence of so-called Schiller-Duval bodies formed by a fibrovascular core and surrounding malignant cells. Immunohistochemically most yolk sac tumors are positive for AFP (Eglen and Ulbright 1987; Jacobsen and Norgaard-Pedersen 1984; Niehans, et al. 1988). Yolk sac tumors are usually negative for hCG (Niehans, et al. 1988).

Pure choriocarcinoma is extremely rare and accounts for less than 1% of testicular tumors (Krag Jacobsen, et al. 1984; Mostofi, et al. 1988), but occurs as a component in about 10–15% of mixed GCTs (Krag Jacobsen, et al. 1984). Histologically, choriocarcinoma is composed of syncytiotrophoblasts and cytotrophoblasts, which are randomly arranged or form villous like structures. Immunostaining for hCG is intensively positive in syncytiotrophoblasts, but may be negative in cytotrophoblasts (Jacobsen and Jacobsen 1983; Kurman, et al. 1977).

Teratomas are composed of various tissue types deriving from the germinal layers, endoderm, mesoderm and/or ectoderm. Mature teratomas are composed of well-differentiated components, but immature teratomas contain immature embryonic or fetal elements. In adults, even mature teratomas are associated with a risk of metastases (Jones, et al. 2006). In adults, pure teratoma is uncommon (2–3% of GCTs), but teratomatous components are present in 50% of mixed GCTs (von Hochstetter and Hedinger 1982). Teratomas account for 14% of prepubertal tumors, but all of them behave in benign manner regardless of their maturity (Weissbach, et al. 1984). Histologically, the most common components are different epithelial components, neural or glandular tissue and cartilage, but virtually any somatic component may be present. The immunohistochemical profile of teratomas depends on the tissue types present in the tumor.

Mixed GCTs comprise 30 to 50% of all GCTs (Krag Jacobsen, et al. 1984; Mostofi, et al. 1987). The most common histological subtypes present in mixed GCTs are seminoma, carcinoma embryonale, teratoma, yolk sac tumor and choriocarcinoma (Mosharafa, et al. 2004).

1.2.6 SERUM TUMOR MARKERS

Tumor markers play a central role in the diagnosis, risk stratification, monitoring of treatment response and surveillance after therapy of testicular cancer. Established markers for testicular cancer are AFP, hCG and lactate dehydrogenase (LDH), the key features of which are represented in Table 3. Because of the excellent prognosis of testicular cancer, it is unlikely that screening with tumor markers could decrease mortality or be cost effective (Gilligan, et al. 2010). Treatment is effective even when tumors became palpable or otherwise detectable.

AFP is a fetal carrier protein produced by the yolk sac and later by the fetal liver during pregnancy (Abelev 1974; Ruoslahti, et al. 1978) and it is the archetype of oncofetal proteins. AFP has a serum half-life of approximately 5–7 days. In testicular tumors, AFP is produced by yolk sac components and occasionally by embryonal carcinomas. Serum AFP is elevated in approximately 40% of NSGCTs (Abelev 1971; Egan, et al. 1977; Sakashita, et al. 1976; Scardino, et al. 1977). Serum AFP is by definition always negative in seminomas. Elevated serum concentrations of AFP may occur in hepatocellular cancer and hepatitis (Germa, et al. 1993; Morris and Bosl 2000), gastrointestinal cancers (Adachi, et al. 2003; Yachida, et al. 2003), and rarely in lung cancer (Okunaka, et al. 1992). Hereditary and persistent moderate elevation of AFP has been reported (Li and Alexander 2009; Schefer, et al. 1998).

Table 3. Key characteristics on established tumor markers for testicular cancer, modified from Gilligan et al. 2010.

	AFP	hCG	LDH
Most common assay method	Double-antibody sandwich assay	Double-antibody sandwich assay measuring “total hCG” i.e. hCG and hCG β simultaneously	Enzyme activity assay
Commonly used decision limit (upper reference limit)	10–15 μ g/l (9 μ g/l <40 years of age, 13 μ g/l >40 years of age)	5–10 IU/l (0.7 IU/l <50 years of age, 2.1 IU/l >50 years of age)	Variable and laboratory-specific; considered elevated if >1.5 x laboratory specific upper reference limit
Recommended detection limit	<1 μ g/l	<1 IU/l and <2% cross-reactivity with LH	Not reported
Half-life	5–7 days	1.5–3 days	Not reported
Elevation in seminoma	Never	10–20%	40–60%
Elevation in NSGCT	10–20% in stage I, 20–40% in stage II and 40–60% in advanced stage II and stage III	10–20% in stage I, 20–30% in stage II and 40% in advanced stage II and stage III	40–60%
Other malignancies associated with elevation	Hepatocellular carcinoma, gastric, colon, pancreatic and lung cancer	Neuroendocrine cancer, bladder, kidney, lung, head, neck, gastrointestinal, cervix, uterus and vulvovaginal cancer, lymphoma and leukemia	Lymphoma, small cell lung cancer, Ewing sarcoma, osteogenic sarcoma
Nonmalignant diseases associated with elevation in men	Hepatic injury: Alcohol abuse, hepatitis, cirrhosis, biliary tract obstruction, toxicity from chemotherapy	Hypogonadism	Cell or tissue damage (e.g., myocardial infarction, liver or muscle disease, hemolysis)
Other causes of false positive findings	Constitutively elevated AFP (hereditary)	Heterophilic antibodies	Sample hemolysis, strenuous exercise

hCG is produced by trophoblastic cells present in choriocarcinomatous components of NSGCTs, i.e. choriocarcinomas, embryonal carcinomas and mixed GCTs, and by large trophoblast-like cells seen in some seminomas. Production of hCG by testicular cancer was described when hCG specific assays were developed (Braunstein, et al. 1973), and within the next ten years hCG was established as a reliable diagnostic and prognostic marker of testicular cancer. Testicular cancer was in fact the first tumor in which marker concentrations were used for prognostic evaluation and staging (Bosl, et al. 1981; Germa-Lluch, et al. 1980; Scardino, et al. 1977). Serum hCG has been found to be elevated in about half of the patients with NSGCT, and in about 10–20 % of those with seminoma (Braunstein, et al. 1973; Lange and Fraley 1977; Norgaard-Pedersen, et al. 1984; Scardino, et al. 1977; Scardino and Skinner 1979; Schultz, et al. 1978). hCG has a half-life of 2–3 days. Small amounts of hCG is also secreted by the pituitary and the serum concentrations correlate with those of LH (Stenman, et al. 1987).

In various studies, 10–80% of seminomas have been found to secrete hCG β (Hoshi, et al. 2000; Madersbacher, et al. 1992; Mann, et al. 1993; Mann and Siddle 1988; Marcillac, et al. 1992). However, assay of hCG β is not routinely used for monitoring of testicular cancer. After term pregnancy, the clearance of hCG β follows a triphasic model with median half-lives of about 1, 23 and 194 hours (Korhonen, et al. 1997), and after injection of purified subunits, the clearance rate is twophasic with median half-lives of 41 and 236 hours (Wehmann, et al. 1979).

LDH is a tetramer composed of various combinations of two subunits, alfa and beta. The various subunit combinations give rise to five isoenzymes, of which LDH-1 is composed of four beta-subunits and especially this isoenzyme may reflect the pathogenesis of testicular cancer. The genes encoding LDH- β subunit are located on chromosome 12 (Li, et al. 1988) and the copy number of (i)12p is associated with serum concentrations of LDH-1 and tumor invasiveness (von Eyben 2001). The serum concentrations of LDH have been found to be elevated in more than half of early stage seminomas (von Eyben 2001; von Eyben, et al. 1983). The half-life of LDH has not been reported. Serum concentrations of LDH are measured enzymatically and the values are method-dependent. Since LDH is a common marker of tissue destruction, any tissue injury and sample hemolysis in vitro cause elevated LDH values (von Eyben 2003). Thus, LDH is a nonspecific marker, but it may be useful for staging and prognostic evaluation of testicular cancer (IGCCCG 1997).

Placental alkaline phosphatase (PLAP) and neuron specific enolase (NSE) are potential markers for testicular cancer. PLAP is produced by various germ cell tumors and ITGCN (Niehans, et al. 1988). Elevated serum concentrations have been reported in up to 70% of seminoma patients (De Broe and Pollet 1988; Lange, et al. 1982). However, smoking may cause up to 10-fold elevations in serum concentrations making the assay more or less useless in smokers (De Broe and Pollet 1988). Also, lack of commercial assays limits the use of PLAP as a serum marker. NSE has been found to be elevated in 30–50% of seminoma patients and less often in NSGCT (Fosså, et al. 1992; Kuzmits, et al. 1987). In spite of this, NSE is not commonly used as a marker of testicular cancer.

1.2.7 DIAGNOSIS

Testicular cancer usually presents as a hard unilateral testicular mass, which is usually painless, but 10–20% of the patients may experience pain. Approximately 5–10% have gynecomastia at diagnosis (Tseng, et al. 1985). About 5–10% of germ cell tumors are located at an extragonadal site, most often in mediastinum or retroperitoneum.

When testicular cancer is suspected, scrotal ultrasound is mandatory. It has a sensitivity of nearly 100% for testicular cancer and it can distinguish between intra- and extratesticular tumors. Magnetic resonance imaging also provides very high sensitivity and specificity. If a testicular mass is verified or strongly suspected, inguinal exploration of the testis and immediate orchiectomy is performed if malignancy is strongly suspected. If the diagnosis remains unclear after exploration, a testicular biopsy can be taken for histological evaluation of frozen sections.

Serum tumor markers (hCG, AFP, LDH) contribute to diagnosis. If serum hCG or AFP is significantly elevated, a germ cell tumor is very likely. Serum AFP is by definition always below upper reference limit in seminomas. Thus, if serum AFP is elevated in a patient with a histologically pure seminoma, the tumor is classified and treated as a NSGCT. Markedly elevated (>900–3000 pmol/l) hCG concentrations are seen almost exclusively in NSGCT.

1.2.8 STAGING AND PROGNOSIS

Disease stage is classified according to the tumor-node-metastases (TNM) classification (Table 4) and staging system (Table 5) of the International Union against Cancer (UICC) (Sobin, et al. 2009). Also, for treatment purposes, patients with metastatic disease are further classified according to the International Germ Cell Cancer Collaborative Group (IGCCCG) staging system, which is based on prognosis (Table 6). Key characteristics are primary tumor site, site of metastases, and serum concentrations of AFP, hCG and LDH.

Table 4. TNM classification of UICC (Sobin, et al. 2009). URL = Upper reference limit.

Primary tumor	Regional lymph nodes	Metastases	Serum markers
T1 Within the testicle. May invade tunica albuginea.	N0 No affected lymph nodes	M0 No metastatic spread to other organs	S0 Normal marker levels
T2 Invades blood or lymph vessels or tunica vaginalis	N1 Affected lymph nodes <2 cm	M1a Metastatic spread to lungs or lymph nodes distant from testicles	S1 hCG <5000 IU/l, LDH <1.5 x URL, AFP <1000 ng/ml
T3 Invades spermatic cord	N2 Affected lymph nodes 2–5 cm	M1b Spread to other body organs than lungs	S2 hCG 5000–50000 IU/l, LDH 1.5-10 x URL, AFP 1000–10000 ng/ml
T4 Invades scrotum	N3 Affected lymph nodes >5 cm		S3 hCG >50000 IU/l, LDH >10 x URL, AFP >10000 ng/ml

Table 5. Staging according to TNM classification of UICC (Sobin, et al. 2009).

