



Katsiaryna Holl

Maternal Risk Factors and Testicular Cancer in the Offspring

A nested case-control study

RESEARCH

Katsiaryna Holl

**Maternal Risk Factors and
Testicular Cancer in the Offspring**

A nested case-control study

ACADEMIC DISSERTATION

*To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public examination in the Auditorium of
Tampere School of Public Health, Medisiinarinkatu 3,
Tampere, on March 28th, 2009, at 14.00.*

National Institute for Health and Welfare, Helsinki, Finland
and
Tampere School of Public Health, University of Tampere, Finland

Helsinki 2009

Acta Electronica Universitatis Tampereensis 820
ISBN 978-951-44-7647-1 (pdf)
ISSN 1456-954X
<http://acta.uta.fi>

© National Institute for Health and Welfare

ISBN 978-952-245-032-6 (print)
ISSN 1798-0054 (print)
ISBN 978-952-245-033-3 (PDF)
ISSN 1798-0062 (PDF)

Yliopistopaino
Helsinki 2009

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To my beloved Mum:

Ms. Hanna Sauchuk

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them. William Lawrence Bragg (1890-1971)

Katsiaryna Holl, Maternal Risk Factors and Testicular Cancer in the Offspring

– A nested case-control study

Publications of the National Institute for Health and Welfare – RESEARCH, 4| 2009, 109 pages

ISBN 978-952-245-032-6 (print), 978-952-245-033-3 (net version)

ISSN 1798-0054, 1798-0062 (net version)

<http://www.thl.fi>

SUMMARY

Testicular cancer (TC) is the most frequently diagnosed cancer in male adults between ages 20 and 40 years and its incidence has dramatically increased over the last 40 years. There are considerable geographic, ethnic and temporal variations in the global incidence of TC. The histological spectrum of TC and the typical incidence peak at very young age suggest that risk factors for the TC operate early in life, possibly already *in utero*. Therefore, we assumed that exposure to herpesvirus infections (Epstein-Barr virus, EBV, and cytomegalovirus, CMV), maternal smoking or to a specific pattern of steroid hormones (high oestrogens and low androgens) *in utero* might contribute to TC development. To ensure the quality and validity of studies investigating the role of endogenous hormones as measured in biological samples stored for many years (up to 22 years) at -20°C , validation studies investigating the effect of storage time should precede the analyses of precious case-control samples.

A case-control study was nested within three Nordic maternity cohorts from Finland, Sweden and Iceland. All three participating cohorts collected serum samples from pregnant women during the first or early second trimester. Over-generation linkages of the maternity cohorts' data with that from nation-wide population census and cancer registries enabled identification of women with offspring diagnosed with histologically verified TC. Eligible TC cases whose mothers had donated a serum sample to one of the maternity cohorts were selected for the study. Control mothers had a male offspring free of TC at the time when the TC case was diagnosed and were matched to the index mothers by date of birth of the son. To assess the effect of storage time on hormonal measurements, a validation study was conducted using samples selected randomly from those donated every other year since the launching of the Finnish Maternity Cohort. Cotinine levels, Immunoglobulin (Ig)M and IgG antibodies to EBV and CMV and hormones levels were measured in serum samples of index and control mothers by the respective laboratory assays.

Deterioration of the serum samples with long-term storage can result in attenuation of the biomarker-disease risk estimates and/or misclassification of exposure among the cases and the controls. Although our results from the validation study did not show a consistent pattern of hormone level variation with storage time, matching for calendar time at blood donation is needed to control for the effect of storage time on measured hormone levels.

We conducted the first study on a possible association of congenital infection with EBV and CMV and risk of TC in the offspring. We found elevated EBV IgG antibody levels more frequently in the mothers of nonseminoma TC cases than in the control mothers. However, further studies are needed to rule out the possibility that EBV reactivation is a marker of a pathogenetic process rather than the causal factor. Decreased risk of TC diagnosed during the prepubertal period in the offspring of CMV IgG seropositive mothers was revealed but lack of specific IgM antibodies ruled out the possibility of congenital infection in CMV seronegatives.

Maternal smoking during pregnancy does not appear to be associated with risk of TC as shown by our direct approach of measuring maternal serum cotinine levels and the meta-analyses of studies with questionnaire data on maternal smoking. Therefore, most probably maternal smoking is not a risk factor for TC. However, further research is needed, especially to understand the role of alcohol.

We provide the first direct evidence that the interplay of maternal steroid hormones (DHEAS, androstenedione, and oestradiol) in early pregnancy is important in the aetiology of TC. Offspring of mothers with the highest DHEAS levels had a decreased risk of TC. Conversely, offspring of mothers with the highest androstenedione levels had an increased risk of TC. High maternal total oestradiol level showed a notably high point estimate for the increased risk of TC. The precise mechanisms underlying these associations remain, however, to be elucidated in future studies. A better knowledge of the impact of populations' lifestyles and environments on endogenous hormone metabolism during pregnancy and its role in the development of TC would shed further light on our understanding of the aetiology of this disease.

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ABBREVIATIONS

AFP	alpha-fetoprotein
ASR	age-standardised incidence rate
BLT	bench-lag time
BMI	body mass index
CCS	case-control study
CI	confidence interval
CIS	carcinoma <i>in situ</i>
CMV	cytomegalovirus
CV	coefficient of variation
DES	diethylstilbestrol
DHEAS	dehydroepiandrosterone sulphate
DMF	dimethylformamide
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EBNA	Epstein-Barr virus-related nuclear antigen
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FMC	Finnish Maternity Cohort
GD	gestational day
hCG	human chorionic gonadotropin
HLA	human leukocyte antigen
IARC	International Agency for Research on Cancer
ICD-O/3	International Classification of Diseases for Oncology, 3rd Edition
IEMA	immunoenzymetric assay
IGF-1	insulin-like growth factor-1
IGFBP-1	insulin-like growth factor binding protein-1
IGFBP-3	insulin-like growth factor binding protein-3
IgG	immunoglobulin G
IgM	immunoglobulin M
IRMA	immunoradiometric assay
LMP	latent membrane protein

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LOQ	limit of quantitation
MIS	Müllerian Inhibiting Substance
NCCS	nested case-control study
np	non-phosphorylated
OR	odds ratio
PAF	population-attributable fraction
PID	personal identifier
pGH	placental growth hormone
RNA	ribonucleic acid
SES	socioeconomic status
SHBG	sex hormone binding globulin
SNP	single-nucleotide polymorphism
TC	testicular cancer
TGS	testicular dysgenesis syndrome
TNM	Classification of Malignant Tumours, T – tumour, N – lymph nodes, M – metastasis
VCA	viral capsid antigen

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

- I. **Holl K**, Lundin E, Kaasila M, Grankvist K, Afanasyeva Y, Hallmans G, Lehtinen M, Pukkala E, Surcel HM, Toniolo P, Zeleniuch-Jacquotte A, Koskela P and Lukanova A (2008): Effect of long-term storage on hormone measurements in samples from pregnant women: the experience of the Finnish Maternity Cohort. *Acta Oncol* 47:406-412.

- II. **Holl K**, Surcel HM, Koskela P, Dillner J, Hallmans G, Wadell G, Kaasila M, Olafsdottir GH, Ögmundsdottir HM, Pukkala E, Stattin P and Lehtinen M (2008): Maternal Epstein-Barr virus and cytomegalovirus infections and risk of testicular cancer in the offspring: a nested case-control study. *APMIS* 116:816-822.

- III. Tuomisto J, **Holl K**, Rantakokko P, Koskela P, Hallmans G, Wadell G, Stattin P, Dillner J, Ögmundsdottir HM, Vartiainen T, Lehtinen M and Pukkala E (2009): Maternal smoking during pregnancy and testicular cancer in the offspring: a nested case-control study. *Eur J Cancer* (in press).

- IV. **Holl K**, Lundin E, Surcel HM, Grankvist K, Koskela P, Dillner J, Hallmans G, Wadell G, Ögmundsdottir HM, Pukkala E, Lehtinen M, Stattin P and Lukanova A (2009): Endogenous steroid hormone levels in early pregnancy and risk of testicular cancer in the offspring: A nested case-control study in the Nordic countries. *Int J Cancer* (in press).

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1. INTRODUCTION

Testicular cancer (TC) is undoubtedly a unique and complex multifactorial disease. Its incidence is increasing markedly worldwide, albeit with geographic and ethnic differences, making it an important source of morbidity for young males. The increasing incidence of TC parallels the great changes in lifestyle and environment around the world. This suggests that TC may be added to the list of so-called “civilization diseases”.

Among the particular biological features of TC is the wide heterogeneity of its various histological types, mimicking virtually any tissue type in the body, including caricatural reflection of early embryo in a teratoma. TC is believed to be result of disturbances in both gonadal development and germ cell differentiation. Therefore, foetal development *in utero* is a critical period when maternal risk factors can contribute to the transformation of the germ cells into (pre)-carcinoma *in situ* (CIS) cells. Remarkably little, however, is known about the risk factors which might contribute to the development of TC.

The attempt to identify the risk factors of TC in the present study stems from the unique opportunities of over-generation linkage and direct exposure assessment in prospectively collected maternal samples.

2. REVIEW OF THE LITERATURE

2.1. Longitudinal approach to epidemiological studies using Nordic population-based registries and biobanks

For the identification of risk factors and causality inference as well as for the determination of population-attributable fraction (PAF) beyond increases/decreases in cancer incidence, longitudinal studies nested in cohorts of stored biological samples are especially suitable. In the Nordic countries, there exists a series of established biobanks with decades of follow-up, and comprehensive, population-based health registries linkable by unique personal identifiers (PID). This setting enables studies with adequate statistical power even for rare diseases and exposures (Pukkala et al. 2007).

2.1.1. Population-based cancer registration

The nationwide Nordic cancer registries have been in operation since the 1950s and have virtually complete country-wide coverage of cancer occurrence. Approximately 950,000 cancer cases diagnosed from 1953 to 2004, 1.6 million cases diagnosed from 1958 to 2004, and 37,000 cancer cases diagnosed from 1955 to 2005 are registered respectively in the Finnish Cancer Registry, the Swedish Cancer Registry, and the Icelandic Cancer Registry. Close to 80,000 new cancer cases are registered annually in these 3 countries: approximately 26,000 in Finland, 51,000 in Sweden, and 1270 in Iceland. The Finnish Cancer Registry since 2007, the Swedish Cancer Registry since 2005, and the Icelandic Cancer Registry since 2003 have followed the International Classification of Diseases for Oncology, third edition (ICD-O/3) for coding the primary site of cancer.

Notification of all diagnosed (mostly >95%) histologically confirmed cancer cases must be sent by physicians, hospitals or laboratories to the cancer registry either in paper or electronic format. Information received is immediately stored in the electronic database of the registry. Visual and automatic checking procedures for any erroneous code combinations or illogical order of dates both at data entry and coding are applied. If information on a cancer case is incomplete or controversial, requests

for further information are sent to the physicians, hospitals or laboratories (Teppo et al. 1994). Furthermore, cancer registry files are regularly linked with population-based registers where the correctness of PID is checked, and the complete name, vital status, possible date of emigration as well as the official place of residence at the time of diagnosis are obtained. Cancer registry files are also annually linked to the causes of death registries (Bray and Parkin 2008, Parkin and Bray 2009).

For each case reported to the cancer registry, information on name, PID, residence, primary site and date of diagnosis, basis of diagnosis, stage, malignancy, histology, treatment and follow-up time is stored. In addition, information about the identity of the notifying hospitals or laboratories, histological specimen numbers, tumour grade, TNM Classification of Malignant Tumours (TNM), site of metastases, characteristics of the treatment, or reason for not being treated are available and can be utilized for various purposes. Each cancer considered to be an independent new primary entity is registered and coded separately.

The national cancer registries are highly regarded both by the scientific and health care communities in the Nordic countries. Furthermore, the output of the registries is widely used in research, administration, or health education on all levels.

2.1.2. Population-based biobanks

Biobanks may be defined as “a system, which will store one or many types of biological specimens for later analysis from single or multiple studies under conditions which permit efficient retrieval and optimum stability of the samples” (Winn et al. 1990). Biobanks have a fundamental role in epidemiological studies because they function as the repository of biological materials from which molecular-based exposure assessment can be made.