Stage	Tumor	Node	Metastases	Serum Marker
I	Any	N0	M0	Not evaluated
IA	T1	N0	M0	S0
IB	T2-4	N0	M0	S0
IS	Any	N0	M0	S1-3
II	Any	N1-3	M0	Not evaluated
IIA	Any	N1	M0	S0-1
IIB	Any	N2	M0	S0-1
IIC	Any	N3	M0	S0-1
III	Any	Any	M1a	Not evaluated
IIIA	Any	Any	M1a	S0-1
IIIB	Any	N1-3	M0	S2
IIIB	Any	Any	M1a	S2
IIIC	Any	N1-3	M0	S3
IIIC	Any	Any	M1a	S3
IIIC	Any	Any	M1b	Any

High preoperative serum tumor marker concentrations are associated with adverse prognosis (Bosl, et al. 1981; Vogelzang 1987; von Eyben, et al. 2001) and are independent predictors of poor survival in NSGCT (Droz, et al. 1988), but not in seminomas (IGCCCG 1997). For evaluation of metastasis, the nodal pathway from the testis, abdominal, mediastinal and supraclavicular nodes, liver and lungs are assessed. If suspicious symptoms are present, brain and bone metastases should also be evaluated. Examination of the brain is also recommended in NSGCT patients with widespread lung metastases (Krege, et al. 2008a; Krege, et al. 2008b). The role of nerve-sparing retroperitoneal lymph node dissection (RPLND) as a staging-procedure after orchiectomy in NSGCT is disputed since the operation may have serious side effects. ITGCN is present in the contralateral testis in 5-8% of patients with testicular tumors (Hoei-Hansen, et al. 2005; von der Maase, et al. 1986). Since ITGCN has a 70% risk of developing into testicular cancer within 7 years (Hoei-Hansen, et al. 2005; von der Maase, et al. 1986), diagnostic biopsy or biopsies of the contralateral testis are recommended in patients with high risk of ITGCN (Krege, et al. 2008a; Krege, et al. 2008b).

Table 6. IGCCCG staging system, modified from International Germ Cell Cancer Collaborative Group consensus classification (IGCCCG 1997).

	NSGCT, Risk			Seminoma, Risk	
	Good	Intermediate	Poor	Good	Intermediate
Primary tumor site	Testis or retro-peritoneum	Testis or retro-peritoneum	Medias-tinum	Any	Any
Sites of metastases	Only pulmonary	Only pulmonary	Extra-pulmonary	Only pulmonary	Extra-pulmonary
AFP µg/l	<1000	1000–10000	>10000	<10	<10
hCG IU/l	<5000	5000–50000	>50000	Any	Any
LDH (x laboratory specific upper reference limit)	<1.5	1.5–10	>10	Any	Any
Approximate proportion of patients	56	28	16	90	10
Predicted 5-year overall survival (%)	92	80	48	86	72
Predicted 5-year progression-free survival (%)	89	75	41	82	67

1.2.9 TREATMENT

The initial treatment of testicular cancer is orchiectomy but in cases with vastly disseminated disease or life-threatening metastases, chemotherapy can be given before orchiectomy. If standard orchiectomy could seriously affect hormonal function, organ-sparing surgery (e.g. tumor resection) is an option.

Because of the high cure rate in early stage disease (Figure 3), treatment can be limited in order to reduce unwanted acute and long-term side-effects. Thus, organ-sparing surgery and reduced doses of irradiation or surveillance instead of staging RPLND or adjuvant chemotherapy can be used. Adjuvant treatment is tailored according to disease stage and prognosis, as outlined in Table 6.

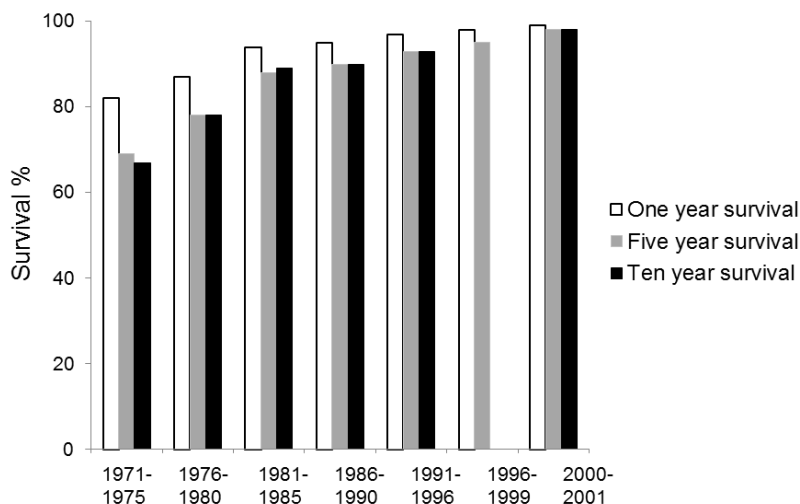


Figure 3. Improvement of survival rates of testicular cancer in the United Kingdom from 1971 to 2001, when cumulative survival rates exceed 95%. Data obtained from the homepage of the foundation Cancer Research UK (Cancer Research UK, 2012).

1.2.9.1 ITGCN

There are three treatment options for ITGCN: namely orchiectomy, radiotherapy or close surveillance. Radiotherapy is curative but will destroy fertility. Orchiectomy is a reasonable choice for patients with unilateral ITGCN and a normal adjacent testis, since scattered radiation could impair the function of the unaffected testis. Surveillance with regular ultrasound imaging is justified for patients wishing to preserve fertility, since the time-course between ITGCN diagnosis and the development of clinical cancer is usually long.

1.2.9.2 Seminoma

After orchiectomy, there are three treatment options for nonmetastazised seminoma: surveillance and adjuvant treatment only at relapse, adjuvant chemotherapy with single-dose carboplatin or adjuvant radiotherapy. The risks and benefits of each treatment option are evaluated individually for each patient. Approximately 15–20% of stage I seminoma patients have occult metastases and will relapse without adjuvant treatment (Warde, et al. 2002), but since the disease is very radiosensitive even at relapse, the overall cancer-specific survival rate reported is 97–100% with surveillance and salvage treatment at relapse (Horwich, et al. 1992; Warde, et al. 1993; Warde, et al. 2002; von der Maase, et al. 1993). The most common adjuvant treatment is 20 Gy para-aortic field radiotherapy (Jones, et al. 2005). However, radiation therapy may have side-effects including a small risk of secondary malignancies (Travis, et al. 2005), gastrointestinal symptoms, (Bamberg, et al. 1999) and impaired fertility owing to scattered radiation to the remaining testicle (Joos, et al. 1997). Single-cycle carboplatin adjuvant chemotherapy has proved equal to radiotherapy concerning recurrence rate (Oliver, et al. 2005).

In stage II seminoma, radiotherapy is the standard treatment. It is delivered as a “hockey-stick”-field comprising the para-aortic region and the ipsilateral iliac region. In stage IIB, chemotherapy is an alternative (Arranz Arijá, et al. 2001), but may carry a higher risk of acute side-effects than radiotherapy. The treatment of choice for advanced, stage III seminoma is chemotherapy (de Wit, et al. 2001).

1.2.9.3 NSGCT

Tailored, risk-adapted treatment is recommended in stage I NSGCT. The risk-assessment is based on the absence or presence of histological vascular invasion (Albers, et al. 2003). Patients with vascular invasion of the tumor are recommended to undergo two cycles of adjuvant chemotherapy and patients without vascular invasion active surveillance (Albers, et al. 2005). Nerve-sparing RPLND is an alternative for high-risk patients unwilling to undergo adjuvant chemotherapy (Albers, et al. 2005). It is notable that if serum tumor markers remain persistently elevated in patients with stage I NSGCT, up to 87% have residual disease in RPLND (Davis, et al. 1994).

Patients with stage II NSGCT without tumor marker elevations can be treated with primary RPLND or close surveillance (Schmoll, et al. 2004), since in these cases, the metastases may represent differentiated, mature teratoma. The treatment of metastatic (stage II and stage III) NSGCT with elevated tumor markers is chemotherapy tailored according to IGCCCG prognostic staging (Table 6) followed by residual tumorectomy if residual masses exist after chemotherapy (Hendry, et al. 1993; Tekgul, et al. 1994).

In metastatic disease, both in seminoma and NSGCT, the disease status is re-evaluated after two chemotherapy cycles. Tumor markers should decline according to half-life kinetics (Schultz, et al. 1978). If they have declined as expected and tumor size has either declined or remained stable, the treatment cycles are completed as planned (Murphy, et al. 1994). If tumor markers are declining but tumor size is growing, residual tumorectomy is mandatory in NSGCT after completion of induction therapy, since mature teratoma is a strong possibility. Further chemotherapy is indicated, if vital cancer tissue is detected after first line chemotherapy by surgery, biopsy or with positron emission tomography, or if tumor marker levels are increasing (Zon, et al. 1998). In NSGCT, persisting AFP and/or hCG elevation during or after treatment is an independent predictor of progression after RPLND with a hazard ratio of 5.6 (Stephenson, et al. 2005). Elevated post orchiectomy hCG and LDH concentrations also predict shorter progression-free survival (Fosså, et al. 1997; Mencil, et al. 1994). However, tumor lysis after adjuvant therapy may result in a transient spike in marker levels (Vogelzang, et al. 1982), which doesn't reflect disease progression. Monitoring of LDH during treatment is not recommended because of high false positive rates due to tissue destruction (Gilligan, et al. 2010).

1.2.9.4 Follow-up after curative therapy and long-term sequelae

The aim of follow-up is to detect relapses and possible side-effects, either physical or psychological, caused by the cancer or its treatment. Most recurrences appear within two years after initial diagnosis, thus follow-up should be intensive during the first two years. However, rare late relapses may occur even after 5 years, and annual follow-up for at least 10 years is recommended (Dieckmann, et al. 2005). During the first years, follow-up consist of clinical examination, serum tumor marker determinations and imaging, after which imaging is not mandatory. AFP and hCG are recommended to be determined at each follow up visit (Gilligan, et al. 2010; Stenman and Alfthan 2002). However, increased serum concentrations of hCG and AFP are not very sensitive for detection of relapse after treatment of NSGCT, being the earliest indicators of relapse in 20–49 % of the cases (Ackers and Rustin 2006; Daugaard, et al. 2003; Divrik, et al. 2006; Gels, et al. 1995; Kausitz, et al. 1992; Trigo, et al. 2000; Venkitaraman, et al. 2007). Determination of LDH during surveillance is not recommended because of the high false positive rate (Gilligan, et al. 2010). Furthermore, addition of LDH to the marker panel does not improve detection of relapse as compared to AFP and hCG alone (Ackers and Rustin 2006; Venkitaraman, et al. 2007). Therapy can sometimes selectively destroy certain histological tumor components and this may alter marker expression (Mostofi, et al. 1987). Therefore, both hCG and AFP should be determined during follow-up even if they weren't elevated at diagnosis (Gilligan, et al. 2010).

After chemotherapy, there is a small long-term risk of secondary malignancies, the risk depending on patient's age, type and duration of treatment and time since therapy (Travis, et al. 2005). Up to 15–25% of long-term survivors may suffer from other potential side effects of chemotherapy such as nephrotoxicity, persistent neurotoxicity, Raynaud's phenomenon and ototoxicity (Brydoy, et al. 2009; Petersen and Hansen 1999). Testicular cancer survivors also have a higher risk of dying of other causes as compared to general population (Fosså, et al. 2007; Zagars, et al. 2004). Side-effects of both radio- and chemotherapy include increased risk of cardiovascular disease and metabolic syndrome (Haugnes, et al. 2007; Nuver, et al. 2005). Abdominal radiotherapy rather often causes persistent, low-grade gastrointestinal symptoms (Yeoh, et al. 1995). RPLND carries risks of surgery-associated side-effects, particularly a 6–8% risk of retrograde ejaculation (Baniel, et al. 1994; Heidenreich, et al. 2003; Spermon, et al. 2002). Due to the disease itself or cytotoxic treatment, there is also substantial risk of hypogonadism, *i.e.* 10–16% (Huddart, et al. 2005; Nord, et al. 2003). Therefore, endocrine function should regularly be evaluated during follow-up.

2 AIMS OF THE STUDY

hCG is an established marker of testicular cancer. However, hCG is a heterogeneous protein consisting of several isoforms, of which hCG β has prognostic value in many nontrophoblastic cancers and hCG-h has been suggested to be the key to malignant transformation of testicular cancer. The present study aimed at evaluating the diagnostic and prognostic value of hCG, hCG β and hCG-h determinations in testicular cancer. In order to do so, we first had to evaluate the reliability of hCG measurement in urine and serum after long-term storage, and to develop an assay specific for hCG-h.

Specific aims of this study were:

I To study the stability of hCG in urine (I) and serum (II, IV) samples during storage.

II To develop an immunofluorometric assay specific for hCG-h (III).

III To study whether simultaneous measurement of serum hCG β and hCG offers additional information in the diagnosis and follow-up of testicular cancer as compared to determination of hCG alone (II).

IV To study whether hCG or hCG β is hyperglycosylated in testicular cancer and whether determination of serum hCG-h has clinical value in the management of the disease (IV).

V To characterize moderate elevation of hCG during follow-up of a testicular cancer patient (V).

3 MATERIALS AND METHODS

3.1 PATIENTS (II, IV, V)

Our material comprised of 351 patients with testicular tumors, treated between 1990 and 2003 in Helsinki University Central Hospital (HUCH). Thirteen patients (4%) had a testicular tumor of other than germ cell origin. Clinical characteristics (Table 7) were retrieved from patient charts in 2006. The staging was re-evaluated and unified according to UICC classification used at the time (Sobin and Wittekind 2002). Seven germ cell tumors with an extratesticular primary site were not staged. Routine follow-up was conducted in HUCH and it consisted of radiographic imaging, serum tumor marker determinations and clinical examination. Serum AFP concentrations, determined with the Delfia assay (PerkinElmer, Wallac), were retrieved from patient records.

3.2 SAMPLES

Serum samples from testicular cancer patients were archival clinical samples (n = 3802). Before 1998, blood was drawn into 7 ml glass tubes without additives and serum was subsequently separated (n = 1004). From 1998 onwards blood was drawn into 5 ml gel separator tubes (BD Vacutainer SST II Plus) and serum separated (n = 2799). Samples were stored at +4 °C for less than a week until assays of hCG and hCG β (II, IV, V), the surplus serum was divided into aliquots and stored at -20 °C for 3–14 years until assays of hCG and hCG-h (II, IV). Samples defined as preoperative or relapse samples were taken less than a month before clinical diagnosis of the disease or relapse and before treatment. Urine samples from patients with hCG-producing tumors (I) were archival clinical samples. Urine and serum samples from pregnant and nonpregnant subjects were donated by apparently healthy women (I, III, IV). Samples of serum and EDTA plasma from 20 men and 20 nonpregnant women were left-over clinical samples stripped of personal identifiers (III).

Table 7. Patient characteristics and outcome. Days are expressed as median (range), ST = stage and RT = radiotherapy.

	Preoperative sample available		Preoperative sample not available	
	Seminoma n = 42	NSGCT n = 52	Seminoma n = 109	NSGCT n = 137
Preoperative sample (Days before treatment)	3 (22–0)	2 (18–0)		
Histology				
Seminoma	41		106	
Spermatocytic seminoma	1		3	
Ca embryonale		23		76
Teratocarcinoma		18		46
Mixed NSGCT		6		5
Choriocarcinoma		1		
Nonseminoma		4		10
Stage				
ST I	38	18	89	75
ST II	3	18	16	29
ST III		15	2	30
Not staged	1	2	2	2
Site of the primary				
Right testicle	21	29	58	76
Left testicle	19	22	50	56
Bilateral testicular tumors	1			1
Retroperitoneum	1	1		1
Mediastinum		1		
Unknown				2
Metastatic site at diagnosis				
Retroperitoneum	2	27	16	55
Mediastinum		10		12
Lung		13	2	23
Liver		4	1	1
Brain		2		2
Marker elevation	1	3		2
Predisposing factors				
Cryptorchidism	4	3	2	7
Familial clustering				2
Outcome				
Relapse	2	5	2	17
Relapse, days after primary treatment	572 (479–664)	207 (66–461)	200 (171–228)	158 (27–5096)
Treatment resistance		1	1	1
Disease-related death		3	1	3
Death, days after primary treatment		440 (9–500)		482 (218–622)
Metastatic site at relapse				
Retroperitoneum	1	4		17
Mediastinum	2	1	1	17
Lung	1	1		2
Liver		1		2
Groin			1	
Adjuvant treatment				
None	6	7	4	14
Only RT	30		87	3
Chemotherapy	6	43	20	106

3.3 DETERMINATION OF DIFFERENT FORMS OF hCG

hCG was determined with a commercial immunofluorometric assay (AutoDelfia, PerkinElmer Wallac). hCG β , hCG-h, hCG α and hCG β cf were determined with highly sensitive and specific in-house time-resolved immunofluorometric assays (IFMA) based on monoclonal antibodies (Table 8). The assays were performed in 96-well plates coated with a capture antibody by incubating 0.2 ml of the respective MAb at a concentration of 10 mg/l in 0.1 mol/l sodium phosphate buffer pH 7.4 for 20 h. Calibrators or samples (25 or 50 μ l) and Delfia assay buffer (PerkinElmer Wallac) to a total volume of 200 μ l were added to the wells. After incubation, the plate was washed with an automatic washer (Delfia Plate Wash, PerkinElmer Wallac) using Delfia wash buffer, after which Eu-labeled detector antibody (200 μ l) in assay buffer was added. Detector antibodies were labeled with a 50-fold molar excess of Eu chelate to give 5–8 Eu chelates per antibody. After further incubation, the wells were washed and Delfia enhancement buffer (PerkinElmer Wallac) was added. After 5 minutes, the fluorescence was measured with a Victor² V 1220 time resolved fluorometer (PerkinElmer Wallac). Samples with concentrations exceeding 15 000 pmol/l were diluted (1:100) and re-assayed.

Table 8. Characteristics of the assay for hCG, hCG β , hCG-h, hCG α and hCG β cf. CV = coefficient of variation.

Assay	hCG	hCG β	hCG-h	hCG α	hCG β cf
Capture Antibody	5008	9C11	B152	2G11	3C11
Label Antibody	5501	1B2	1B2	7E10	1B2
Sample volume	25 μ l	50 μ l	50 μ l	50 μ l	50 μ l
Incubation times	1 and 0,5h	2 and 0,5h	2,5 and 0,5h	2 and 1h	1 and 0,5h
CV	<10% above 2.1 pmol/l	2–10% above 3 pmol/l; <15% at 1–3 pmol/l	<10% above 10 pmol/l		2–10 % above 3 pmol/l
Functional detection limit	1.5 pmol/l (0,5 IU/l)	0.5 pmol/l	2 pmol/l	2.8 pmol/l	0.4 pmol/l
Hook effect	None, up to 500 000 IU/l	None, up to 50 000 pmol/l	None in functional assay range		
Cut-off value used in this study	15 pmol/l (5 IU/l)	2 pmol/l	10 pmol/l	Not applicable	Not applicable
References	Pettersson, et al. 1983; Alfthan, et al. 1992a	Alfthan, et al. 1992a	Stenman et al. 2010	H. Alfthan, unpublished results	Alfthan, et al. 1992a

The commercial hCG IFMA employs a MAb against hCG β for capture and a MAb against hCG α for detection (Pettersson, et al. 1983), and thus only intact hCG is detected. hCG-h was determined with an assays that uses a MAb B152 recognizing a type-2 O-glycan at Ser 132 (III). hCG β (I, II, IV) and hCG α (I) were determined using MAbs against epitopes hidden in intact hCG. The assay for hCG β cf (I) uses a specific capture MAb that recognizes the so-called core fragment of hCG β . For comparison of the concentrations of various forms of hCG, the hCG concentrations expressed in IU/l were converted to molar concentrations (pmol/l) by multiplying with 2.93.

The hCG assay was calibrated against the 4th international standard (IS) 75/589. The hCG β assay was initially calibrated with an in-house preparation of hCG β (Alfthan, et al. 1988). After 2005, the hCG β assay was calibrated against World Health Organization (WHO) International Reference Reagent (IRR) 99/650. This did not change the results. hCG α was calibrated against IRR 99/720 and hCG β cf assay against IRR 99/708 (Birken, et al. 2003a; Bristow, et al. 2005). Provisional calibrators for hCG-h were prepared from the cell medium of the choriocarcinoma cell line JEG-3 (American Type Culture Collection code HTB-36), concentrated 20-fold by ultrafiltration. The hCG concentration determined by the Delfia assay was used to assign values to the provisional standard for hCG-h. Calibrators with concentrations between 8.8 and 8800 pmol/l were prepared in assay buffer or male EDTA plasma (III).

The recognition of various forms of hCG in the assays for hCG and hCG β was determined using the WHO IRR preparations. The cross reaction of hCG β in hCG assay and of hCG in the hCG β assay is low but since the value for the provisional standard of hCG-h was assigned with the hCG IFMA, the recognition of hCG-h in the hCG assay is by definition 100%. Also, since IRR 99/642 is purified from pregnancy urine, it contains hyperglycosylated hCG (about 20%, but the ratio varies between batches), which is recognized in the hCG-h assay (Table 9).

Table 9. Crossreaction (in percentages) of different hCG forms in the assays used.

	hCG	hCG β	hCG-h
IRR 99/642 (hCG)	100	0.4	20
IRR 99/642 (hCGβ)	0.4	100	8.7
JEG-3 medium (hCG-h)	100	2.2	100

3.4 OTHER ASSAYS

Urine density was measured with a refractometer (UG-1, Atago CO Ltd) and pH with PHM92 (Radiometer Analytical). Urine dip stick test (Multistix® 8 SG, Siemens AG) was used to rule out urinary tract infection (I). The IgG isotype of the antibodies was determined with the Mouse Monoclonal Isotype kit (Serotec) (III). In one sample with discordant results between hCG and hCG-h assays, hCG was re-determined with two other commonly used commercial assays: Siemens Immulite 2000 (Siemens Medical) and the Roche Elecsys total hCG (Roche Diagnostics) assays, according to instructions (IV). These assays recognize hCG, hCG β and hCG β cf (Sturgeon, et al. 2009) together, and are thus of the so-called total hCG assay type.

3.5 STUDY DESIGNS

3.5.1 STABILITY OF hCG IN SERUM AND IN URINE (I, II, IV)

To study the effect of freezing, long-term storage and thawing, we re-determined hCG after 3–14 years of storage at -20 °C in 152 serum and in 74 hCG-containing urine samples from cancer patients. The results were compared to the hCG concentration that were determined with the same commercial AutoDelfia assay before storage. We analyzed the effect of urine density, which was determined before storage in 28 of 74 samples and re-determined after storage in 12 samples, and of urine creatinine concentration, which was retrieved from the patient charts and was available in 30 of 74 samples.