There are 17 biobanks in the Nordic countries (Finland, Sweden, Norway, and Iceland, Figure 2.1). Since 1966 altogether two million donors have donated more than four million biological samples, stored at $-10\text{ }^{\circ}\text{C}$ to $-135\text{ }^{\circ}\text{C}$ (Pukkala et al. 2007). In the past ten years, informed consent has been collected from all persons

donating biological samples, clearly indicating to the donors that the material will be used for research purposes. For older samples either pertinent legislation or opting-out announcement have formalized their use for research purposes. In addition to stored biological samples, data on date of sample collection, PID, demographic, reproductive or medical history, and lifestyle factors may be available in the biobanks' data files.

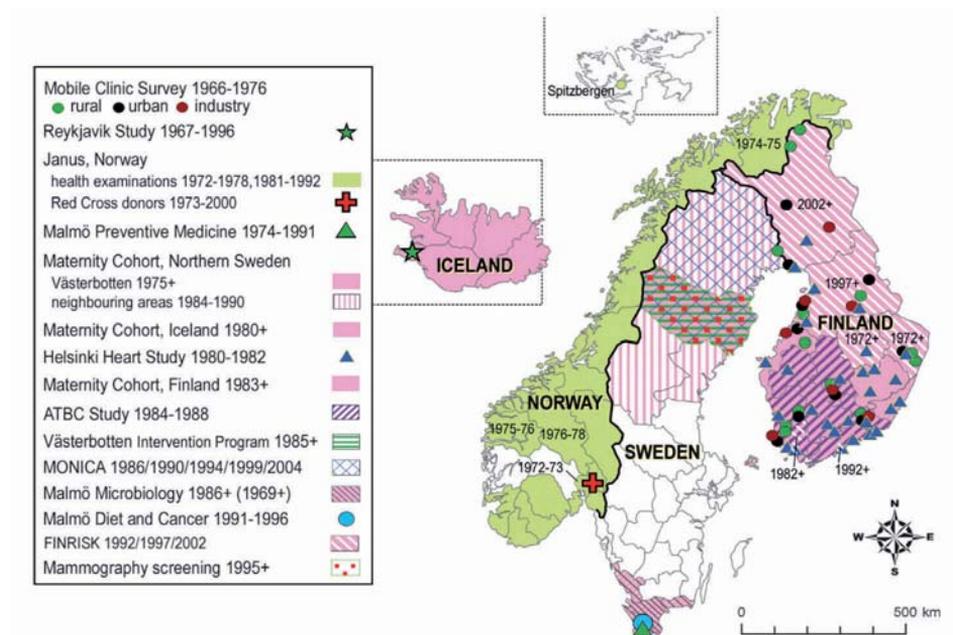


Figure 2.1. Map of the Nordic countries indicating the coverage areas of the serum banks (with permission of Pukkala et al. 2007).

All Nordic biobanks are independent entities that make their own decisions, but since 1994 have committed to a wide range of collaborative initiatives. To facilitate joint studies similar policies for quality assurance, logistics, study designs as well as for terms of collaboration have been adopted. Furthermore, research projects using these biobanks need appropriate permission from the boards of the biobanks.

2.1.3. Over-generation linkage

Every resident of the Nordic countries has a unique PID that is used in all official registries in these countries. The Nordic health data infrastructure and PID are utilised to allow electronic linkages to obtain data on numerous registry-based health

indicators. Over-generation linkage of the cancer registry, population census registry, and the biobank data enable identification of parents with offspring who have been diagnosed with malignant disease. Additionally, hospital discharge registries, perinatal outcome registries, cause of death registries, registries of infectious diseases and other disease specific registries can be used for over-generation linkage due to the availability of PID.

Permission for linkage information between the biobanks, population-census registries and cancer registries can be obtained from the national data protection authorities, national or local ethical committees. Identification and access of the individuals who have donated samples to the Nordic biobanks is not allowed in the Nordic countries, but according to the informed consent the samples can be used for medical research.

2.1.4. Case-control study nested with population-based registries and biobanks

Nested case-control design is commonly used in molecular epidemiological studies within a cohort. This approach is particularly useful when complex and expensive measurements are conducted. In essence, initially blood samples for all members of the cohort are collected at the time of entry and frozen. The cohort is defined and followed up until a sufficient number of diseased individuals (cases) are identified. Exposure assessment and other pertinent information is thereafter obtained from biobank and registry files, but only for cases and a sub-sample of disease-free individuals (controls), but not for all members of the cohort (Santos Silva 1999, pp.182–183).

In a nested case-control design, for each cancer case of interest, typically one to four matching controls of the same gender are randomly selected following the incidence density sampling principle from the population-based registry (Doll and Peto 1981). However, controls have to be alive at the time of case's diagnosis, to be free of the disease of interest and to be at risk of being cases at the time of the case's diagnosis, to have donated a sample and to be born around the same time as the case. The relative risk estimated as the odds ratio (OR) will then be based on the incidence rate

ratio in the source population between those exposed and not exposed. This holds true regardless of a disease rarity assumption, provided that the control sampling is independent of the exposure assessment given the matching criteria (Kass and Gold 2005, pp. 327–329).

The goal of matching is to balance the ratio of cases to controls within matched sets, and to make distributions of the potentially confounding variables among controls more like those of cases. In case of heterogeneous biobanks, matching for sub-cohort is essential. A difference of one to two months in sampling date is sometimes necessary due to seasonal variation of the biomarkers tested in biological samples (Agborsangaya et al. 2009, to be published). As freezing and thawing can affect a number of biomarkers it is also strongly recommended to match by the number of freeze-thaw cycles of a given sample. Matching may increase random error, e.g., matching on a non-confounder or variables intermediate in the causal pathway between exposure and disease reduces the point estimates.

There are certain practices in control selection that are bound to specific features of the sample materials unique for the biobanks. First, because most biobank data bases do not include variables indicating how many times a sample has been used as a control and how much serum is left, it is often necessary to pick up one or two extra control candidates that will be used if the actual controls are missing or do not contain enough material. In some studies based on biobanks persons who have been diagnosed with other cancers have not been accepted as controls, although formally they would be eligible at least until the date of cancer diagnosis of the respective case. However, this causes only a negligible theoretical error, because the pool of eligible controls for each case normally includes hundreds of subjects (Pukkala et al. 2007).

2.1.5. Quality assurance of biobank-based studies

A potential weakness of studies based on historical biobank samples is the stability and quality of the old samples. The oldest samples in the Nordic biobanks are more than 30 years old and many are stored at the relatively high temperature of -25°C . Therefore, an appropriate set of validation studies for monitoring deterioration of

samples over time including testing for desiccation, integrity of the container, and stability of the analytes over long storage periods is needed. Although there is some scientific understanding of stability for many biochemical analytes, data is limited for newer molecular-level assays (Gislefoss et al. 2008). Furthermore, enzymes and vitamins may be less stable under storage conditions compared to antibodies, organic and fatty acids, carbohydrates and trace metals.

A better understanding of intra- and inter-individual variability and the natural history of newer biomarker is needed. In addition, greater interchange between laboratory specialists, biobank keepers and epidemiologists is also needed. The numbers of freeze-thaw cycles need to be recorded. The effect of freeze-thaw cycles could in some cases be prevented by sufficient aliquoting or other suitable methods.

The quality of the samples stored in biobanks always needs to be assured by the following procedures: (a) serum is obtained by a standardized procedure and all aliquots are prepared in the same way, (b) samples are aliquoted into appropriate airtight tubes to prevent freeze-drying effect with long-term storage, (c) storage temperature is monitored regularly, (d) the time between sample collection and freezing for long-term storage is kept as short as possible, (e) laboratory assays must ensure high specificity and sensitivity. As a part of the quality assurance of the studies based on biobank material, it is recommended to conduct pilot studies to validate proposed analyte measurements (hormones, antibodies, trace elements) for each particular research purpose.

2.2. Descriptive epidemiology of TC

The worldwide incidence of TC has dramatically increased over the last 40 years (Huyghe et al. 2003, Huyghe et al. 2007). This includes the Nordic countries (Figure 2.2). Epidemiological studies suggest that the risk of TC is strongly associated with the year of birth and that the younger generations have an increased risk compared to previous ones (Bergström et al. 1996, McKierman et al. 1999, Richiardi et al. 2004).

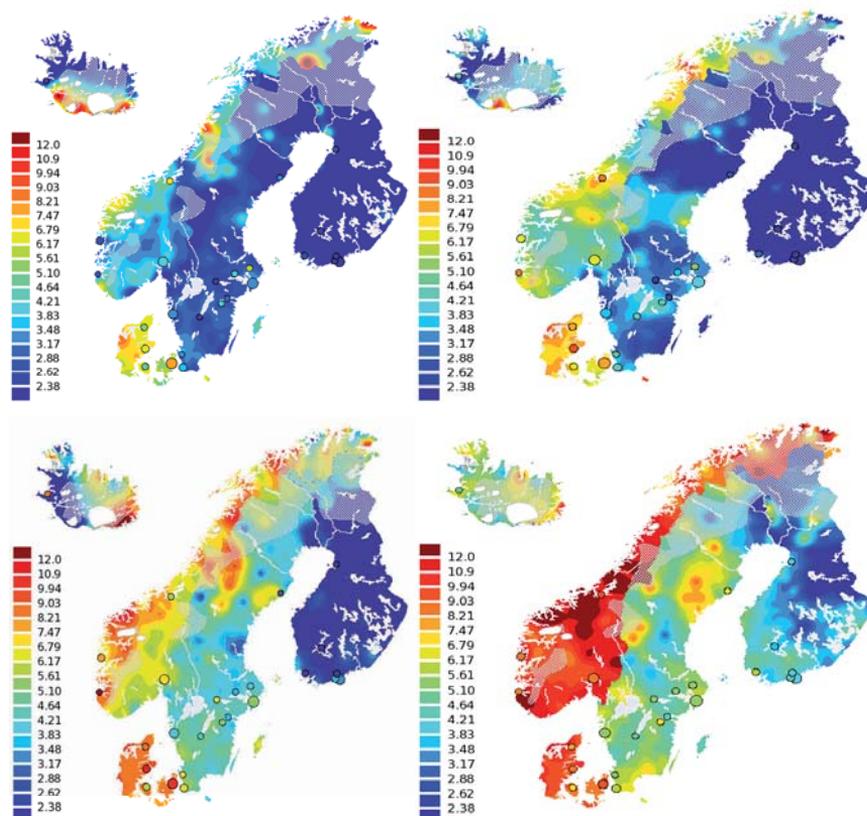


Figure 2.2. Age-standardised incidence rate (ASR) of TC in Nordic countries for years 1970-2006 (Pukkala, 2007, <http://astra.cancer.fi/cancermaps/Nordic>).

There are considerable geographic and ethnic variations in the global incidence of TC. The disease mainly affects Western populations, with average rates in developed areas of the world six times higher than those in developing areas (Bray et al. 2006a). TC incidence exhibits extremely low rates in black and other non-white races in comparison to white population (Shah et al. 2007). This can be seen in the world age-standardised incidence rate (ASR) data from the 1998 and 2002 for most developed

countries (Figure 2.3). ASR of TC ranges from about 1/100,000 in Asian and African/African-American populations to approximately 9/100,000 in Denmark and Norway (Curado et al. 2007).

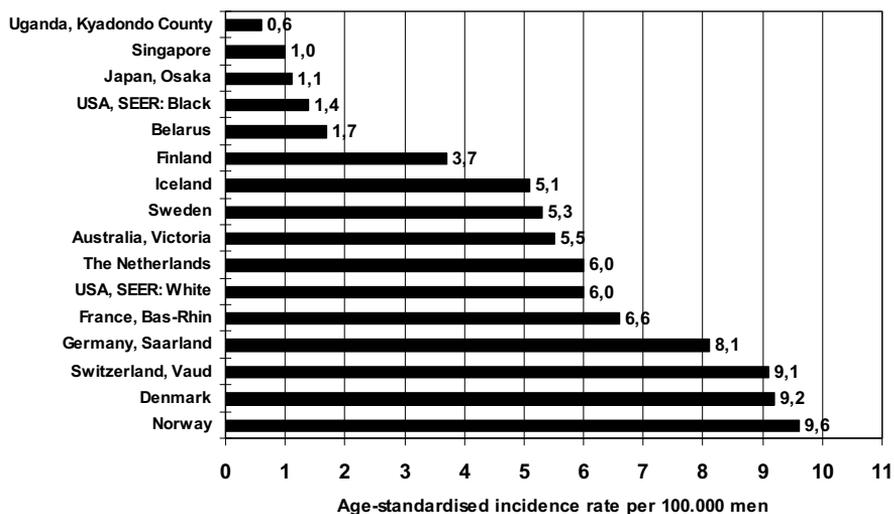


Figure 2.3. Age-standardised incidence rate of TC for years 1998-2002.

In most countries TC is rare, representing about 0.5-1.5% of all malignancies in the males. About 500,000 new cases were diagnosed worldwide in 2002 (GLOBOCAN 2002), with the vast majority being germ cell tumours and occurring in young adult males. In populations considered to have a high frequency of the disease, the life time risk (to the age of 75 years of age) is approximately 0.5–1% (Bray et al. 2006c). Age-specific incidence rate of TC in 1997-2006 in Finland, Sweden and Iceland shows an incidence peak between the ages of 20 and 40 years (Figure 2.4). While the majority of TC occurs between 20 and 40 years of age and accounts for 20% of all male cancers in these ages, the peak incidence for teratoma and embryonal carcinoma is at ages 20-35 years, and for seminoma is at ages 25-45 years (Figure 2.4). The incidences of the various types of TC decline rapidly after the peak age.

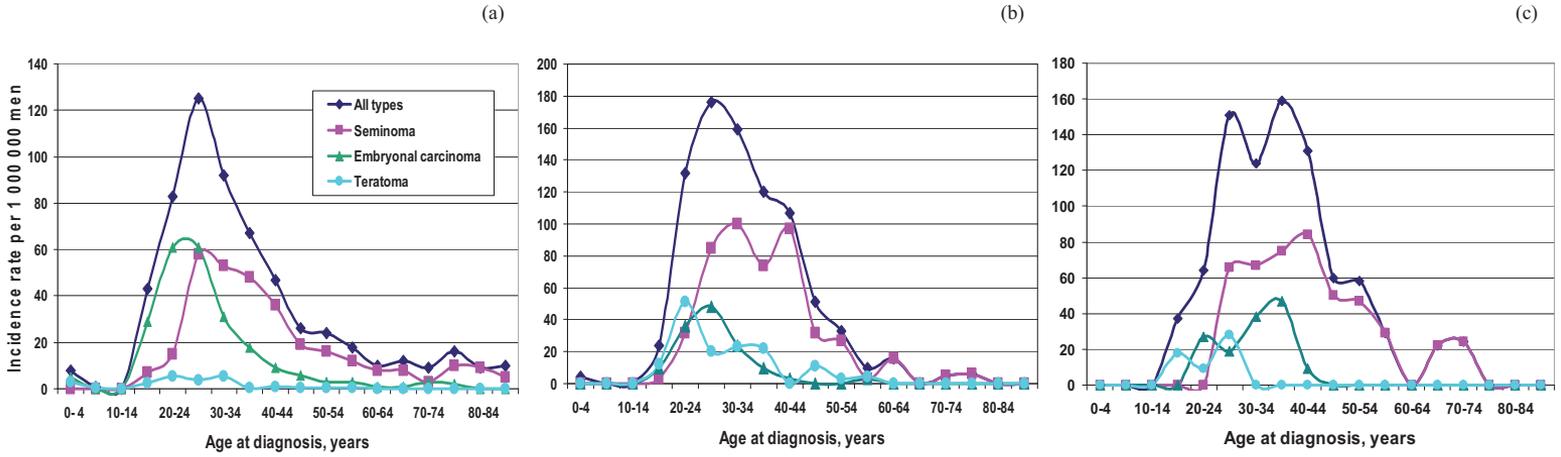


Figure 2.4. Age-specific incidence rate of major TC types in Finland (a), Sweden (b) and Iceland (c) for years 1997-2006.

2.3. Histological occurrence of TC and its subtypes

In general, TC has been divided into three biologically distinct categories of prepubertal (infantile and childhood) TC, postpubertal (adult) TC, and spermatocytic seminoma. There is some evidence that the origin and biology of prepubertal and postpubertal TC are distinct from each other. Approximately 75% of prepubertal TCs are of germ cell origin, as compared to more than 90% of those TC cases found in the postpubertal period (Cushing et al. 2006). Non-germ cell testicular neoplasms comprise one third of TC cases in children and less than 10% in adults. In children, yolk sac tumour and teratoma are the most frequent tumours, while seminoma and embryonal carcinoma are rare. Testicular neoplasms in adults often consist of seminoma, embryonal carcinoma, or mixed testicular germ cell tumours. Furthermore, there is a strong association between postpubertal TC and CIS. No such association has been observed in any prepubertal TC (Bahrami et al. 2007).

Genetic studies have shown that postpubertal TCs are often aneuploid with consistent chromosomal abnormality, i.e., gain of short arm of chromosome 12, usually in the form of an isochromosome, i(12p) (Reuter 2005). By contrast tumours arising in prepubertal gonads tend to be diploid with structural abnormalities of chromosomes 1, 3, 6 and without the 12p amplification (van Echten et al. 2002).

For clinical purposes TCs have traditionally been separated into seminomatous and nonseminomatous tumours. Seminomatous tumours grow more slowly and have better prognosis than nonseminomatous tumours.

2.3.1. Seminomatous testicular tumours

Classic seminoma and spermatocytic seminoma are the two types of seminomatous tumours (Table 2.1, p. 26).

Seminoma

Seminoma is the most common testicular germ cell neoplasm, accounting for approximately 50% of these tumours. It is also a recognizable component in a large proportion of mixed germ cell tumours. In seminomas, the malignant cells have many similarities with primordial germ cells, and the tumour may simply reflect the malignant expansion of a clone of primordial germ cells (Ulbright 2004). The peak incidence is between 25 and 40 years of age. Before puberty, seminoma is extremely rare.

Spermatocytic seminoma

Spermatocytic seminoma is an uncommon germ cell neoplasm that accounts for 1% to 2% of all testicular tumours. Classic seminoma and spermatocytic seminoma are believed to have a different pathogenesis and a separate cell of origin. The cellular origins of spermatocytic seminoma appear to be more differentiated than those of seminoma and capable of spermatogenesis (Stoop et al. 2001). Spermatocytic seminoma typically occurs in men older than 50 years of age, and has only occasionally been seen in younger patients (Chung et al. 2004).

2.3.2. Nonseminomatous testicular tumours

These types of tumours are composed of embryonal carcinoma, yolk sac tumour, immature or mature teratoma, choriocarcinoma, and other rare testicular tumours (Table 2.1, p. 26). They are often seen in various combinations, referred to as mixed testicular germ cell tumours, which may also include seminoma.

Embryonal carcinoma

Pure embryonal carcinoma, even if relatively rare, is the second most common single-cell-type testicular germ cell tumour after seminoma. Embryonal carcinoma as a component, however, is present in more than 80% of mixed germ cell tumours (Cushing et al. 2006). Pure embryonal carcinoma occurs most frequently between 25 and 35 years of age, which is ten years earlier than the peak age for seminoma. Embryonal carcinoma is uncommon in young children and rare after the age of 50 years.

Yolk sac tumour

Yolk sac tumour is a germ cell neoplasm that differentiates in the direction of the embryonic yolk sac and extraembryonic mesenchyme. It is the most common testicular neoplasm in the juvenile population, accounting for approximately half of prepubertal testicular tumours (Ross et al. 2002). In children, the median age at presentation is 16 month (range, newborn to 11 years). Childhood yolk sac tumour does not appear to be associated with cryptorchidism (Ro et al. 2000) or CIS (Bahrami et al. 2007). Yolk sac tumour in children is almost always seen in the pure form. By contrast, in adults it is seen only admixed with other neoplastic germ cell elements and the pure form is extremely rare. Foci of yolk sac tumours are found in 40% of mixed germ cell tumours in adults.

Choriocarcinoma

Choriocarcinoma is extremely rare and accounts for less than 1% of testicular tumours (Ro et al. 2000, Ulbright 2004). However, as a component, choriocarcinoma is seen in approximately 8% of mixed germ cell tumours. Choriocarcinoma typically occurs in the second or third decades of life (Bahrami et al. 2007).

Teratoma

Teratomas are tumours composed of variable types of tissue representing one or more of the germinal layers of endoderm, mesoderm, and ectoderm. A tumour composed of only one of the three germ cell layers is defined as monodermal teratoma. Mature teratomas are composed of entirely mature and well-differentiated components. By contrast, immature teratomas contain embryonic or foetal-like tissues, which are typically accompanied by mature elements (Bahrami et al. 2007).

Teratoma occurs in both children and adults, but the biological behaviour of the tumour is substantially different between the two groups. In adults, teratoma as a pure neoplasm is uncommon (only 2%–3% of all germ cell tumours); however, as a component, it is present in almost 50% of mixed germ cell tumours. By contrast, in

children almost all teratomas are pure neoplasms. Teratoma is the second most common prepubertal testicular neoplasm after yolk sac tumour, accounting for 14% of these tumours. Most prepubertal teratomas are exclusively composed of mature tissues; nevertheless, almost all of them behave in a benign manner regardless of the maturity of their components. On the other hand, most postpubertal teratomas are immature, although completely mature teratomas in adults are at risk of metastases. In other words, the immaturity of teratomous components is not an indicator of poor biological behaviour in the primary tumour; rather, the age of the patients is important. Most prepubertal teratomas are seen in children younger than 4 years with a mean age of 20 months. Postpubertal teratomas occur after the age of 15 years (Cushing et al. 2006).

Mixed germ cell tumours

Mixed germ cell tumours of the testis are the second most common testicular germ cell tumours in adults (following seminoma), comprising 30% to 50% of cases (Cushing et al. 2006). Tumours that contain seminoma occur at a later age than tumours without a seminomatous component. Mixed germ cell tumours are rarely seen in prepubertal gonads.

Table 2.1. Characteristics of TC histological subtypes.

Histological type	Frequency	Age at diagnosis, years
<i>Seminomatous testicular tumours</i>		
Seminoma	50% of all TC	25–40
Spermatocytic seminoma	1–2% of all TC	>50
<i>Nonseminomatous testicular tumours</i>		
Embryonal carcinoma	relatively uncommon as a pure form; > 80% of all mixed germ cell tumours	25–35
Yolk sac tumour	50% of all prepubertal TC; 40% of all mixed germ cell tumours	0–11
Choriocarcinoma	< 1% of all TC; ~8% of all mixed germ cell tumours	20–30
Teratoma	10–14% of all prepubertal TC; 2–3% of all postpubertal TC; ~50% of all mixed germ cell tumours	<4 and >15
Mixed germ cell tumours	30–50% of all TC in adults	>15

2.4. Natural history of TC

TC has its origin in foetal life as a result of a defect in the early development of primordial germ cells or gonocytes into spermatogenic cells (Skakkebaek et al. 1987, Rajpert-De Meyts et al. 1998, Skakkebaek et al. 2001). In the human foetus, differentiation of gonocytes into infantile spermatogonia is a relatively long and slow process. If testicular differentiation is impaired, either due to an inherent genetic defect or an exposure to environmental factors, this process may be delayed or arrested. This probably happens shortly after the arrival of the migrating primordial germ cells in the genital ridge (Adamah et al. 2006). Hormonal imbalances in the cellular microenvironment may lead to errors in mitosis-meiosis switch in “sexually confused” germ cells and can result in polyploidization. Other errors in cell division and progressive genomic aberrations would lead to further genomic instability and formation of transformed “pre-CIS cells” (Rajpert-De Meyts 2006). These cells remain quiescent until they are stimulated to proliferate by gonadotropin-mediated signals and form germ cell tumours either in infancy or after puberty in young adulthood (Figure 2.5).

Ninety-five percent of TC derives from germ cells that do not have gonadotropin receptors (Skakkebaek et al. 2003) but depend on testicular growth factors that are produced under gonadotropin stimulation. Gonadotropin levels are high during infancy, decline rapidly after 3 months of age (Andersson et al. 1998), and remain very low until puberty, when they rise to adult levels. The age-adjusted incidence of TC closely follows the changes in the gonadotropin levels, which suggests that tumourogenesis depends on permissive action of gonadotropins in the testis (Rajpert-De Meyts and Skakkebaek 2000, Golub et al. 2008).