To study the short-term stability of hCG and hCG β in serum, we determined hCG and hCG β at sample arrival, after 1, 3, 6, 24, 48 hours and one week of storage at +4 °C in three pregnancy serum samples. To study the short-term stability of hCG in urine, we stored aliquots of fourteen consecutive urine samples from a single pregnancy for 3–10 months, and compared the hCG concentrations in samples stored at -20 °C to those stored at +4 °C. Urine hCG has been found to be stable at +4 °C for at least a month (de Medeiros, et al. 1991; McChesney et al. 2005). To study the inter-individual variations in hCG recovery and to verify the stability of hCG at +4 °C, we stored pregnancy urine samples from three different pregnancies at +4, -20 and -80 °C. The concentrations of hCG, hCG β , hCG α and hCG β cf were determined before storage, after one week, and one, three and 6 months of storage.

We studied the effect of urinary pH, urea concentration and preservatives on hCG immunoreactivity in urine samples spiked with pregnancy urine. We adjusted aliquots to either pH 6, pH 7 or pH 9 and added urea (0, 0.2 mol/l or 1 mol/l) and preservatives - glycerol, ethylene diamine (EDA), ethylene diamine tetra-acetic acid (EDTA) or bovine serum albumin (BSA) - to aliquots, which were then stored at +4 °C, -20 °C and -80 °C. The concentrations of hCG, hCG β , hCG α and hCG β cf were determined before storage, after one week and one month of storage. We also studied, whether the loss of immunoreactivity was dependent on hCG concentration by spiking samples with different concentrations of purified hCG (80–20 000 000 pmol/l, Pregnyl®), and whether the subunits were able to re-associate within one week at +4 °C after reduction of added urea to concentrations less than 0.01 mol/l.

3.5.2 DEVELOPMENT OF AN ASSAY SPECIFIC FOR HCG-H (III)

MAb B152 is the only antibody specific for hCG-h. The isotype of MAb B152 is IgG2a, and complement can interfere with the reactivity of this Ab (Børner 1989). Therefore we evaluated the degree of complement interference and methods to overcome it. We compared the recovery of hCG-h in 6 parallel samples of early pregnancy EDTA-plasma and serum. We also studied the addition of EDTA containing buffer to serum samples, to a final concentration of 5 mmol/l. We studied the effect of heating for 60 min at 56 °C with serum and EDTA plasma samples spiked with well characterized hCG-h isolated from a testicular cancer patient (Valmu, et al. 2006).

3.5.3 HCG, HCG β AND HCG-H IN SERUM OF TESTICULAR CANCER PATIENTS (II, IV, V)

We determined the concentrations of hCG, hCG β and hCG-h in serum samples from patients with various testicular germ cell tumors. hCG and hCG β were determined in 94 serum samples taken before treatment, 22 samples taken at relapse (four seminoma and 18 NSGCT patients), and 3687 samples taken during disease-free follow-up. hCG-h was determined in 67 serum samples taken before treatment, 20 samples at relapse (four seminoma and 16 NSGCT patients), and 89 were consecutive follow-up samples taken during or shortly after treatment. The proportion of hCG consisting of hCG-h (%hCG-h) was calculated by dividing the concentration of hCG-h with the concentration of hCG (hCG-h/hCG*100%).

3.6 STATISTICAL METHODS

Statistical analyses were performed using SPSS (versions 14.0 and 16.0) and PASW software. Student's paired t-test (III), Wilcoxon paired samples test (I), Spearman correlation (IV), Kruskal-Wallis test, Kendall's tau_b and the Mann-Whitney U (II) test were used to compare the results of repeated determinations and differences between patients groups. Survival curves were plotted using the Kaplan-Meier method, and comparison of progression free survival time was performed with the Mantel-Cox log-rank test (IV). Progression free survival time was analyzed in all patients, including patients with metastases at diagnoses. An event was defined as the first detection of recurrence in patients in complete remission after therapy. For two patients, who never achieved remission, the event was defined as the first occurrence of progression. The predictive value of various markers on survival was analysed by uni- and multivariate analysis using the Cox proportional hazard model (IV). All tests were two-sided and *P*-values below 0.05 were considered significant.

3.7 ETHICAL ASPECTS

This study was approved by the Ethics committee of HUCH. Permission to study archival serum and urine samples from cancer patients and their patient charts was granted by the National Supervisory Authority for Welfare and Health. Apparently healthy non-pregnant and pregnant subjects providing serum and urine samples gave verbal informed consent. The sampling of biologic material was carried out according to the Helsinki declaration.

4 RESULTS AND DISCUSSION

4.1 STABILITY OF hCG AND hCG β IN SERUM

During storage at +4 °C for up to 1 week, the recovery of hCG and hCG β in pregnancy serum remained within $100 \pm 10\%$ of the concentrations determined before storage. The correlation between hCG concentrations determined before and after 3–14 years of storage at -20 °C was excellent ($R^2 = 0.98$) but the concentrations were slightly higher after long-term storage ($y = 1.036x$), probably due to sublimation of water causing sample concentration. Also, there were four outliers at low concentrations (Figure 4). In order to avoid bias caused by long storage, we used hCG concentrations determined after storage and samples with elevated concentrations before and after storage to calculate the %hCG-h. The long-term stability of hCG-h was not studied since the assay for hCG-h was developed after sample collection.

Our findings confirm earlier ones, in which hCG in serum has been found to be stable for years of storage at -20 °C (Spencer, et al. 1993). Archival serum samples can be used to evaluate the clinical utility of hCG determination in testicular cancer. Although we could not evaluate the stability of hCG-h in serum during long term storage, it is unlikely that storage would have specifically affected the unique carbohydrate structure of hCG-h. This was also supported by the fact that the correlation coefficient of hCG and hCG-h was as high as 0.96 in preoperative serum samples from NSGCT patients stored at -20 °C (See Figure 11). Furthermore, there were practically no outliers (See Figure 11). If hCG-h had degraded more than hCG during storage, we would most likely have observed more major differences and discrepancies in the concentration of hCG-h and hCG.

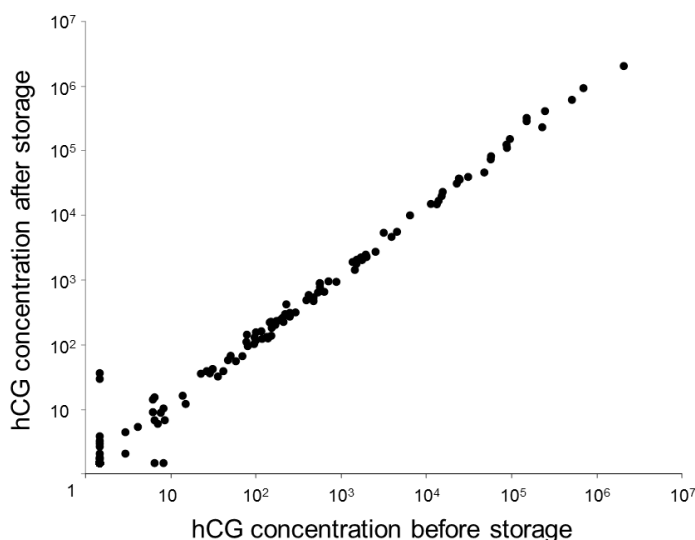


Figure 4. hCG concentrations (expressed as pmol/l) in serum before (x-axis) and after (y-axis) storage at -20 °C for 3–14 years.

4.2 STABILITY OF hCG IN URINE

4.2.1 STABILITY IN URINE DURING LONG-TERM STORAGE

A significant proportion of hCG immunoreactivity was lost (>20%) in a majority (58 %) of cancer urine samples during years of storage at -20 °C (Figure 5). The loss did not correlate with storage time ($P = 0.210$), initial hCG concentration ($P = 0.189$), urine density ($P = 0.099$), but tended to correlate with urine creatinine concentration ($P = 0.056$). The apparent recovery of hCG exceeded 100% in one third of samples. This is most likely explained by sample concentration due to sublimation of water during storage, a phenomenon that has been described before (Wilcox, et al. 1985). Urine density indeed had increased during storage in 6 of 12 samples indicating sample concentration. The frequency and magnitude of the hCG loss was probably underestimated because of sample concentration causing increase in hCG concentration.

There were discrepancies between hCG concentrations before and after storage in most samples (Figure 5). This variation in hCG recovery after storage may explain some unexpected results of studies, in which urinary hCG concentrations have been determined after long term storage at -20 °C. Very large day-to-day variation in pregnancy urine hCG concentrations observed in a study by McChesney et al. (McChesney, et al. 2005) is likely to reflect the variable loss of hCG immunoreactivity during storage at -20 °C rather than differences in hCG excretion.

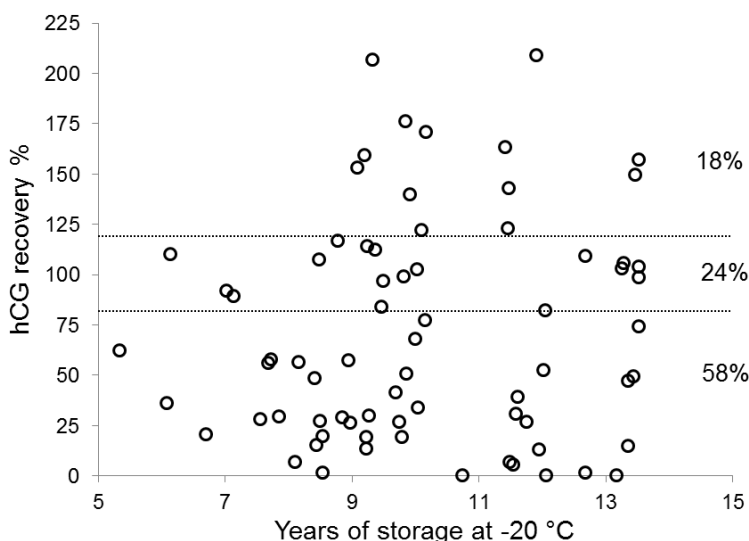


Figure 5. Recovery of hCG in urine after up to 14 years of storage at -20 °C as compared to hCG concentrations before storage. The dashed lines depict recovery of $100 \pm 20\%$.

Because urine is a noninvasive, readily feasible sample matrix, it would have been interesting to evaluate, whether urine hCG concentrations reflect the disease course in testicular cancer and whether quantitative urine measurements or even the new very sensitive (detection limit of 10 IU/l) qualitative hCG assays potentially employed at home by the patient themselves, have clinical utility during follow-up of testicular cancer. However, since the recovery of hCG in urine after years of storage at $-20\text{ }^{\circ}\text{C}$ was highly variable, it was obvious, that archival urine samples could not be used to assess the clinical value of urine hCG determinations in testicular cancer.

4.2.2 STABILITY IN URINE DURING SHORT-TERM STORAGE

A highly variable proportion of hCG was lost at $-20\text{ }^{\circ}\text{C}$ (recovery varied between 13-77%, median 43%, $P = 0.019$) during storage for 3–10 months in all 14 consecutive urine samples from a single pregnancy. Storage time between 3–10 months did not correlate with recovery ($P = 0.464$). In one of three samples from different pregnancies, hCG was nearly completely lost after three months at $-20\text{ }^{\circ}\text{C}$ (Figure 6). In the other two samples, hCG remained fairly stable. Thus, hCG is lost at $-20\text{ }^{\circ}\text{C}$ within months. The loss varies between individuals and between different samples from the same individual. When stored at $+4\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$ hCG was stable for six months, but the recoveries of hCG β , hCG α and hCG β cf varied ($n = 3$).

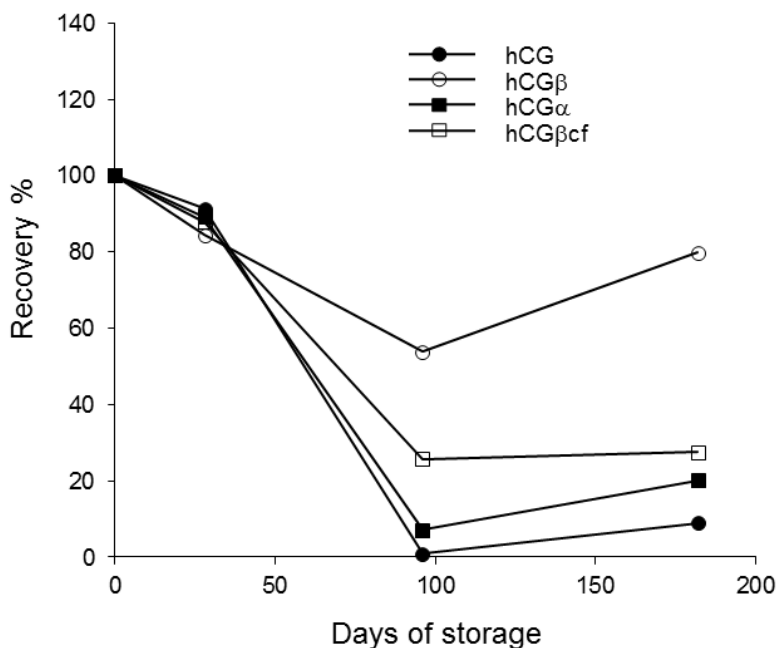


Figure 6. Recovery of different forms of hCG in a single pregnancy urine sample stored at $-20\text{ }^{\circ}\text{C}$ for six months.

The stability of hCG under freezing conditions is important when hCG determinations are used for doping control. Samples taken for this purpose are generally stored at -20 °C before analysis. Two urine samples (A- and B-samples) are dispatched to the doping laboratories for analysis. The A-sample is analyzed immediately, while the B-sample is stored frozen at -20 °C until used to confirm a positive finding in the A-sample (WADA 2010). This may be done weeks later. Our findings show, that in order to make determination reliable, urine samples should not be stored at -20 °C before analysis of hCG, but at -80 °C or at +4 °C for short time (less than a month) before analysis of hCG. In this study the recovery of hCG decreased within 3 months of storage at -20 °C, but we have observed loss of hCG even after one week (data not shown).

Our findings differ from previous studies, in which hCG has been considered stable at -20 °C (Wilcox, et. al. 1985; McCready, et al. 1978). In the study by Wilcox et al., hCG was re-determined in 88 urine samples stored at -20 °C for 7–9 months after initial assay. They concluded that hCG concentrations did not decrease but increased over time probably due to evaporation (Wilcox, et al. 1985). However, the initial concentration was not determined before storage, but after variable times of storage at -20 °C, and hCG could have already been lost before the initial assay. Furthermore, they used a total hCG assay detecting hCG, hCG β and hCG β cf together. Dissociation of hCG into subunits would not have been detected, since dissociation leads to an equimolar increase of hCG β and thus unaltered hCG immunoreactivity in total hCG assays. McCready et al. studied the stability of hCG in five urine samples, which were either frozen at -20 °C without delay or after one week of storage at room temperature or +4 °C. In their study hCG activity decreased in samples stored at +4 °C, but hCG was considered stable at -20 °C (McCready, et al. 1978). However, since the study material was very limited (n = 5) and since hCG degradation is highly variable, it is possible that none of these five samples had a composition leading to hCG loss at -20 °C. Furthermore, they also used a total hCG radioimmunoassay. Recently, the recovery of urine hCG was reported to be “inconsistent”, but the rate or magnitude of the loss were not described (Robinson, et. al. 2010). hCG has been found to be stable at +4 °C for at least for three to four weeks (de Medeiros, et al. 1991; McChesney, et al. 2005), which our study confirms.

4.2.3 EFFECT OF UREA AND PROTECTIVE ADDITIVES ON THE LOSS OF HCG IN URINE

We studied the effect of added urea because it has been suggested to cause degradation of urinary LH at -20 °C (Livesey, et al. 1983). Addition of urea to urine caused an almost complete loss of hCG in urine stored at -20 °C after one week (Figure 7). The loss was greater at high urea concentrations ($P < 0.005$), and at low pH (pH 6 and 7 as compared to pH 9) ($P = 0.031$). The loss was accompanied by an almost equimolar increase in the concentrations of hCG β and hCG α . Thus added urea induced dissociation of hCG into free subunits. The urea-induced loss was not dependent on the initial hCG concentration ($P = 0.256$), nor was it reversed after reduction of urea concentration to less than 0.01 mol/l ($P = 0.898$). Thus, urea most likely causes permanent structural changes preventing re-association.

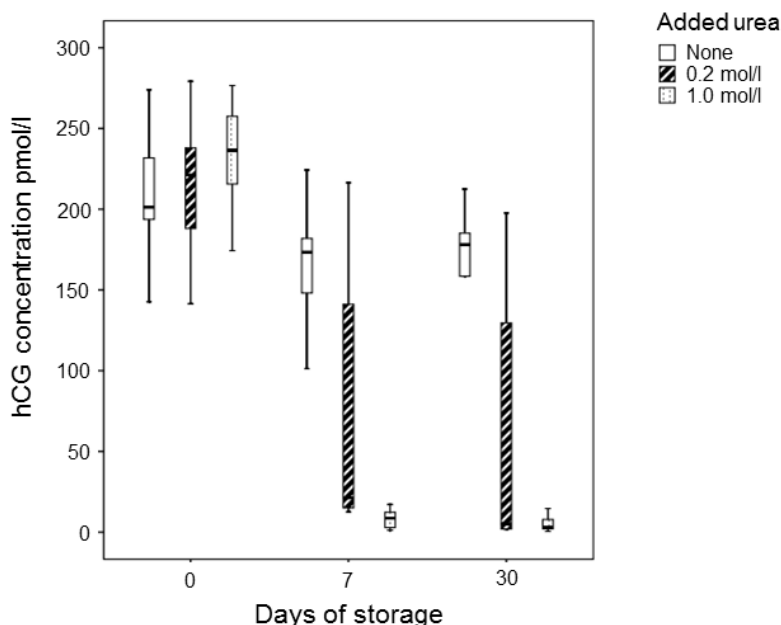


Figure 7. Added urea causes a rapid and substantial loss of hCG immunoreactivity when urine is stored at -20 °C (n = 81, nine in each group). The solid line represents median value, the whiskers represent range, and the boxes 25th and 75th percentile.

The urea-induced changes could have been caused by protein carbamylation. At -20 °C, urine contains liquid regions with high concentration of urea (Livesey, et al. 1983). Decomposition of urea leads to formation of cyanate (Dirnhuber and Schutz 1948; Marier and Rose 1964) that reacts with functional groups on proteins leading to carbamylation (Stark 1965; Stark, et al. 1960), which causes irreversible alterations in protein structure (Cole and Mecham 1966; Frantzen 1997; Salinas, et al. 1974). However, since the loss of hCG at -20 °C was very variable and was also observed in dilute samples, it is likely that mechanisms other than carbamylation contribute to the loss of urinary hCG at -20 °C.

This study reveals that hCG can be lost when stored at -20 °C, but it does not clarify the role of various factors in this process. Addition of urea even at physiological concentration (0.2 mol/l) resulted in a loss of urine hCG immunoreactivity at -20 °C, but since we did not measure the urea concentration in native samples, we cannot fully evaluate its impact. However, the correlation between hCG loss and urine density, which correlates with urea concentration, was nearly significant ($P = 0.056$). The concentrations of hCG remained fairly stable in pregnancy urine stored at +4 and -80 °C, but the recoveries of hCG β , hCG α and hCG β cf varied between individual samples – increasing in some while decreasing in others. It is likely that several mechanisms contribute to the observed changes in the immunoreactivity of different forms of hCG.

Addition of glycerol to 5% and 10% prevented much of the urea-induced loss of hCG ($P = 0.021$ for both). EDA, EDTA or BSA did not prevent degradation (Figure 8), even though EDA is used to protect proteins against carbamylation when manufacturing recombinant

proteins in *E. coli*. Glycerol has been shown to protect urinary FSH and LH against degradation at $-20\text{ }^{\circ}\text{C}$ by increasing the proportion of liquid in partially frozen urine (Livesey, et al. 1983). It also stabilizes protein conformation, which can reduce reaction with cyanate (Gekko and Timasheff 1981). However, glycerol cannot be used as a preservative in doping control, since it can be used as a plasma expander and is included in the WADA list of prohibited substances (WADA 2011).

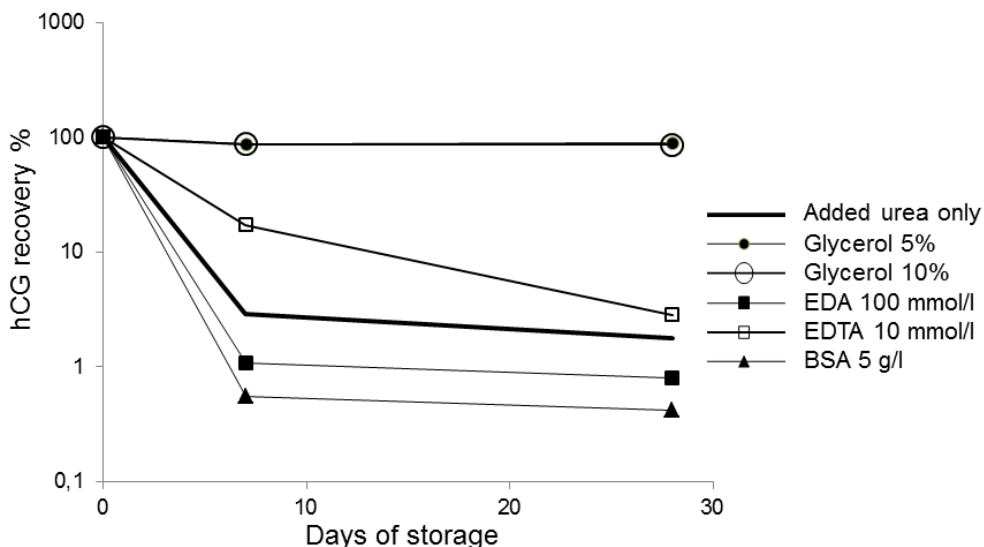


Figure 8. Added urea causes a rapid and complete loss of hCG immunoreactivity at $-20\text{ }^{\circ}\text{C}$, which glycerol prevents. Note the logarithmic scale on y-axis. hCG recovery is less than 10% in other samples except for those stored with glycerol.