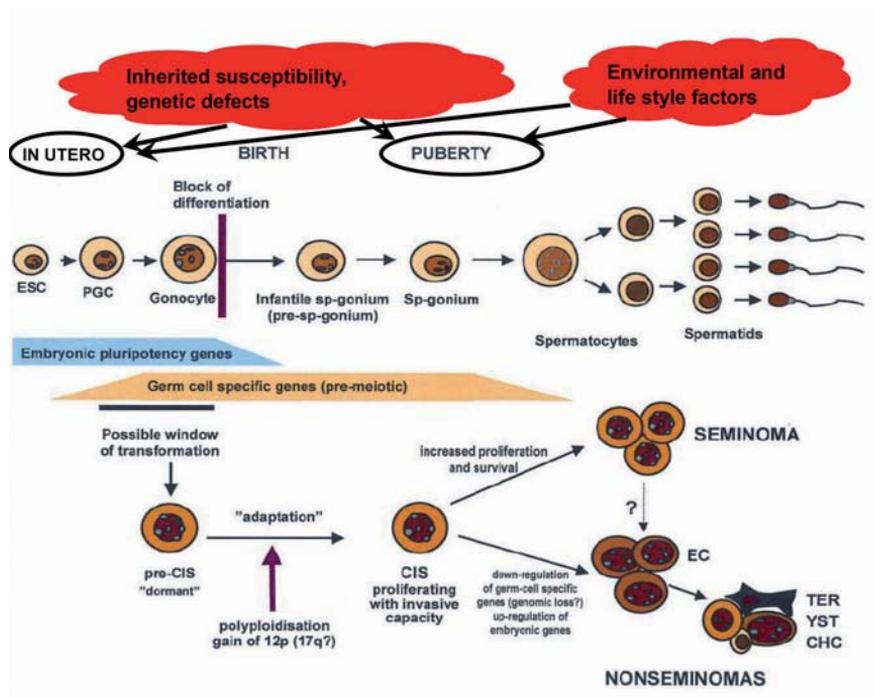


Figure 2.5. Scheme illustrating the current understanding of the pathogenesis of testicular CIS in relation to germ cell differentiation. EC, embryonal carcinoma; TER, teratoma; YST, yolk sac tumour; CHC, choriocarcinoma (adapted from Rajpert-De Meyts 2006, Skakkebaek et al. 2001).

2.5. Aetiology of TC

Histology and the early age-specific incidence peak of TC suggest that its risk factors operate early in life, possibly already *in utero*. Increase of incidence of TC within a generation suggests that changes in the occurrence of exogenous aetiological factors may contribute to the development of TC. However, genetic predisposition probably sets the stage for TC (Table 2.2, p. 45).

2.5.1. Cryptorchidism and other disorders of testicular differentiation

The most established factor associated with the risk of TC is cryptorchidism. Some studies have reported a two to four-fold increased risk of TC and a PAF of 10% associated with cryptorchidism (Boyle and Zaridze 1993, Moller et al. 1996, Walschaerts et al. 2007, Akre et al. 2009), other studies even a five to ten-fold increased risk of TC associated with cryptorchidism (Moss et al. 1986, Giwercman et al. 1987, Swerdlow et al. 1997b, Weir et al. 2000, Dieckmann and Pichlmeier 2004, Akre et al. 2009). Recently several studies have reported that surgical treatment of cryptorchidism before puberty decreases the risk of TC from five to one or two-fold (Herriton et al. 2003, Pettersson et al. 2007b) whereas another study did not find such an association (Myrup et al. 2007). It has been hypothesized that cryptorchidism may be the first step in (a partially genetic determined) a causal chain of events that can lead to TC or a less severe stage of testicular dysgenesis syndrome (TGS) (Garner et al. 2005). The hypothesis proposed that abnormal testicular development, which could have numerous primary causes, leads secondarily to hormonal or other malfunctions of the Leydig and/or Sertoli cells during male sexual differentiation, leading in turn to increased risk of the reproductive disorders (Sharpe and Skakkebaek 2008).

A significant increase in the prevalence of cryptorchidism over the last 40 years has been observed in Denmark (Boisen et al. 2004) but not in its neighbour, Finland (Toppari et al. 2001, Boisen et al. 2004). Differences in semen quality (Jørgensen, 2002) and in prevalence of cryptorchidism (Boisen et al. 2004) at birth and at 3 months of age as well as differences in TC incidence (Adami et al. 1994, Curado et al.

2007) have been noted between Denmark and Finland. This contrast could be reflected by environmental/lifestyle differences between the two countries and/or ethnic differences in susceptibility (Sharpe and Skakkebaek 2008).

Other testicular abnormalities such as testicular atrophy (Dieckmann and Pichlmeier 2004), gonadal dysgenesis (Dieckmann and Skakkebaek 1999, Dieckmann and Pichlmeier 2004), and subfertility/infertility (Dieckmann and Skakkebaek 1999, Fossa and Kravdal 2000, Jacobsen et al. 2000, Dieckmann and Pichlmeier 2004, Doria-Rose et al. 2005, Walsh et al. 2009) have been reported as risk factors for TC.

2.5.2. Carcinoma *in situ* (CIS)

CIS of the testis is a distinct histological pattern preceding the development of seminomatous and nonseminomatous germ cell tumours of the testis (Skakkebaek et al. 1982). The link between TC and CIS may also be related to a condition referred to as TGS, which occurs already *in utero* with varying levels of severity. TC represents the most severe form of the TGS, whereas CIS is a less severe form of TGS (Boisen et al. 2001, Sharpe and Skakkebaek 2008).

Fifty percent of patients diagnosed with CIS of the testis develop invasive TC within 5 years of diagnosis. It is thought that all patients harbouring CIS cells at puberty will eventually develop TC (Jørgensen et al. 1990). It is now generally agreed that nearly all TC cases including seminoma or nonseminoma are preceded by the presence of CIS cells, with the exception of two rare tumour types: infantile germ cell tumours and spermatocytic seminoma (Reuter 2005, Bahrami et al. 2007). There is a higher probability of developing nonseminomatous lesions if the transition from CIS to invasive cancer occurs at a young age (Rorth et al. 2000). By contrast, seminomatous lesions are more likely to result from the progression of CIS at older ages.

2.5.3. Inherited susceptibility

Familial clustering of the TC with affected first degree relatives occurs in 1 to 2.8% of all TC cases (Sonneveld et al. 1999, Dieckmann and Pichlmeier 2004). Case reports

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of familial TC support family history as a possible TC risk factor independent of cryptorchidism (Fuller and Plenk 1986, Yoshimura et al. 1993, Han and Perschel 2000, Gupta et al. 2005). Genetic anticipation may also be responsible for many father-son TCs (Han and Perschel 2000). Inherited susceptibility is especially strong in bilateral TC (Heimdal et al. 1996a).

Familial studies have shown that brothers of TC patients have an eight to ten-fold increased risk of TC. Among fathers and sons the point estimates were considerably lower, approximately four-fold (Heimdal et al. 1996a, Heimdal et al. 1996b, Hemminki and Li 2004). A significantly higher risk of TC in dizygotic twins has been observed compared to monozygotic twins (Swerdlow et al. 1997a), suggesting that an environmental component may also act *in utero* to cause TC. A possible genetic effect in the twin studies can be found only in a case of assessment of TC risk in the twin brothers of affected patients. However, the numbers have been too small in most studies for any conclusions to be drawn (Braun et al. 1995, Swerdlow et al. 1997a).

Positive associations between TC and leukaemia, distal colon cancer, kidney cancer, melanoma, connective tissue tumours, prostate cancer, lung cancer, breast cancer, and non-Hodgkin's or Hodgkin's lymphoma in the family have been reported (Dong et al. 2001, Spermon et al. 2001, Hemminki and Li 2004, Hemminki and Chen 2006, Walschaerts et al. 2007).

A segregation analysis suggested a recessive mode of inheritance, with penetrance among homozygotes of about 45%. However, it has been conceded that there may be more than one predisposition gene involved in TC and that the mode of inheritance is probably more complex (Nicholson and Harland 1995, Heimdal et al. 1996b).

A linkage study based on families with at least two affected cases of TC did not yield clear evidence for linkage of any autosomal gene (Rapley et al. 2000, Rapley et al. 2002, Crockford et al. 2006), indicating that, like many other cancers and chronic diseases, there are several moderate-risk genes involved. However, X-chromosome linkage (i.e. no male-to-male transmission) provided the first statistically significant evidence for a TC predisposition locus. The gene called TGCT1 is located at Xq27 and seems to be associated with the risk of bilateral TC and perhaps cryptorchidism

(Rapley et al. 2000, Rapley et al. 2003). However, the set of pedigrees in these studies was quite small.

Genetic association studies have been mostly focused on the role of human leukocyte antigen (HLA) genes (Lutke Holzik et al. 2004). However, the results have been inconsistent. For instance, one study found evidence of an association between HLA Bw41 and seminoma (Dieckmann et al. 1993). Two other studies also reported HLA-associations, but not with the same loci (Kratzik et al. 1991, Özdemir et al. 1997). Conversely, no HLA class II associations were found in another study (Sonneveld et al. 2002). Genes involved in modulating the action of steroid hormones have been considered without conclusive evidence in some of the studies (Rajpert-De Meyts et al. 2002, Giwercman et al. 2004, Starr et al. 2005, Figueroa et al. 2008). One study showed that genetic variation in insulin-like growth factor (IGF) loci is not associated with risk of TC (Chia et al. 2008). Moreover, another study revealed little evidence of an association between 8q24 single-nucleotide polymorphisms (SNP) and risk of TC, especially nonseminoma (Cook et al. 2008a).

Microdeletions in the Y chromosome associated with decreased fertility have been investigated with regard to risk of TC. However, most of the studies have yielded negative results (Frydelund-Larsen et al. 2003, Lutke Holzik et al. 2005, Bor et al. 2006). An increased risk of TC was associated with some deletions in Y chromosome, which were associated with subfertility but could, however, be transmitted from father to son (Repping et al. 2003, Nathanson et al. 2005).

2.5.4. Ethnic and migration factors

The effects of genetic susceptibility and environmental exposure on the risk of TC development have also been distinguished in migration studies (Parkin and Khlal 1996). TC continues to rise in most populations but with remarkable ethnic differences, indicating the influence of genetic background (McGlynn et al. 2005a, Jack et al. 2007, Shah et al. 2007). For instance, Finland is a relatively low TC incidence country, Sweden is a middle-incidence and Denmark a high-incidence country (Richiardi et al. 2004). Men migrating from one country to another tended to

retain their country of origin risk of TC, whereas their children tended to approach the risk of the new home country (Hemminki and Li 2002, Ekbom et al. 2003, Montgomery et al. 2005) or had an intermediate TC risk between those observed in the first generation of immigrants and in the population to which they migrated (Parkin and Iscovich 1997).

2.5.5. Prenatal and perinatal exposures

Prenatal exposure

Nausea. There are conflicting findings on whether excessive nausea during pregnancy is a risk factor for TC. Some studies have found excessive nausea to be associated with two to four-fold increased risk of TC (Depue et al. 1983, Petridou et al. 1997, Sonke et al. 2007), a decreased risk of TC (Moller and Skakkebaek 1997, Coupland et al. 2004), or no risk of TC (Brown et al. 1986, Weir et al. 2000, Cook et al. 2008b). As excessive nausea during early pregnancy may indicate a rapid rise in endogenous hormone levels such as human chorionic gonadotropin (hCG) and oestrogens (Furieux et al. 2001) it is plausible that hormone levels in early pregnancy are involved in the mechanism ultimately leading to TC in early adulthood. However, the overall effect may be modest and difficult to establish in epidemiological studies (Sonke et al. 2007).

Birth weight. Low birth weight has been investigated mainly as an indicator of foetal growth retardation. Some studies have found low birth weight to be associated with two-fold increased risk of TC (Akre et al. 1996, Moller and Skakkebaek 1997, English et al. 2003, Coupland et al. 2004, Cook et al. 2008b, Dusek et al. 2008). Other studies have found high birth weight to be associated with one and half-fold increased risk of TC (Akre et al. 1996, Richiardi et al. 2002, Richiardi et al. 2003), or no risk of TC (Sabroe and Olsen 1998, Wanderås et al. 1998, Weir et al. 2000, Rasmussen et al. 2003). Due to the inconsistency of these results meta-analyses were conducted on birth weight and risk of TC (Michos et al. 2007, Richiardi et al. 2007). These ultimately revealed an increased risk of TC, but both for children with low birth weight (<2500-3000g) and high birth weight (>4000g) supporting a proposed U-

shaped relation between birth weight and TC (Michos et al. 2007). The length of the child at birth was found to be a non-significant risk factor for TC (Moller and Skakkebaek 1997, Sabroe and Olsen 1998, English et al. 2003, Cook et al. 2008b).

Timing of delivery. The relation between preterm birth and risk of TC may involve elevated exposure to oestrogens *in utero* (Weir et al. 2000). An almost 50% increased risk of TC was observed in males born at least two weeks early, whereas those born late were at reduced risk (Weir et al. 2000, Richiardi et al. 2002, Coupland et al. 2004). There was, however, no association between preterm birth and risk of TC in other studies (Wanderås et al. 1998, Sonke et al. 2007, Cook et al. 2008b).

Parity. Several studies (Prener et al. 1992, Moller and Skakkebaek 1997, Sabroe and Olsen 1998, Westergaard et al. 1998, Cook et al. 2008b) but not all (Wanderås et al. 1998, Weir et al. 2000, Coupland et al. 2004, Sonke et al. 2007) have indicated that children of mothers with high parity have decreased risk of TC when compared to children of nulliparous mothers. It has previously been suggested that maternal endogenous oestrogen levels are higher in first pregnancies than in subsequent pregnancies (Arslan et al. 2006). More recently, a significant trend for decreased risk of TC both with increasing birth order and sib-ship size in male offspring was shown (Richiardi et al. 2004). However, neither elevated maternal hormone levels nor parental subfertility in the TC cases alone can explain the results (Richiardi et al. 2004).

Twin pregnancies. The significant from one and half to two-fold increased risk of TC in dizygotic than in monozygotic twins supports a prenatal aetiology with raised maternal oestrogen levels during the pregnancy (Braun et al. 1995, Swerdlow et al. 1997a). Prenatal oestrogen levels may be higher in males with a female co-twin than in males with a male co-twin. However, the finding of elevated risk of TC in male/female twins was restricted to instances where the male twin was born after his female twin (Swerdlow et al. 1996).

Maternal age. There is contradictory evidence on whether maternal age is a risk factor for TC. Some studies have found older maternal age to be associated with a one and half or two-fold increased risk of TC (Moller and Skakkebaek 1997, Sabroe and Olsen 1998, Wanderås et al. 1998, English et al. 2003), a decreased risk of TC (Coupland et

al. 2004, Cook et al. 2008b, Dusek et al. 2008), or no risk of TC (Weir et al. 2000, Richiardi et al. 2002, Sonke et al. 2007). The effect of maternal age on the risk of TC remains unclear, although it is possible that an impact of maternal age may be presented only in women with no prior children (Wanderås et al. 1998).

Retained placenta. Retained placenta, presence of Rh-antibodies and foetal presentation has been associated with the risk of TC, although, other than a dysfunctional *in utero* environment, the reason for this is unclear (Moller and Skakkebaek 1997, Wanderås et al. 1998).

Maternal hormone exposure

According to the leading hypothesis on TC aetiology, exposure to a specific pattern of steroid hormones *in utero*, in particular to high levels of oestrogens and low levels of androgens, is the major determinant of TC risk in the offspring (Henderson et al. 1979, Henderson et al. 1988a, Henderson et al. 1988b, Sharpe and Skakkebaek 1993, Sharpe 2003).

High levels of oestrogens during embryogenesis may cause developmental arrest of foetal germ cells, which remains dormant until puberty. At puberty proliferation to testicular CIS and ultimately to invasive cancer (Rajpert-De Meyts and Skakkebaek 1993, Dieckmann and Skakkebaek 1999) may then be stimulated by gonadotropins from the pituitary (Henderson et al. 1988a). Oestrogens also inhibit the secretion of Müllerian Inhibiting Substance (MIS) by the Sertoli cells, which is responsible for the regression of the Müllerian duct and the first phase of testicular descent. MIS also exerts tumour-suppressing activities. Therefore, MIS production or action in the critical early foetal period may lead to the appearance of abnormal gonocytes or CIS cells (Rajpert-De Meyts and Skakkebaek 1993, Sharpe and Skakkebaek 1993).

Exposure of pregnant mice *in utero* to oestrogens has been found to increase the frequency of TGS (Yasuda et al. 1985), cryptorchidism (Walker et al. 1990), hypospadias (Kim et al. 2004), and TC (Newbold et al. 1987); and there is also evidence of impaired Leydig cell development and reduced Sertoli cell numbers,

resulting in impaired spermatogenesis (Yasuda et al. 1985). The same associations have been suggested to take place in humans (Buetow 1995, Toppari et al. 1996), although they are still under debate (Sharpe 2003, Sharpe and Skakkebaek 2008).

Maternal exposure to pharmacological oestrogens e.g. diethylstilbestrol (DES) etc. during pregnancy may lead to an increased risk of TC in the offspring. Several studies have found from five to eight-fold increased risk of TC in relation to hormone consumption during pregnancy (Henderson et al. 1979, Depue et al. 1983, Toppari et al. 1996, Weir et al. 2000). However, the hormones studied have been varied and in most studies were not specified. One study found that the relationship remained significant even when stratifying by histology of TC (Weir et al. 2000) whereas other studies found no increased risk of TC with exogenous hormone consumption during pregnancy (Brown et al. 1986, Moss et al. 1986). There is also insufficient evidence to support an increased risk of TC in men exposed to DES *in utero* (Leary et al. 1984, Jensen et al. 1995, Strohsnitter et al. 2001).

The most straightforward way by which the foetus is exposed to high levels of oestrogens, is through increased bioavailability of maternal high oestradiol levels naturally occurring during pregnancy (Sharpe 2003). Although this epidemiological data reported so far is inconsistent, increased oestrogen levels during pregnancy are associated with several maternal or foetal conditions such as high maternal age, first birth order, excessive nausea, twin pregnancy, premature birth, low and high birth weight (Prener et al. 1992, Braun et al. 1995, Moller and Skakkebaek 1997, Petridou et al. 1997, Swerdlow et al. 1997a, Sabroe and Olsen 1998, Wanderås et al. 1998, Westergaard et al. 1998, Weir et al. 2000, Richiardi et al. 2002, Coupland et al. 2004, Michos et al. 2007, Sonke et al. 2007, Cook et al. 2008b). On the other hand, conditions related to decreased oestrogen levels during pregnancy such as increased parity, smoking, pre-eclampsia, and eclampsia appear to protect against TC (Weir et al. 2000, Akre et al. 1996, Cook et al. 2008b). These evidences have been largely interpreted within the framework of the steroid hormone imbalance hypothesis. Although the available evidence supports the hypothesis, it is, however, far from being proven (Garner et al. 2008). Furthermore, it has been proposed that indicators of higher levels of pregnancy oestrogens are more related to seminomas, while indicators of oestrogen exposures associated with intrauterine growth retardation are more

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related to nonseminomas (Akre et al. 1996). However, these issues have not been supported by another study (Weir et al. 2000).

The amount of androgens in circulating maternal blood is also an important factor for the normal development of the testis and its descent during foetal life (Henderson et al. 1988b). Androgens have been shown to play a decisive role of Sertoli cell production during foetal life (Sharpe and Skakkebaek 2008) and to increase the cleavage and probable activation of MIS (Rajpert-De Meyts and Skakkebaek 1993). This was supported by animal studies where treatment with androgens reversed oestrogens inhibiting testicular descent (Henderson et al. 1979) and *in utero* exposure of rats to the anti-androgen flutamide resulted in cryptorchidism (van der Schoot 1992) or in a reduced number of Sertoli cells (Atanassova et al. 2005). Furthermore, germ cell cancer and CIS are frequently observed in low-androgen states of various reproductive abnormalities, e.g., androgen insensitivity syndrome (Muller and Skakkebaek 1984, Cassio et al. 1990, Hannema et al. 2006, Aguilar-Ponce et al. 2008), mixed gonadal dysgenesis (Muller et al. 1985), Klinefelter's syndrome (Rajpert-De Meyts and Skakkebaek 1993). A relative excess of maternal androgens during pregnancy was observed in black women when compared to Caucasian women (48% higher testosterone levels). This provides a possible explanation for the three-fold lower incidence of cryptorchidism and TC in black men (Henderson et al. 1988b). On the other hand, women in China, where the incidence of TC is low, have increased levels of oestradiol, unconjugated oestriol and sex hormone binding globulin (SHBG) during early pregnancy compared to black women (Lipworth et al. 1999, O'Brien et al. 1997). However, one study did not support either the oestrogen or the androgen hypothesis. Rather, lower oestrogens in case mothers may indicate that a placental defect increases the risk of cryptorchidism and, possibly, TC (McGlynn et al. 2005b).

Maternal infection exposure

Exposure to Epstein-Barr virus (EBV) with known oncogenic potential (Shibata et al. 1991, Iezzoni et al. 1995, Lehtinen et al. 2003, Rezk and Weiss 2007) may be related to the risk of TC (Algood et al. 1988, Heinzer et al. 1993, Shimakage et al. 1996).

Further support for EBV aetiology of TC comes from histopathological studies showing that all seminomas and embryonal carcinomas expressed EBV ribonucleic acid (RNA), whereas the non-malignant testes did not (Shimakage et al. 1996). The occurrence of TC in young patients and high levels of wild-type (not mutated) p53 protein in testicular tumour cells (Guillou et al. 1996, Gray et al. 1998) also fit the role of EBV in TC pathogenesis as the former may have been inactivated by Epstein-Barr virus-related nuclear antigen (EBNA) 5, one of the EBV oncogenes (Rajpert-De Meyts et al. 1994). A putative passenger role for EBV in the virus transformed lymphocytes, which are not infrequently present in the stromal tissues of TC (Rajpert-De Meyts et al. 1994), however, cannot be excluded.

EBV has been considered as a possible cause of TC also by analogy with its association with Hodgkin's lymphoma (Evans et al. 1980, Herbst et al. 1990, Brousset et al. 1993). Indeed, Hodgkin's lymphoma shows epidemiological similarities to TC with a peak age in adolescents and young adults (Newell et al. 1984). The occurrence of both diseases is frequent in high socioeconomic class with increase in white urbanised populations and most notably in Europe, particularly in Denmark. Both cancers with a variety of histological types have a low but definite familial occurrence with lifetime risk determined at a young age and irrespective of geographic migration later in life.

Cytomegalovirus (CMV) may also be related to the risk of TC (Mueller et al. 1988). However, this study suggested that risk of TC may be related to a relatively late age of exposure to CMV resulting in damage to the development of the testis. Confounding by immune status of TC cases or by other risk factors of TC was not taken into account for this observation. So far there is no evidence showing an association between maternal CMV infection and risk of TC in the offspring.

Maternal exposure to smoking and alcohol consumption

An association of maternal smoking with the risk of TC in the offspring has been suggested by two ecological studies (Clemmesen 1997, Pettersson et al. 2004) reporting a strong geographical and temporal correlation between female smoking prevalence and TC incidence in the Nordic countries. A positive association between

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parental lung cancer and TC in the offspring has been also suggested (Heimdal et al. 1996b, Dong et al. 2001, Kaijser et al. 2003). Besides containing numerous potent carcinogens, tobacco smoke has several physiological effects, such as reducing placental blood flow and interfering with pregnancy oestrogens (Bernstein et al. 1989, Petridou et al. 1990) that could disturb normal testicular cell differentiation (Mochizuki et al. 1984). However, a number of questionnaire-based case-control studies have found no association between maternal smoking and risk of TC (Swerdlow et al. 1987, Moller and Skakkebaek 1996, Weir et al. 2000, Coupland et al. 2004, McGlynn et al. 2006, Pettersson et al. 2007a, Sonke et al. 2007).

Smoking and alcohol consumption have been associated with alterations in endogenous oestrogen levels (MacMahon et al. 1982, Reichman et al. 1993, Gill 2000). Parental drinking has been linked to the risk of other childhood cancers (Everson et al. 1986, Moller 1993, Ji et al. 1997, Sorahan et al. 2001) or cryptorchidism (Damgaard et al. 2007). One study did not, however, find any evidence of germ cell tumours being related to maternal exposure to alcohol consumption (Chen et al. 2005a).

Maternal exposure to endocrine disrupting chemicals

Exposure to endocrine disrupters has received considerable attention as potential risk factors for TC over the past few decades (Joffe 2001, Skakkebaek et al. 2007). Some of these exposures may lead to the mutation in germ cells (Giri 2002) or malfunctions of the Leydig and/or Sertoli cells during male sexual differentiation (Sharpe and Skakkebaek 2008). In the earlier studies, exposure was estimated based on parental employment in exposure-prone jobs, especially jobs involving pesticide exposure. However, results from these studies are inconsistent, showing either a positive association between parental exposure-prone jobs and risk of TC (Shu 1995), or no association (Rodvall et al. 2003, Chen et al. 2005b, Chen et al. 2006). In fact, substantial exposure to pesticides can also occur in and around the home. Increased breast milk or blood levels of some endocrine disrupting chemicals have been found in case mothers compared to the control mothers (Hardell et al. 2003, Damgaard et al. 2006, McGlynn et al. 2008). Further, significant geographic differences were revealed

between Denmark and Finland in the level of certain persistent organochlorine compounds in human placenta and breast milk (Shen et al. 2008).