4.3 DEVELOPMENT OF IMMUNOFUOROMETRIC ASSAY FOR HCG-H

4.3.1 ASSAY DESIGN AND PERFORMANCE

Two (1B2 and 6G5) of 13 studied Abs formed good sandwiches with MAb B152. When MA B152 was used as a capture antibody and either 6G5 or 1B2 as detection antibody, the maximal signal was 5-times higher than when B152 was used as tracer. Therefore, B152 was used as a capture and 1B2 as a detection Ab. The optimal incubation time was 2.5 h in the first step and 0.5 h in the second step. The limit of detection was 2 pmol/l based on the mean + 2 CV of the signal of the zero calibrator. The limit of quantitation was 5 pmol/l, which was the concentration corresponding to a correlation coefficient (CV) of 20% in plasma samples. The CV was below 10% at concentrations above 10 pmol/l. This concentration was also used as a cut-off limit to define elevated values in testicular cancer patients. Analysis of hyperglycosylated hCG β (hCG β -h) prepared by dissociating hCG-h purified from JEG medium into subunits showed that the recognition was approximately 25% of that for hCG expressed in molar concentration [unpublished results].

4.3.2 COMPLEMENT INTERFERENCE

The median recovery of endogenous hCG-h in serum samples from early pregnancies was 33% (range 22–46%) as compared to that in EDTA plasma, and the median recovery of purified hCG-h standard spiked into serum was 37% (range 16–38%) of that in EDTA plasma. Dilution of hCG-h spiked serum 100-fold increased recovery of hCG-h to the same level as in diluted EDTA plasma. The low recovery of hCG-h in serum is attributable to complement interference in the assay (the mechanism is described in paragraph 1.1.8).

Børmer has recommended that the use of IgG2 antibodies in immunoassays should be avoided because of the interference caused by complement, which may cause false low results in serum samples (Børmer 1989). However, B152 has unique specificity against hCG-h, and thus had to be used in the assay. The interference could also have been avoided by using B152 as tracer rather than for capture, but the maximal signal obtained with this design was much lower. Furthermore, an excess of normally glycosylated hCG would have reduced the binding capacity of the capture antibody binding to all glycoforms of hCG.

Calcium chelators like EDTA inactivate complement, and as expected, addition of EDTA to serum normalized the recovery. If serum was first added to the assay well and EDTA-containing buffer afterwards, the recovery of hCG-h was about 15% lower than when serum first was diluted with EDTA-containing buffer and added to the assay well afterwards. Because heating of serum is used to inactivate complement, we studied whether it affected recovery of hCG-h. Incubation of undiluted serum at 56 °C for 60 min increased the recovery of hCG-h from 35% to 85% but the recovery of hCG decreased by 15–20% due to dissociation of hCG into subunits. Thus, heating was not an option for pretreatment of archival serum samples from testicular cancer patients. We therefore diluted 60 µl of serum with 180 µl of assay buffer containing 7 mmol/l EDTA yielding final concentration of 5 mmol/l, incubated it for at least 1 h and used 200 µl for the assay of hCG-h in archival serum samples.

The problem of complement interference with the use of MAb B152 for assay of hCG-h in serum has not been described before. On the contrary, a report on an earlier available commercial assay, which also used B152 as capture antibody, stated that dilution of serum gave expected results and that results for serum and EDTA plasma were identical (Pandian, et al. 2003). However, in many earlier studies on hCG-h, urine samples have been used (Bahado-Singh, et al. 2002; Birken, et al. 2001; Cole, et al. 1998; Kovalevskaya, et al. 1999). In urine, the content of complement is insignificant and therefore assay of hCG-h in urine with MAb B152 is not problematic. Assay of hCG-h in serum has been shown to be useful for screening of Down's syndrome (Palomaki, et al. 2007) and for monitoring of patients with trophoblastic disease (Cole, et al. 2006a), but in these conditions the concentrations of hCG-h are high enough to allow dilution of the sample and thereby reduction of the complement interference in serum.

4.3.3 PREPARATION OF PROVISIONAL STANDARD

Because there is no commercial standard available for hCG-h, we used hCG-h derived from JEG-3 cells to calibrate the assay and assigned the value to this calibrator with the Delfia hCG assay for hCG. The glycan structure of hCG-h produced by these cells has been very well characterized by mass spectrometry and virtually all of it is hyperglycosylated (Valmu, et al. 2006). The use of hCG-h from JEG-3 as a calibrator is a provisional solution: the carbohydrate structure of hCG-h is heterogeneous (Valmu, et al. 2006) and thus some hCG from JEG-3 cells may not react with MAb B152. Furthermore, the hCG assay used for value assignment may not recognize hCG and hCG-h equally. JEG-3 medium also contains hCG β that is not recognized in the hCG assay but may react to some extent in the assay for hCG-h. Thus the use of JEG medium is a temporary solution until an international standard for hCG-h is available.

At 4–5 weeks of pregnancy, almost all hCG in serum (>90%) was hyperglycosylated. The proportion decreased to 5–10% at 10 weeks and less than 3% after 20 weeks (Figure 9). In pregnancy urine, the apparent proportion of hCG-h exceeded 100% at 4–5 weeks of pregnancy and at 10 weeks it was 20%. These findings are in line with earlier ones (Kovalevskaya, et al. 2002a) indicating that the calibration of the hCG-h assay is comparable to that of the earlier commercial hCG-h assay by Nichols Advantage (Pandian, et al. 2003).

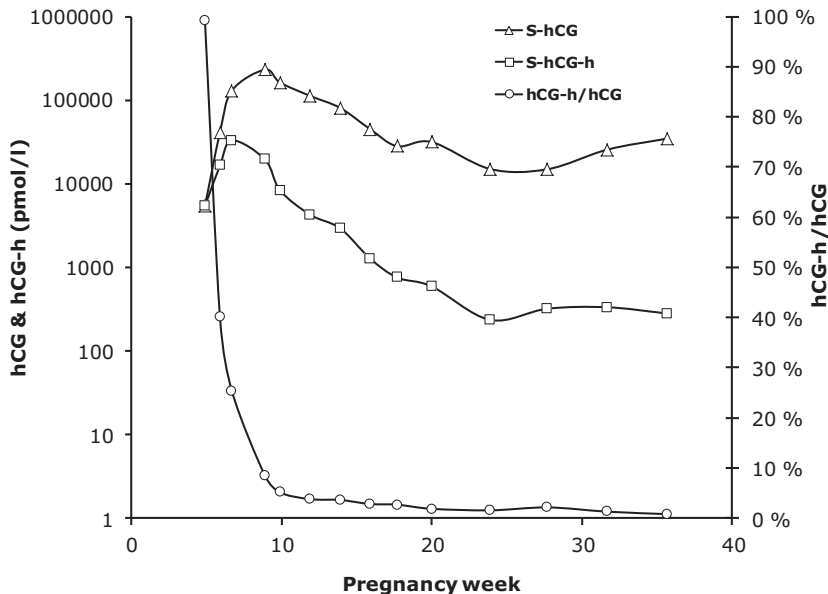


Figure 9. Concentrations of hCG and hCG-h in serial serum samples obtained from one woman during the 5th to the 37th weeks of pregnancy.

4.4 HCG, hCG β AND hCG-H IN SERUM OF TESTICULAR CANCER PATIENTS

4.4.1 PREOPERATIVE SERUM CONCENTRATIONS OF HCG, hCG β AND hCG-H IN SEMINOMA PATIENTS

In seminoma, the preoperative serum concentration of hCG β was elevated in 22 of 42 patients (52%) and that of hCG in seven (17%). In five patients (12%) both hCG and hCG β were increased, while in 17 (40%) only hCG β , and in two (5%) patients only hCG was increased. In 18 (43%) patients both markers were negative (Figure 10). Separate measurement of hCG β increased the frequency of marker positive seminomas from 17% detected with hCG alone to 57%. Thus, in seminomas hCG β is a better diagnostic marker than hCG and simultaneous measurement of hCG β with hCG offers additional diagnostic value.

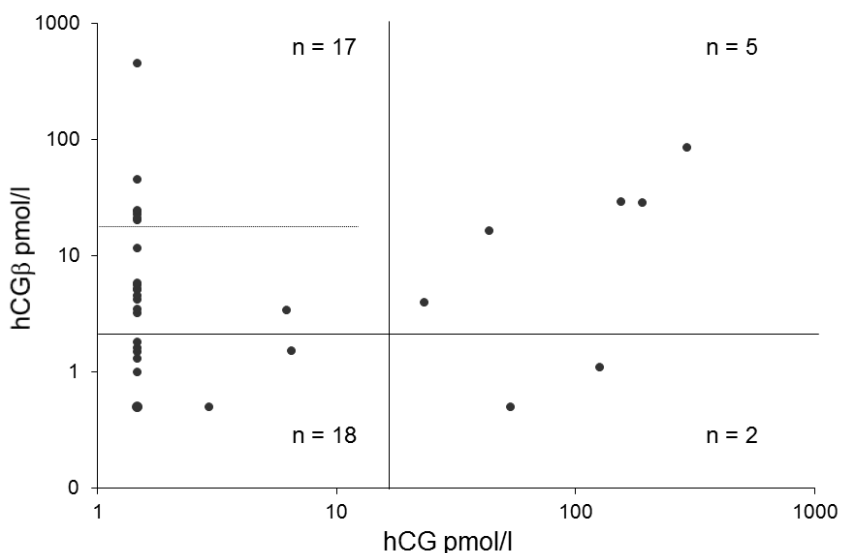


Figure 10. Preoperative concentrations of hCG and hCG β in seminoma patients. The solid lines indicate decision making limits (2pmol/l for hCG β and 15 pmol/l for hCG). The short dashed line on y-axis indicates the cut-off limit for total hCG assays (15 pmol/l).

In some previous studies, serum concentration of hCG β has been increased without an increase of hCG in seminoma patients (Hoshi, et al. 2000; Mann and Siddle 1988; Saller, et al. 1990). However, separate determinations of hCG β have not been used in clinical routine. On the contrary, assays recognizing hCG and hCG β together have been recommended for monitoring of testicular cancer (Mann, et al. 1993; Mann and Siddle 1988; Saller, et al. 1990) and most presently available hCG assays are so-called total hCG assays using antibodies against two epitopes on hCG β which are exposed both on intact hCG and hCG β . However, since the decision making limit for total hCG (15 pmol/l or 5 IU/l) is much higher than that for hCG β (2 pmol/l), tumors causing only a slight increase of serum hCG β will not be detected

with an assay measuring hCG and hCG β together. Based on the calculated sum of hCG + hCG β , an assay measuring hCG and hCG β together would have missed 42% of the marker-positive seminomas (Figure 10).

In our study, the frequency of an isolated increase of serum hCG β (without concomitant hCG increase) was higher than in previous reports (Hoshi, et al. 2000; Madersbacher, et al. 1992; Mann and Karl 1983; Mann and Siddle 1988; Marcillac, et al. 1992; Saller, et al. 1990). In a recent Japanese study, an isolated increase of hCG β was detected in 37%, while hCG was detected in 50% of the seminoma patients (Hoshi, et al. 2000). The high frequency of increased hCG values observed in that study is most likely explained by differences in patient material, i.e., a larger proportion of seminoma patients with advanced disease, i.e. 44% with stage II and III disease, as compared to 7% in our study. Also, there were fewer patients with an elevated hCG and a normal hCG β than in previous studies. This may be explained by the high sensitivity of our assay, which allowed us to use a lower cut-off level for hCG β , i.e. 2 pmol/l as compared to 9–900 pmol/l in earlier studies (Hoshi, et al. 2000; Madersbacher, et al. 1992; Mann and Karl 1983; Mann and Siddle 1988; Marcillac, et al. 1992; Saller, et al. 1990).

Only two seminoma patients had an increased concentration of hCG-h. One patient with elevated hCG-h (37 pmol/l) had significantly increased hCG β concentration (450 pmol/l), but normal hCG concentration. This result could be explained by partial recognition of hyperglycosylated hCG β in the hCG-h assay. No hCG-h was detected in 13 patients with undetectable hCG and moderately elevated hCG β concentrations (3.2–46 pmol/l). This is probably explained by the low recognition of hCG β -h in our assay (~25%), or by absence of hCG β -h. Thus, even though we aimed to evaluate the glycosylation of hCG β by analyzing samples with elevated hCG β and normal hCG concentration (n = 13 preoperatively, n = 6 during relapse), we could not make definitive conclusions on the glycosylation of hCG β , since the concentrations were mostly too low to be detectable by our hCG-h assay.

Another patient with increased concentration of hCG-h (23 pmol/l) had undetectable concentrations of hCG and hCG β , which is surprising, since our hCG and hCG β assays measure hyperglycosylated forms as well. When determined with the Immulite assay, the hCG concentration was 200 pmol/l (68 IU/l) and with the Elecsys assay 120 pmol/l (41 IU/l). This suggests that the hCG immunoreactivity of this patient consisted of somehow degraded or aberrantly glycosylated hCG or hCG β that was not recognized by our assays for hCG and hCG β . Large between-assay differences in recognition of hCG produced by tumors has been described before (Cole and Sutton 2004; Harvey, et al. 2010).

4.4.2 PREOPERATIVE SERUM CONCENTRATIONS OF HCG, HCG β AND HCG-H IN NSGCT PATIENTS

In NSGCT patients, the preoperative serum concentration of hCG β was elevated in 40 of 51 (78%) and that of hCG in 37 of 51 patients (73%). hCG and hCG-h were simultaneously elevated in 28 (74%) and that of hCG β in 30 (79%) of 38 patients (Figure 11). Median %hCG-h was 84% (95% confidence interval (CI) 83–93%, range 38–130%), which shows that a major part of hCG is hyperglycosylated in NSGCT patients with elevated serum hCG

concentrations. The median ratio of hCG β to hCG was 0.08 (95% CI 0.08–0.17, range 0.03–0.51).

The preoperative concentration of AFP was determined in 39 of 51 NSGCT patients and it was elevated in 30 (77%) patients. Both hCG and AFP were increased in 22 (56%) of 39 patients, hCG alone in six (8%) and AFP alone in eight (21%) patients. Both hCG and AFP remained negative in three patients, but one of these had an elevated concentration of hCG β . Thus only two of 39 (5%) NSGCT patients were marker-negative.

hCG-h is underestimated by some hCG assays (Cole and Sutton 2004). Our results show that a major part of hCG is hyperglycosylated in testicular cancer patients. Therefore it is important that hCG assays used in the management of testicular cancer detect this form and hCG equally.

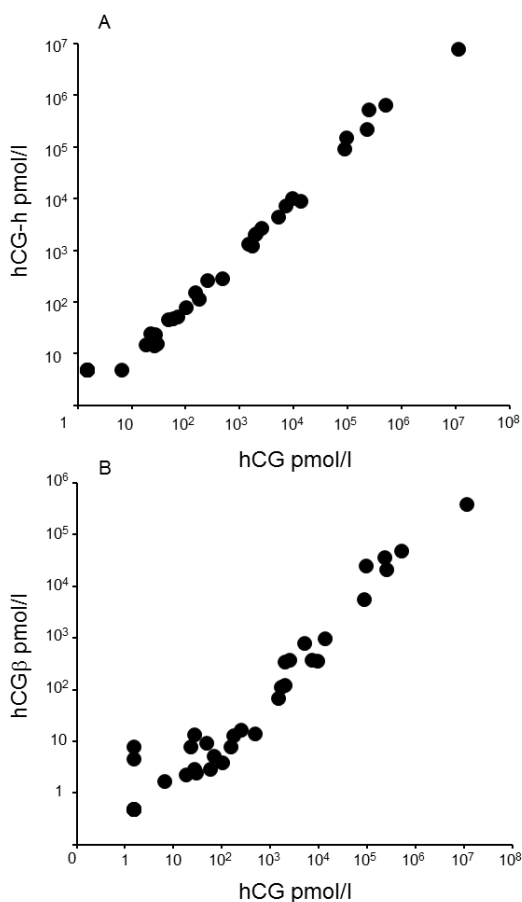


Figure 11. The preoperative serum concentrations of hCG and of hCG-h (A) and of hCG and hCG β (B) in NSGCT patients.

Surprisingly, %hCG-h exceeded 100% in about one fourth of the NSGCT cases, even though the AutoDelfia hCG assay should recognize hCG and hCG-h fairly equally. This is probably explained by the fact that the assay of hCG-h also measures hyperglycosylated hCG β , although with a lower recognition. In NSGCT the observed concentrations of hCG β , which has previously been found to be hyperglycosylated in testicular cancer (Valmu, et al. 2006), were up to half of those of hCG. A high concentration of hCG β -h will increase the results for hCG-h accordingly. Also, the glycosylation pattern of hCG is highly variable between individual patients (Valmu, Alfthan et al. 2006) and these variations may affect recognition in antibody-based assays. Presently, hCG detected by the B152 antibody is defined as hyperglycosylated, but B152-based assays may not equally detect all types of hyperglycosylated hCG. We used hCG-h derived from JEG-3 choriocarcinoma cells for calibration of our assay for hCG-h assuming that its recognition was similar to hCG-h derived from tumor tissue, but this may not always be the case. Thus, the calculated fraction of hCG-h cannot be considered exact. Despite the problems in calculating the ratio of hCG-h to hCG, it has recently been suggested to have significant clinical value in being able to distinguish between quiescent and active gestational trophoblastic disease in females. Cole and Muller even recommended that if hCG-h/hCG –ratio is <0.4, hCG concentration could be allowed to increase up to >3000 IU/l before initiating chemotherapy (Cole and Muller 2010). This suggestion has been criticized (Kohorn 2010; Seckl, et al. 2010).

4.3.3 DETECTION OF RELAPSE

Serum samples taken within a month before diagnosis of a relapse and before further treatment were available from four seminoma and 18 NSGCT patients. An isolated increase in hCG β was seen in one seminoma and five (28%) NSGCT patients. Both hCG and hCG β were increased in one of four seminoma patients and in six of 18 (33%) NSGCT patients. All hCG isoforms remained negative in two seminoma patients and seven (39%) NSGCT patients. Thus, separate determination of hCG β increased the number of marker-positive relapses from seven to thirteen (46%) and markedly enhanced the detection of relapses. AFP was available from nine of 18 NSGCT patients and the concentration was elevated in four of these.

For analysis of hCG-h during relapse, 20 serum samples (16 from NSGCT patients and 4 from seminoma patients) were available. Serum hCG and hCG-h were both elevated in five of 16 NSGCT and one of four seminoma patients. Median %hCG-h was 77% (95% CI 57–112%) in NSGCT patients and 60% in the seminoma patient. An increase in serum hCG and hCG-h detected the disease and relapses and followed the disease course equally. This suggests that separate measurement of hCG-h at diagnosis or during follow-up does not provide clinical information additional to that provided by hCG. However, since most of serum hCG was hyperglycosylated at relapse and since serum concentrations of hCG are often only moderately elevated at relapse, it is important that hCG assays used in the follow-up of testicular cancer recognize hCG-h with roughly equal sensitivity.

4.3.4 PROGNOSTIC VALUE

Five of 67 patients relapsed and two never achieved remission. Three NSGCT patients died of the disease. All relapses and deaths occurred within two years of diagnosis (Table 7).

The preoperative serum concentrations of hCG, hCG β and hCG-h strongly correlated with each other and with disease stage (Figure 12, Table 10), which is a known prognostic factor (Table 6). As expected, the hCG concentrations were higher in NSGCT than in seminoma patients, the highest concentrations being observed in a choriocarcinoma patient (95 400 pmol/l) and in a patient with a choriocarcinoma component in a mixed tumor (11 200 000 pmol/l) (Figure 13).

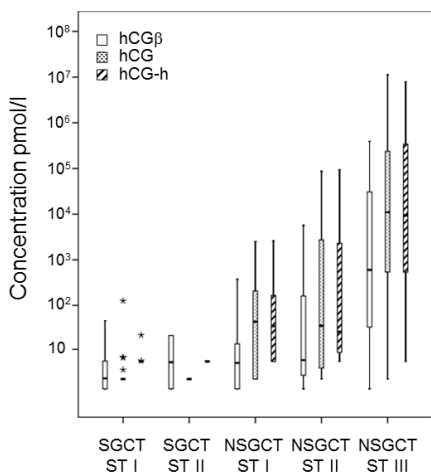


Figure 12. Different forms of hCG in relation to disease stage in preoperative serum samples. The line represents median value, the whiskers represent range, the boxes 25th and 75th percentile and the asterisks outliers. Please see Table 7. for number of patients in each group.

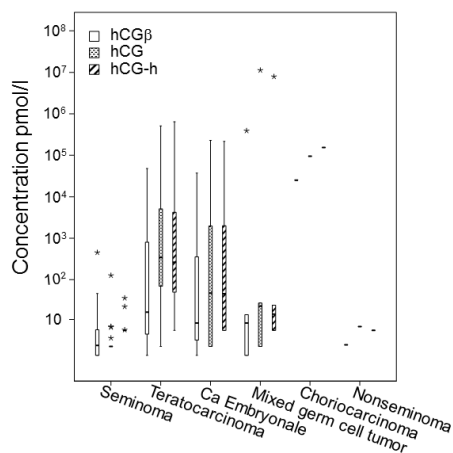


Figure 13. Different hCG forms in relation to histological tumor type. The line represents median value, the whiskers represent range, the boxes 25th and 75th percentile and the asterisks outliers. Please see Table 7 for number of patients in each group.

In univariate Cox regression analyses, preoperative hCG β concentration and extragonadal primary site predicted shorter progression free survival (Table 10), but only extragonadal primary site remained an independent prognostic factor (odds ratio 6.04 (95% CI 1.16–31.4) and $P = 0.032$) in multivariate analysis (odds ratio for hCG β 1.59 (0.98–2.57) and $P = 0.062$). In Kaplan Meier (Mantel-Cox) analyses, preoperative marker positivity was not a statistically significant predictor of progression free survival time. %hCG-h did not correlate with outcome variables or with progression free survival time.

In pregnancy, hCG-h is secreted by cytotrophoblasts (Kovalevskaya, Genbacev et al. 2002) that participate in decidual invasion at implantation. In choriocarcinoma, cytotrophoblasts participate in cancer invasion (Lala and Graham 1990; Strickland and Richards 1992). hCG-h has been shown to enhance growth and invasion of cytotrophoblasts in a choriocarcinoma cell line (Hamada, Nakabayashi et al. 2005) and Cole et al. further demonstrated that the monoclonal antibody B152 prevents growth of human choriocarcinoma cells transplanted

into athymic mice (Cole, et al. 2006b). hCG-h has been suggested to be essential for growth and metastasis formation of malignant cytotrophoblast cells of testicular germ cell tumors (Cole 2007) and the ratio of hCG-h to hCG has been suggested to be the discriminator of malignancy in female trophoblastic disease (Cole and Muller 2010). However, our findings do not demonstrate a prognostic role of %hCG-h in testicular cancer: The concentrations of hCG and hCG-h detected the disease and its progression equally. Preoperative serum concentrations of hCG and hCG-h correlated with known prognostic factors and progression-free survival in the same way and thus %hCG-h did not have prognostic value. This suggests that measurement of serum hCG-h does not provide additional prognostic information compared to that obtained by commonly used hCG assays. However, most patients received adjuvant therapy and relapses were rare, which limited the power to observe statistically significant differences in our study. Expression of hCG β is associated with adverse prognosis in many nontrophoblastic tumors and an elevated serum hCG β has been shown to be a strong prognostic factor in renal cell carcinoma, bladder, gastrointestinal, and head and neck cancers (Stenman, et al. 2004). In our study, a high preoperative hCG β concentration predicted shorter progression free survival but it was not an independent prognostic factor in multivariate analysis.