2.5.6. Postnatal exposure

Although several findings suggest that TC originates *in utero*, it cannot be ruled out that events later during childhood or adolescence initiate or promote the development of TC. However, the possible mechanisms behind these associations remain unexplained.

Personal risk factors

Age. The peak incidence of TC occurs between the ages of 20 and 40 years (Bray et al. 2006b, Figure 2.4) which is different from that for most other cancers with peak normally much later in life. The incidence of TC appears to be related to steroid hormone activity either *in utero* or during the puberty. The steeply increased incidence of TC observed following puberty is thought to reflect the promotion of cells initiated during gestation to CIS by adult steroid hormones (Moller 1993, Moller and Evans 2003).

Race. The incidence of TC varies with race. Black and other non-white races have extremely low rates of TC in comparison to Caucasian populations (Curado et al. 2007). Although the reasons for the differing rates are unknown, certain hormonally related racial differences have been observed. Pregnancy levels of androstenedione and testosterone were seen to be higher in black mothers than in white mothers (Troisi et al. 2003a). Higher free testosterone levels have also been seen in young black men compared to young white men (Ellis and Nyborg 1992, Morrison et al. 2003). However, the possibility that other genetic, lifestyle, or environmental influences may contribute to this association should not be excluded.

Androgen levels. The role of androgen levels during adolescence and early adult life has received little attention, although acne (Depue et al. 1983) and baldness (Petridou

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et al. 1997), which are linked to higher androgen levels, have been found to be inversely associated with the risk of TC.

Puberty. Puberty can be considered as a critical time for TC development. It is a period of high replication. Spermatogonia, which remain rather inactive during infancy and childhood, start undergoing meiosis to form spermatocytes and sperm at puberty, stimulated by a surge of steroid and peptide hormones. Although age at puberty is difficult to measure, it would appear that late age at puberty is associated with a marked decrease (up to 50%) in the risk of TC (Moss et al. 1986, Moller and Skakkebaek 1996, Weir et al. 1998, Coupland et al. 1999). Other studies did not support puberty as a risk factor of TC (Swerdlow et al. 1989) based on ages at shaving, voice changing and nocturnal emission (McGlynn et al. 2007).

Body mass index (BMI). Studies of BMI and risk of TC have yielded inconsistent results, with some finding an almost two-fold increased risk of TC with a low BMI (Petridou et al. 1997, Akre et al. 2000), a three-fold increased risk of TC with high BMI (Garner et al. 2003) or no risk of TC (Davies et al. 1990, Dieckmann and Pichlmeier 2002, Rasmussen et al. 2003, Richiardi et al. 2003, Bjorge et al. 2006, McGlynn et al. 2007). Studies examining weight separately from BMI found no association between increased body weight and risk of TC (Swerdlow et al. 1989, Davies et al. 1990, Dieckmann and Pichlmeier 2002).

Height. Many studies have examined height as a risk factor for TC (Swerdlow et al. 1989, Gallagher et al. 1995, Akre et al. 2000, Dieckmann and Pichlmeier 2002, Richiardi et al. 2003, Bjorge et al. 2006, McGlynn et al. 2007, Dieckmann et al. 2008) showing up to three-fold increased risk of TC among men taller than 185-195cm. Several potential mechanisms have been proposed to explain a positive association of height with the risk of TC, including childhood nutrition (Dieckmann and Pichlmeier 2002, Dieckmann et al. 2008) increased levels of IGF (Juul et al. 1994, Khandwala et al. 2000) or an earlier age at puberty in those of greater height (Hagg and Taranger 1992, Karlberg 2002).

Lifestyle factors

So far a wide variety of lifestyle factors have been examined in the research searching for clues in the aetiology of TC. The results have been inconsistent, with strong associations found with diet but weak and varied associations for socio-demographic indicators.

Socioeconomic status (SES). The majority of the studies on the effect of SES and risk of TC have used SES indicators based on social class or education. Some studies (Hayes et al. 1990, Swerdlow et al. 1991, Moller and Skakkebaek 1996), but not all (Van den Eeden et al. 1991, Prener et al. 1992), have found positive association between high social class and the risk of TC. TC seems to occur much more frequently in the higher social classes than in the lower, perhaps due to other factors, such as diet or occupation. Education has also been used as an indicator of SES. One study found a positive association between TC and increasing educational attainment, although the data were not explicitly presented (Srivastava and Kreiger 2000). A recent study has revealed that a wide disparity in the risk of TC between high and low social classes (higher incidence among men with higher SES) has diminished over time (Pukkala and Weiderpass 2002).

Physical activity. The effect of physical activity on the risk of TC is unclear. Some studies have found that higher levels of physical activity have a protective effect (Gallagher et al. 1995, UK Testicular Cancer Study Group, 1994a; UK Testicular Cancer Study Group, 1994b), whereas others have found an adverse effect (Srivastava and Kreiger 2000) or no effect (Thune and Lund 1994). However, these studies did not control for dietary factors. One study showed that the analysis of the TC cases responses found no relationship between physical activity and risk of TC, while the maternal responses found an inverse association (Cook et al. 2008c).

Diet. Diet has been associated with the risk of TC with inconclusive results. For instance, meat (Sigurdson et al. 1999, Garner et al. 2003), high fat consumption (Sigurdson et al. 1999), milk and cheese (Davies et al. 1996, Ganmaa et al. 2002, Garner et al. 2003) have been found to have a positive association with TC. There was, however, no association between risk of TC and dietary consumption at any age

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between birth and 18 years of age (McGlynn et al. 2007) and between risk of TC and dietary intake of phytoestrogens (Walcott et al. 2002).

Dietary products (i.e. milk, meat and eggs) may contain the female steroid hormones such as oestrogens and progesterone (Hartmann et al. 1998). It is reasonable to hypothesize that these hormones in dietary products may be associated with the risk of TC (Joffe 2001, Ganmaa et al. 2002). The strength of this association is further supported by the recent increasing trend in TC incidence and the increased consumption of dietary products in developed countries starting in the 1940s and 1950s (Sharpe and Skakkebaek 1993). Another factor contributing to the level of oestrogens in dietary products may be the treatment of cattle with hormones and antibiotics, although the impact of this practice on carcinogenesis is unknown.

Urban/rural living. There have been several conflicting studies on rural and urban patterns of TC suggesting either a rural preponderance of TC (Talerman et al. 1974, Walschaerts et al. 2007), or no rural/urban differences in the rates of TC (Schouten et al. 1996, Petridou et al. 1997, Sonneveld et al. 1999).

Late infection exposure. The secular increase of TC in developed countries could reflect an increasingly larger proportion of boys who are susceptible to relatively late infections (i.e., at or after puberty) with common childhood viruses (Mueller et al. 1988) having gonadotropic properties. An association of TC with higher social class (Hayes et al. 1990, Swerdlow et al. 1991, Moller and Skakkebaek 1996) also points to the possibility of late infections with common childhood viruses. At a later age these infections are often more severe and may include testicular inflammation. For example, in the case of mumps, the occurrence of orchitis increases with age of infection in boys (Feldman 1982, p.419-440) and there have been reports of TC developing following atrophy of mumps orchitis (Kaufman and Bruce 1963, Beard et al. 1977). The eradication of mumps from the Nordic countries in 1996 indicates that mumps (Peltola et al. 2000), however, can not be related to the ever increasing incidence of TC in the Nordic countries.

Occupational exposure

Although many studies have found positive associations between occupation and risk of TC (Hayes et al. 1990, Marshall et al. 1990, Tarone et al. 1991, Van den Eeden et al. 1991, Davis and Mostofi 1993, Rhomberg et al. 1995, Knight et al. 1996, Hardell et al. 1998, Finkelstein 1998, Fleming et al. 1999, Andersson et al. 2003, Walschaerts et al. 2007), no occupation has emerged as a clear risk factor for this lesion. Occupational exposures to several chemical substances have also been associated with the risk of TC. Dimethylformamide (DMF) was implicated as a risk factor in case-reports of TC in leather tanners (Levin et al. 1987) and aircraft repairmen (Ducatman et al. 1986). However, a review on DMF conducted by the International Agency for Research on Cancer (IARC) concluded that inadequate evidence exists to implicate DMF as a human carcinogen (IARC 1999). Other chemical exposures of potential concern include: fertilizers high in nitrogen (Kristensen et al. 1996), non-specified fertilizers (Haughey et al. 1989), fumes or smoke (Haughey et al. 1989) and pesticides (Hardell et al. 1998, Fleming et al. 1999). All these studies focused on occupational exposures, except for fertilizers high in nitrogen, where exposure occurred as a result of nutrient run-off (Kristensen et al. 1996).

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Table 2.2. Aetiology of TC

Risk factor	Comments
Cryptorchidism	Established risk factor: two to ten-fold increased risk of TC.
CIS	Established risk factor: 50% of patients diagnosed with CIS develop TC within 5 years.
Inherited susceptibility	Established risk factor: about four-fold increased risk of TC among father/son and more than eight-fold increased risk of TC in brothers of TC cases. It only occurs in 1-2.8% of all TC cases. No single gene or SNP was established so far.
Ethnicity/migration	Men migrating from one country to another tended to retain their country of origin risk of TC, whereas their children tended to approach the risk of the new home country.
<i>Prenatal and perinatal exposures</i>	
Nausea	Inconsistent findings but the effect of excessive nausea on risk of TC during early pregnancy may be due to rapid rise in oestrogen levels.
Birth weight	Inconsistent findings. U-shaped relation: low and high birth weight may be a risk factor for TC.
Timing of delivery	Inconsistent findings but preterm birth may be a risk factor of TC involving elevated exposure to maternal oestrogens during pregnancy.
Parity	Inconsistent findings but high parity shows decreased risk of TC compared to nulliparous due to higher maternal oestrogen levels in first pregnancies than in subsequent pregnancies.
Twin pregnancies	Up to two-fold increased risk of TC in dizygotic compared to monozygotic twins supports a prenatal aetiology with raised maternal oestrogen levels during pregnancy.
Maternal age	The effect of maternal age is unclear, probably based on the oestrogen excess theory.
Retained placenta	The effect of retained placenta on risk of TC is unclear.
Maternal steroid hormones (high oestrogens and low androgens)	The findings mostly come from studies using surrogates of steroid hormone imbalance: maternal exposure to pharmacological oestrogens, pregnancy characteristics, ethnicity, and reproductive abnormalities.
Maternal EBV infection	There is some support from epidemiological and histopathological studies; however, the real effect is unclear.
Maternal CMV infection	No studies on maternal CMV infection and risk of TC in the offspring have been conducted so far.
Maternal smoking	Ecological studies but no other questionnaire-based case-control studies have shown the association with risk of TC.
Maternal alcohol consumption	The association with risk of TC is unclear.

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Endocrine disrupting chemicals Inconsistent findings, however, exposure to endocrine disrupting chemicals has recently received considerable attention as potential risk factors for TC.

Postnatal exposure

Age	Peak age is between 20 to 40 years of age.
Race	Risk of TC is higher in Caucasian population compared to black or other non-white races.
Androgen levels	Increasing male baldness, as a surrogate for elevated androgen levels, was associated with 20% reduction in the risk of TC.
Puberty	Late age at puberty is associated with a 50% decreased risk of TC but not all studies supported this finding.
BMI	Inconsistent findings suggesting two-fold increased risk of TC with low BMI, three-fold increased risk of TC with high BMI or no risk of TC.
Height	Up to three-fold increased risk of TC among men taller than 185-195cm. Several potential mechanisms have been proposed: childhood nutrition, increased levels of IGF or an earlier age at puberty.
SES	Inconsistent findings but TC seems to occur much more frequently in the higher social classes than in the lower.
Physical activity	Inconsistent findings suggesting that higher levels of physical activity have a protective effect, an adverse effect or no effect.
Diet	Inconsistent findings but the risk of TC may be due to female steroid hormones added to dietary products (milk, meat, cheese etc).
Urban/rural living	Inconsistent findings suggesting either a rural preponderance of TC or no rural/urban differences in the rates of TC
Late infection	No late infection has been established as a risk factor for TC.
Occupation	No occupation has been established as a risk factor for TC.

3. AIMS OF THE STUDY

The histological spectrum and the typical incidence peak at very young age suggest that risk factors for TC operate early in life, possibly already *in utero*. The objective of this study is, therefore, to assess maternal risk factors during pregnancy and the risk of TC in the offspring. The aims of this study were specifically to estimate:

- the effect of storage on the serum levels of three groups of hormones and associated proteins: steroid hormones [oestradiol, progesterone, testosterone, dihydroepiandrosterone sulphate (DHEAS), sex hormone-binding globulin (SHBG)], pregnancy-specific hormones [human chorionic gonadotropin (hCG), placental growth hormone (pGH), alpha-fetoprotein (AFP)], and insulin-like growth factor (IGF) family hormones
- the association of maternal smoking with risk of TC in the offspring
- the association of maternal EBV and CMV infections with risk of TC in the offspring
- the association of early pregnancy maternal serum of steroid hormones: oestrogens (oestradiol, oestrone) and androgens (testosterone, androstenedione, DHEAS) and SHBG with risk of TC in the offspring.

4. MATERIALS AND METHODS

4.1. Serum banks

There are three long-standing Maternity cohort serum banks in the Nordic countries: the Finnish Maternity Cohort (FMC) (Oulu, Finland), the Northern Sweden Maternity Cohort (Umeå, Sweden), and the Rubella Screening Serum Bank (Reykjavik, Iceland) (Figure 2.1, p. 15; Table 4.1). In general, these biobanks offer a unique opportunity to study the effect of *in utero* exposures to the health of the offspring.

The *Finnish Maternity Cohort* of the National Institute for Health and Welfare possesses serum samples from almost all pregnant Finnish women (~98%). Following informed consent for screening of congenital infections the samples were collected from women during the first trimester of pregnancy (weeks 8 to 13). Since 1983 the left-over sera have been stored at -25°C.

The *Northern Sweden Maternity Cohort* is based at the University Hospital in Umeå, including residents of the four northernmost counties of Sweden. Blood samples are drawn from more than 95% of pregnant women in these counties during the first trimester or the early weeks of the second trimester (weeks 7 to 18) as a part of screening for infectious diseases. Since 1975 the serum samples have been stored at -20°C.

The *Rubella Screening Serum Bank* at the Department of Virology, University of Iceland, contains serum samples collected from more than 95% of pregnant women in Iceland during the first trimester of pregnancy (8 to 13 weeks). Since 1975 the serum samples have been stored at -20°C (Pukkala et al. 2007).

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Table 4.1. Main characteristics of the Nordic maternity cohorts

Characteristics	Finnish Maternity Cohort	Northern Sweden Maternity Cohort	Rubella Screening Serum Bank
Location	Finnish National Institute for Health and Welfare, Oulu	University Hospital, Umeå	Department of Virology, University of Iceland, Reykjavik
First serum donation	1983	1975	1975
Number of samples	1.71 million from ~98% pregnant women	~121,400 from >95% pregnant women (residents of the 4 northernmost counties)	120,000 from >95% pregnant women
Biological material	serum samples (1st trimester of pregnancy: 8–13 weeks of gestation)	serum samples (1st or the early 2nd trimester of pregnancy: 7–18 weeks of gestation)	serum samples (1st trimester of pregnancy: 8–13 weeks of gestation)
Storage temperature	–25 C	–20 C	–20 C
Aim of collection	screening for congenital infections	screening for congenital infections	screening for congenital infections
Aim of storage	epidemiological research	epidemiological research	epidemiological research

4.2. Cancer registries

The Finnish and Icelandic cancer registries are nationwide, while the regional cancer registry at the Oncological Center in Umeå covers the four northernmost counties in Sweden. Cancer registries receive notifications from hospitals, pathology laboratories and physicians, achieving almost 100% reporting coverage.

Multiple generation linkage of the population census registries, cancer registries and the maternity cohort data enabled identification of women with offspring who had been diagnosed with TC. Approval to collate information between the FMC, the Population Registry and the Cancer Registry was obtained from the Ministry of Social Affairs and Health and the Population Census Registry of Finland (#1422/54/94). Relevant permissions were obtained from the Icelandic National Bioethics Committee (#03-013) and the Icelandic Data Protection Authority (#2003/308). Similar approvals were also available in Sweden from research ethics committees.

4. 3. Study population

Study I

Fourteen random samples were collected every other year from 1984 to 2004 from the FMC storage. Altogether 154 women during the first trimester of their first pregnancy were selected for the hormone analyses. Both mean and median age of the women at sample withdrawal was 29 years (range 25 to 34 years), (Table 4.2).

The following variables have been studied:

- Storage time was defined as the time between sample freezing for storage and sample withdrawal from the storage for the hormone analyses. The mean storage time for the study population was 12 years (range 2 to 22 years).
- Bench-lag time (BLT) was defined as the time between sample collection and freezing for storage. The mean BLT for the study population was 3.5 ± 2.2 days (range 1 to 14 days). Forty-one percent of the samples had a BLT of one to two days, 35% a BLT of three to four days and 24% a BLT greater than four days.
- Gestational day (GD) was defined as the time between the first day of the last menstruation and sample withdrawal of the subjects, and calculated using the formula: $280 - (\text{date of expected delivery} - \text{date of blood sampling at Maternity Care Units})$. The mean GD for the study population was 77.7 ± 26.7 days (range 42 to 222 days).

Studies II, III, IV

The study was conducted as a pair-matched, case-control study nested within Finnish, Swedish and Icelandic maternity cohorts. TC cases diagnosed between 1985 and 2003, between 1976 and 2006, and between 1979 and 2006 were identified respectively from the Finnish, Swedish and Icelandic cancer registries. Initially 68 TC cases in Finland, 34 TC cases in Northern Sweden and 13 TC cases in Iceland were

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diagnosed with a histologically verified testicular germ cell tumour. Altogether 42 TC cases, 45 TC cases and 49 TC cases were excluded from the Study IV, Study III and Study II, respectively. The main reasons for the exclusion of 42 TC cases were absence of maternal serum sample in one of the maternity cohorts (36 cases) or the pregnancy began before the maternity cohort was established (2 cases). Among excluded cases one case had seminoma, three cases had teratoma, five cases had embryonal carcinoma, one case had yolk sac tumour, two cases had mixed germ cell tumour, and 26 cases (from Sweden) had no available information on histology. In addition, four TC cases had somatic malignancy of the testis (i.e. embryonal rhabdomyosarcoma). Seven TC cases (17%) were diagnosed at under 10 years of age (infantile/prepubertal cases), and 35 TC cases (83%) were diagnosed between 15 and 26 years of age (postpubertal cases). Additionally, three TC cases and seven TC cases were excluded from Study III and Study II respectively due to these being no remaining maternal serum samples stored at the FMC (Table 4.2).

Thus, altogether 73 TC cases, 70 TC cases and 66 TC cases whose index mothers had donated serum sample to one of the maternity cohorts were selected for Study IV, Study III and Study II respectively. Approximately nine (Study III) or four control mothers (Study II, IV) in Finland, four control mothers in Sweden and three control mothers in Iceland with male offspring free of TC at the time of diagnosis of the case were matched with the index mothers. The matching criterion was date of birth of the son (\pm one month). The control group comprised 286 (Study IV), 519 (Study III) and 258 (Study II) control mothers. Descriptive statistics for maternal and offspring's variables are presented in Table 4.2.

Table 4.2. Study population

Study	Country	Time of diagnosis	Initial number of cases	Final number of cases	Number of matched controls	Total	Median maternal age (range), years	Median/mean age at diagnosis (range), years	Number of prepubertal TC cases (%)	Number of postpubertal TC cases (%)	Histology – number of cases (%)
I	Finland	1984–2004	–	–	–	154	29 (25–34)	–	–	–	
II	Finland	1985–2003	68	52	208						seminoma – 11 (16.7); teratoma – 21 (31.8); embryonal carcinoma – 15 (22.7);
	Sweden	1976–2006	34	8	32						yolk sac tumours – 14 (21.2); mixed germ cell tumour – 4 (6.1); choriocarcinoma – 1 (1.5%)
	Iceland	1979–2006	13	6	18						
	Total		115	66	258	324	28 (17–45)	17/11 (0–26)	29 (44)	37 (56)	
III	Finland	1985–2003	68	56	469						seminoma – 11 (16.7); teratoma – 24 (31.8); embryonal carcinoma – 16 (22.7);
	Sweden	1976–2006	34	8	32						yolk sac tumours – 14 (21.2); mixed germ cell tumour – 4 (6.1); choriocarcinoma – 1 (1.5%)
	Iceland	1979–2006	13	6	18						
	Total		115	70	519	589	28 (17–47)	15/10 (0–25)	31 (44)	39 (56)	
IV	Finland	1985–2003	68	59	236						seminoma – 11 (16.7); teratoma – 24 (31.8); embryonal carcinoma – 16 (22.7);
	Sweden	1976–2006	34	8	32						yolk sac tumours – 14 (21.2); mixed germ cell tumour – 4 (6.1); choriocarcinoma – 1 (1.5%)*
	Iceland	1979–2006	13	6	18						
	Total		115	73	286	359	28 (17–45)	17/11 (0–25)	31 (44)*	39 (56)*	

* Three TC cases had unknown histology and age at diagnosis

4. 4. Laboratory methods

All study samples were blinded before they were sent to the analysing laboratory in order to ensure protection of integrity and similar processing of cases and controls. Each case and control had a code indicating its case-control status but did not include personal information that could connect the samples to the donors. Assays were performed in batches of 76 serum samples, analysed together on the same day and with the same laboratory kit. Serum samples of case and control subjects that were matched together were systematically analysed within the same batch.

Study I

A total volume of 370 µL from 154 previously unfrozen serum samples was aliquoted from each sample and sent to the Department of Medical Bioscience, University of Umeå, Sweden for the hormone and protein measurements. Quantification of serum hormones and associated binding-proteins was performed in the same series of analyses using commercial competitive or non-competitive enzyme-linked or radio- immunoassays. Specifications of the hormone assays and intra- and inter-assay coefficients of variation (CV) obtained from the manufacturers are given in Table 4.3.

Table 4.3. Assay methods used

Analyte	Method	Range of coefficient of variation (%)	
		Intra assay	Inter assay
Oestradiol	ALPCO Diagnostics Oestradiol EIA	7.5-12.2	8.2-12.1
Progesterone	R&D Systems' Progesterone EIA	4.9-7.6	2.7-8.3
Testosterone	DPC IMMULITE 2000 Total testosterone Chemiluminescent EIA	5.1-16.3	7.2-24.3
DHEAS	DPC IMMULITE 2000 DHEA-SO ₄ Chemiluminescent EIA	4.9-9.8	7.9-13
SHBG	DPC IMMULITE 2000 SHBG Chemiluminescent EIA	0.03-4.2	4.0-6.6
hCG	DPC hCG Coat-A-Count IRMA	2.6-5.8	4.6-7.0
AFP	DPC IMMULITE 2000 AFP Chemiluminescent EIA	2.1-6.3	4.5-12
pGH	BIOCODE-HYCEL pGH IRMA	3.0-5.5	5.0-7.9
IGF-1	DSL IGF-1 ELISA (DSL-10-2800)	4.5-8.6	3. 3-6.8
IGFBP-3	DSL IGFBP-3 ELISA (DSL-10-6600)	7.3-9.6	8.2-11.4
total IGFBP-1	DSL Total IGFBP-1 ELISA (DSL-10-7800)	1.7-4.6	6.2-7.6
np IGFBP-1	Medix Biochemica IGFBP-1 IEMA	4.4-5.4	4.4-5.9

Note: EIA – enzyme immunoassay; IRMA - immunoradiometric assay; ELISA - enzyme-linked immunosorbent assay; IEMA - immunoenzymetric assay.

Study II

Immunoglobulin (Ig)M and IgG antibodies to EBV and CMV were determined according to manufacturers' instructions by means of commercial standard enzyme-linked immunosorbent assays (ELISAs). These measurements were performed at the National Institute for Health and Welfare, Oulu, Finland. For EBV IgM and IgG antibodies to viral capsid antigen (VCA), a commercially available ELISA (Panbio Diagnostics, Brisbane, Australia) was used with reported >85.7% and 100% sensitivity, >87.5% and >87.2% specificity respectively. For CMV IgM and IgG, ELISA (Biokit, Barcelona, Spain) was used with reported 93.8% and 97.6% sensitivity and 97.8% and 99.5% specificity respectively. The cutoff levels were preassigned by following the manufacturers' recommendations relative to internal positive and negative reference sera used on all plates. Median IgG antibody level was used as an alternative cutoff for high IgG antibody levels. Specificity of the response was controlled for by separately considering IgM positives that were positive for only one of the two IgM tests.