Table 10. Spearman correlation between preoperative concentrations of hCG, hCG β and hCG-h and known prognostic variables and Cox univariate regression analysis of the prognostic value of the serum concentrations and prognostic parameters on progression free survival.

	Spearman correlation				Cox univariate regression analysis	
	hCG	hCG β	hCG-h	%hCG-h	Odds ratio (95% CI)	P
Stage	<0.001	<0.001	<0.001	0.187	1.90 (0.69–5.22)	0.213
Nonseminomatous vs seminomatous tumor	<0.001	0.003	<0.001		3.95 (0.47–32.9)	0.204
Extragenital (n = 3) vs gonadal primary site	0.704	0.136	0.660	0.506	6.86 (1.32–35.6)	0.022
Tumor size	0.181	<0.001	0.054	0.311	1.01 (0.99–1.03)	0.628
Vascular invasion (12 of 38)	0.864	0.276	0.537	0.298	2.81 (0.17–46.4)	0.469
Tunica albuginea invasion (6 of 48)	0.086	0.212	0.078	0.238	4.27 (0.71–25.6)	0.112
hCG		<0.001	<0.001	0.035	1.34 (0.93–1.93)	0.113
hCG β	<0.001		<0.001	0.037	1.55 (1.01–2.40)	0.046
hCG-h	<0.001	<0.001		0.010	1.39 (0.94–2.04)	0.097

4.3.5 FOLLOW-UP SAMPLES AND FALSE-POSITIVE RESULTS

The concentrations of hCG, hCG β and hCG-h followed the disease course in the same way (Figure 14). We observed slightly elevated hCG β levels (2–8.5 pmol/l) without other signs of disease in 28 (1.1%) of 2540 follow-up samples. This is less than expected, since our upper reference and cut-off limit was based on 97.5 percentile and thus expected false positive rate was 2.5%. Furthermore, hCG β normalized in 22 of 23 samples, which were re-assayed using a buffer with higher concentration of HAMA blocking antibodies. This indicates the presence of assay interfering anti-reagent Abs or a decay of some other assay interfering substance during long-term storage at -20 °C. As discussed in paragraph 1.1.8, endogenous interferences in immunoassays may cause false-positive or false-negative results, which can be clinically misleading. They are very difficult to detect by the laboratory, and thus it is important that clinicians are aware of and know to suspect possible false laboratory values. In cases with spurious elevations, the next follow-up samples were normal, which, though, may not always be the case. It should be kept in mind that slightly elevated hCG β values do not need to be pathological and it is important not to initiate treatment on the basis of a single elevated value (Cole and Khanlian 2004). We observed false-positive hCG values above 15 pmol/l in one of 2063 samples. hCG values above 3 pmol/l (upper reference limit of healthy young males is 2.1 pmol/l) were observed in 61 of 2063 (3.0%) follow-up samples without other evidence of relapse. No false positive results for hCG-h were observed, but too few samples were available ($n = 81$) for meaningful analyzes of false-positive rate. Interestingly, hCG β (but not hCG) was elevated in three patients with non-germ cell testicular tumors (Leydig cell tumor, sarcoma and neuroendocrine tumor).

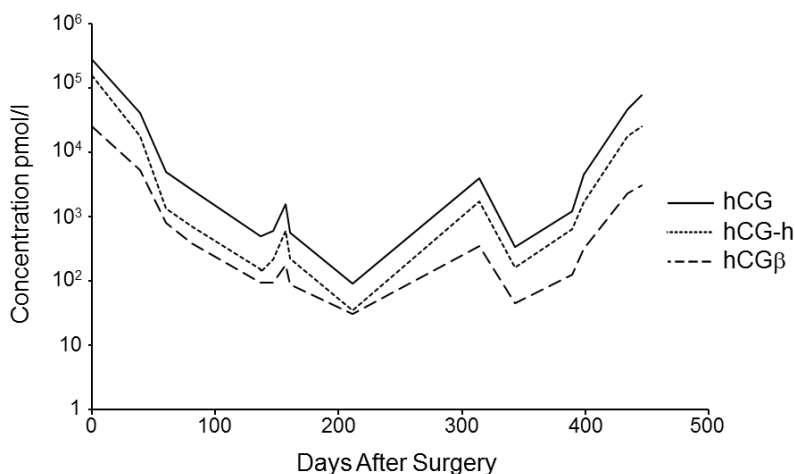


Figure 14. The concentrations of hCG, hCG-h and hCG β in a choriocarcinoma patient, whose disease was treatment resistant and progressed to death within two years of diagnosis.

4.3.6 HCG EXPRESSION IS NOT ALWAYS CAUSED BY TUMOR TISSUE

In testicular cancer, treatment of a suspected relapse is often initiated on the basis of marker elevation alone and slightly elevated hCG concentrations have led to inappropriate chemotherapy (Hoshi, et al. 2000). We describe a case with increasing hCG levels almost ten years after therapy causing suspicion of a relapse.

A 27-year-old man underwent left radical orchiectomy because of stage I seminoma. Preoperative serum concentrations of hCG and AFP were normal, as was serum testosterone (10.2 nmol/l). However, FSH was elevated, 27.5 IU/l, suggesting partially compensated hypogonadism. A year later examinations revealed a subnormal serum testosterone concentration, elevated FSH (50 IU/l) and LH (20 IU/l) and a moderate increase of hCG (10.8 pmol/l, 3.7 IU/l). Oral testosterone replacement therapy was administered, but the patient discontinued its use within a few weeks. During the next years, when he did not receive substitution therapy, the serum concentration of hCG remained slightly elevated. Intramuscular replacement therapy was re-instituted 3.5 years after surgery and serum concentrations of hCG, FSH and LH normalized. Ten years after surgery, the patient ceased the testosterone medication because of side-effects. Serum hCG increased to 13.2 pmol/l (4.5 IU/l), which raised a suspicion of tumor relapse. At this point, serum concentration of testosterone was 2.9 nmol/l, that of FSH 61.5 IU/l and of LH 31.4 IU/l indicating hypogonadism. Intramuscular testosterone replacement therapy was re-instituted and hCG, FSH and LH concentrations decreased rapidly (Figure 15). Apart from the increasing serum hCG concentration, there were no other signs of relapse during follow-up.

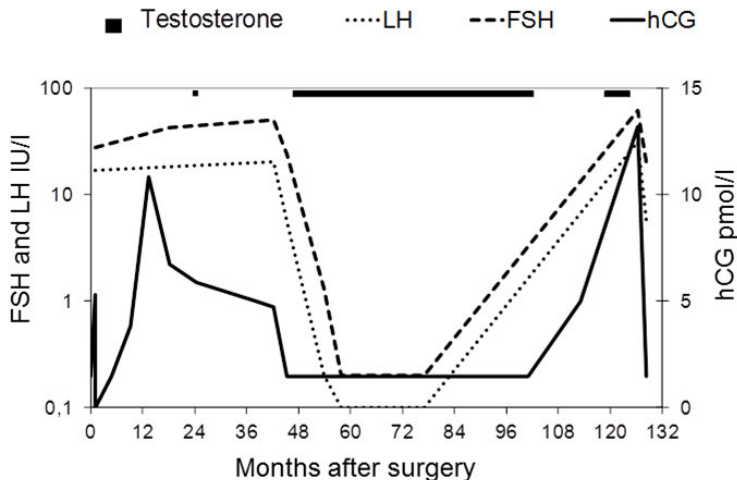


Figure 15. Serum concentrations of hCG, LH and FSH in relation to anamnestic use of testosterone replacement therapy in a hypogonadal testicular cancer patient.

Hypogonadism induces pituitary secretion of gonadotropins, including hCG, and hormone replacement therapy in postmenopausal women causes suppression of the hCG concentrations (Stenman, et al. 1987). In nonpregnant women and in men the hCG concentrations are about 3-10% of those of LH (Stenman, et al. 2004). Concentrations of hCG up to 38 pmol/l (13 IU/l) have been observed in postmenopausal women (Cole 2005). Hypogonadism is a common finding in testicular cancer patients due to the disease itself and its treatment, especially cytotoxic chemotherapy. Elevation of hCG immunoreactivity after treatment of testicular cancer has been described previously (Catalona, et al. 1979; Germa, et al. 1987; Hoshi, et al. 2000), but the elevation was ascribed to cross reaction of LH in the hCG assay rather than to hCG itself. Since we determined serum hCG with an assay, which has negligible crossreactivity with LH (Alfthan, et al. 1992a), this could be ruled out, and hCG elevation could be attributed to physiological hCG secretion by the pituitary.

Not surprisingly, moderately elevated hCG concentrations have led to inappropriate chemotherapy in testicular cancer patients (Hoshi, et al. 2000) and in women because of suspected trophoblastic cancers (Cole and Khanlian 2004; Cole, et al. 1999a). Understanding of the behavior of tumor markers and the possibility of misleading values in various clinical conditions or due to assay interfering factors, like heterophilic antibodies, is important, since unnecessary and potentially harmful chemotherapy must be avoided. A moderately increased hCG concentration in testicular cancer patients is not always caused by a relapse.

5 SUMMARY AND CONCLUSIONS

This study addressed the clinical utility of hCG, hCG β and hCG-h determinations in the management of testicular cancer. We also evaluated the stability of hCG in urine and serum and developed an assay specific for hCG-h.

We showed that hCG remains stable in serum for years when stored at -20 °C (II). hCG and hCG β are stable at +4 °C for at least one week (IV). In urine, hCG immunoreactivity is often lost during storage at -20 °C and the loss is highly variable. Before assay of hCG, urine should not be stored at -20 °C but either at +4 °C for a few weeks or at -80 °C for longer times. Urea probably contributes to the degradation of hCG, but other mechanisms most likely participate in the process. Addition of glycerol significantly reduces the urea-induced loss of hCG (I).

When developing an assay specific for hCG-h, we found that complement causes interference in the determination of hCG-h in serum with assays using antibody B152, which is of the IgG2a isotype. This can be eliminated by using EDTA plasma rather than serum or by inactivating complement in serum with EDTA before the assay (III).

hCG β in serum is a sensitive marker for all types of testicular cancer and in seminomas it is more useful than intact hCG. Separate determination of hCG β provides clinically valuable information compared to the commonly used assays for total hCG, since in approximately one third of marker-positive seminomas and relapses, serum concentrations would have been within reference limits with an assay measuring hCG and hCG β together (II).

Most of the hCG in patients with NSGCT is hyperglycosylated, but determination of hCG-h does not appear to provide clinical information additional to that obtained by separate determinations of hCG and hCG β . However, hCG assays used for diagnosis and monitoring of testicular cancer should recognize hCG-h (IV).

A moderate increase in serum hCG can be a physiological reaction to hypogonadism, which is common in testicular cancer survivors. It is important to recognize this condition in order to avoid useless and potentially harmful therapy (V).

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Helsinki, August 2012

A handwritten signature in cursive script, reading "Anna Lempiäinen". The signature is written in black ink on a white background.

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8 ORIGINAL PUBLICATIONS