Study III

The laboratory analysis was performed at the National Institute for Health and Welfare, Kuopio, Finland. Serum cotinine levels were measured by novel HPLC-MS/MS system. HPCL-MS/MS system used was Waters 2695 Separations System (Milford, MA, USA) connected to Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (San Jose, CA, USA) operated in the atmospheric pressure chemical ionization-mode. In the HPLC system Waters XTerra C18 column was used (50 mm length, 2.1 mm diameter, 3.5 μ m particles size). Limit of quantitation (LOQ) was 2.0 ng/ml calculated as six times standard deviation of blank. The linear range of the method was from LOQ to 1000 ng/ml and expanded measurement uncertainty was estimated to be 35%.

Study IV

Measurements of serum levels of steroid hormones and associated binding protein were performed at the Department of Medical Biosciences, University of Umeå, Umeå, Sweden. Serum DHEAS and androstenedione levels were measured by competitive chemiluminescent enzyme-immunoassay (DPC Immulite 2000 DHEA-SO₄ Chemiluminescent EIA, UK; DPC Immulite 2000 Androstenedione Chemiluminescent EIA, UK respectively). Serum total testosterone, total oestradiol and oestrone levels were measured by competitive radioimmunoassay (DPC Coat-A-Count Total Testosterone RIA, USA; Spectria Oestradiol Sensitive RIA, Orion Diagnostica, Finland; DSL Oestrone RIA, DSL-8700, USA, respectively). Serum SHBG level was measured by chemiluminescent immunometric assay (DPC Immulite 2000 SHBG, UK).

The inter-assay CV calculated for the laboratory determined commercially available control sera included in each assay run were 4.1% for DHEAS at 5.1 µmol/L, 5.8% for androstenedione at 4.3 ng/mL, 5.5% for testosterone at 3.9 nmol/L, 13.4% for oestradiol at 530 pmol/L, 12.2% for oestrone at 310 pg/mL and 6.2% for SHBG at 74 nmol/L. Serum free testosterone and free oestradiol levels, unbound to SHBG or albumin, were calculated from the absolute levels of each of the steroids and SHBG using mass action equations, and assuming a constant serum albumin level of 43 g/L (Rinaldi et al. 2002).

4.5. Statistical methods

Descriptive statistics were calculated for each variable separately in each Study. The seroprevalence was determined for EBV and CMV. Differences in seroprevalences between groups were tested by chi-square test (Study II). All measured hormone and other protein levels were ln-transformed to reduce departures from the normal distribution (Study I, IV). Box-plots were drawn for the hormone levels and storage time (Study I). Spearman's partial ranked correlation coefficient (r_s) was used to test for correlation between the serum hormone levels and 1) maternal smoking, 2) maternal age (Study IV), 3) storage time, 4) BLT, 5) GD at serum sampling (Study I).

The effect of BLT on hormone levels was evaluated on a continuous scale by multivariate regression analysis. Additionally, a nonparametric local regression model with smoothing parameters set from 0.1 to 1 was fitted to describe the effect of GD on variation of hormone levels. The best smoothing parameter was chosen for every hormone according to the smallest AIC_{c1} statistics for all the smoothing parameters that were specified in the model statement. These analyses were done using (GLM), (CORR) and (LOESS) procedures of the SAS program version 9.1 (Study I).

Relative risks, expressed as OR and their 95% confidence intervals (CI) were estimated by conditional logistic regression (Studies II, III, IV). Quartile cut points for hormone levels were used to estimate the relative risks. Linear-by-linear association test was used to present p-value for trend in OR for hormone levels divided by quartile cut points. Multivariable models were adjusted for GD, maternal age and maternal smoking status (Study IV).

Separate analyses were performed for the two main histological sub-groups of TC, seminomas and nonseminomas; for two groups according to age at diagnosis (≤ 8 years of age and ≥ 15 years of age) (Studies II, III, IV); for the two groups of maternal age (17–28, 29–47 years of age); and for the four groups of cotinine level: non-smokers (< 5 ng/ml), passive smokers (5–14.9 ng/ml), light smokers (15–99.9 ng/ml) and heavy smokers (≥ 100 ng/ml), (Study III). Homogeneity of ORs across age at primary diagnosis and histological sub-groups was tested according to Breslow and Day (Breslow and Day 1980; Study II). Two-sided $p < 0.05$ was considered statistically significant. The relative risk analyses were performed using SPSS for Windows 15.1 (SPSS, Inc., Chicago, Illinois).

In the meta-analysis Mantel–Haenszel fixed-effects model was used to calculate the summary estimate. Two-sided $p < 0.05$ was considered statistically significant. Chi-square test for heterogeneity was evaluated ($p < 0.1$ was considered representative of significant statistical heterogeneity) Statistical analysis for meta-analysis was performed with R version 2.5–1 (R Development Core Team, Study III).

5. RESULTS

Study I

Stored FMC serum samples can be used to study hormone-disease associations

A decrease by storage time was shown for serum levels of progesterone ($r_s=-0.36$), IGF-I ($r_s=-0.23$) and IGFBP-3 ($r_s=-0.38$), whereas an increase by storage time was found for oestradiol ($r_s=0.23$), SHBG ($r_s=0.16$), and AFP ($r_s=0.20$) levels, and serum levels of non-phosphorylated (np) IGF binding protein (BP)-1 ($r_s=0.27$). No correlation was observed between the storage time and serum levels of testosterone, DHEAS, hCG, pGH and total IGFBP-1.

There was no correlation between BLT and any of the hormones analysed.

Samples from individuals with GD between 40 and 120 days (145 women) were used for the analysis of the impact of GD. Hormone levels by GD followed the dynamics of the pregnancy. Serum levels of oestradiol ($r_s=0.42$), progesterone ($r_s=0.21$), SHBG ($r_s=0.54$), pGH ($r_s=0.66$), AFP ($r_s=0.7$), total IGFBP-1 ($r_s=0.54$), np IGFBP-1 ($r_s=0.49$) increased with increasing GD, while levels of DHEAS ($r_s=-0.25$) and hCG ($r_s=-0.37$) levels decreased with increasing GD.

Study II

Increased risk of TC in the offspring of mothers with high antibody levels to EBV – but no consistent association with antibodies to CMV

Almost all index and control mothers were positive for IgG EBV. Therefore, median IgG antibody level was used as a cutoff for low and high IgG antibody levels. A tentative association was identified in the high EBV IgG antibody group (OR, 2.50; 95% CI, 1.15–5.40) compared to low EBV IgG antibody group. The association was especially true for high EBV IgG antibody level and nonseminoma TC (OR, 2.73; 95% CI, 1.25–5.99). Further division of the nonseminoma cases by age at diagnosis yielded a statistically significant high EBV IgG antibody associated risk of TC occurring in the infantile/prepubertal period (OR, 2.72; 95% CI, 1.05–7.04) (Table

5.1).

By contrast, the risk of TC in cases diagnosed in the infantile/prepubertal period tended to be decreased among the offspring of CMV seropositive women (OR, 0.35; 95% CI, 0.14–0.89) (Table 5.1).

Table 5.1. Odds ratio (95% CI) of TC associated with maternal EBV and CMV antibodies, stratified by histological type and age at diagnosis.

Category	Number (cases/ controls)	Epstein-Barr virus		Cytomegalovirus	
		IgM	IgG [†]	IgM	IgG
		OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
Age at diagnosis, years [‡]					
Infantile/prepubertal period, (≤ 8)	29/115	n.a.	2.72 (1.05–7.04)	n.a.	0.35 (0.14–0.89)
Postpubertal period, (≥ 15 ; ≤ 26)	37/143	n.a.	2.96 (0.88–9.39)	1.0 (0.11–8.94)	2.97 (0.87–10.17)
Histology					
Non-seminoma	55/214	n.a.	2.73 (1.25–5.99)	0.66 (0.08–5.53)	0.80 (0.39–1.64)
Infantile/prepubertal period, (≤ 8)	29/115	n.a.	2.72 (1.05–7.04)	n.a.	0.35 (0.14–0.89)
Postpubertal period, (≥ 15 ; ≤ 26)	26/99	n.a.	2.75 (0.69–11.01)	1.33 (0.14–12.81)	3.08 (0.68–13.94)
Seminoma	11/44	n.a.	3.68 (0.31–43.26)	n.a.	2.75 (0.33–23.05)
Postpubertal period, (≥ 15 ; ≤ 26)	11/44	n.a.	3.68 (0.31–43.26)	n.a.	2.75 (0.33–23.05)

Note: There were no TC cases between 8 and 15 years of age.

[†] Subjects with low and high IgG antibody levels to EBV included in the model.

[‡] Included cases with all histological types.

Study III

No excess risk of TC in the offspring of mothers smoking during pregnancy

No statistically significant association between maternal serum cotinine level and risk of TC in the offspring was found (OR, 0.68; 95% CI, 0.35–1.34). The value of 15 ng/ml was used as a cutoff level for smokers and non-smokers. The point estimate was also similar for infantile/prepubertal and postpubertal TCs. There was no statistically significant difference between the seminoma and nonseminoma cases.

A literature search for the meta-analysis was carry out in PubMed up to January 2008 using search terms “maternal”, “smoking”, “epidemiology”, “offspring”, (“testicular” or “testis”), (“cancer” or “tumour” or “malignancy”) and with no language restrictions. Additionally, references from relevant publications and review articles were checked. Out of a total of 17 publications, seven were eligible for further

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analysis fulfilling the selection criteria of sufficient information on exposure (obtained via questionnaires/interviews with answer “Yes/No” or number of cigarettes smoked per day), outcome (obtained via hospital or population based registries) and study design (case-control or nested case-control studies). To be included in meta-analysis, studies had to present number of cases and controls and information on measures of relative risk (unadjusted and/or adjusted ORs). Data concerning study design, population characteristics, matching factors for cases and controls, adjusted factors for the multivariate analysis were extracted from these studies using a standardized data abstraction form (Table 5.2). Meta-analysis included a total of 2,149 TC cases. The summary estimate of meta-analysis supported our results and indicated no association between maternal smoking and risk of TC in the offspring (OR, 1.0; 95% CI, 0.88–1.12, Table 5.2).

Table 5.2. Selected characteristics of studies on the association between maternal smoking and TC in the offspring.

Author, year	Study design	Source of cases/controls	Source of smoking data	Case recruitment period	Matching factors	Adjusting factors	Cases (No) [†]	Controls (No) [†]	OR (95% CI)
Swerdlow, 1987	CCS	Radiotherapy centres/hospital controls	Interview, case notes	1977–1981	Age (the same)	–	218	404	1.01 (0.71–1.45)
Moller, 1996	CCS	Cancer Registry/Central Person Register	Interview, questionnaire	1986–1988	Year of birth	–	296	287	0.97 (0.69–1.36)
Weir, 2000	CCS	Cancer Registry/Ministry of Revenue’s Enumeration Composite Records	Interview, self-administered questionnaire	1987–1989, 16–59 yrs of age	Age (the same within 5-yrs age groups)	Age, exogenous hormone use during pregnancy, bleeding/threatened miscarriage, pregnancy length, treatment for undescended testicle	339	511	0.86 (0.64–1.17)
Coupland, 2004	CCS	Cancer treatment centres or regional cancer registries/hospital controls	Interview, Questionnaire	1984–1987, 15–49 yrs of age	Date of birth (within 1 yr)	Age, place of residence	446	420	1.22 (0.91–1.63)
McGlynn, 2006	CCS	Military medical databases/Defence Serum Repository	Interview, questionnaire	2002–2005, 0–46 yrs of age	Age (within 1 yr), race, date of serum sample drawn (within 30 days)	Son’s age, race, family history of TC	514	560	1.01 (0.80–1.29)
Pettersson, 2007a	NCCS [§]	Cancer Registry/hospital controls	Birth registry (records in standardised manner)	1973–2002, ≥15 yrs of age	Date of birth (first three control children born after case child)	Age, histology, maternal age, birth order, gestational duration.	192	494	0.90 (0.64–1.26)
Sonke, 2007	CCS [*]	Registry, hospital based/case nominated	Interview, self-administered questionnaire	1990–1996, 18–50 yrs of age	Race, age (±5 yrs), state of residence	Mother’s race, education, body mass index, son’s birth weight, age, history of cryptorchidism, nausea, length of pregnancy	144	86	1.1 (0.50–2.2)

Summary estimate, Mantel-Haenszel OR: 1.0; 95% CI: (0.88, 1.12)

Test for heterogeneity: $\chi^2 = 3.02$; (*p*-value, 0.8)

^{*} Case-control study;

[§] Nested case-control study;

[†] Number of cases and controls eligible for the smoking exposure data;

Study IV

The interplay of maternal steroid hormones in early pregnancy is important in the aetiology of TC in the offspring

Quartile cut points for hormone levels were used to estimate the ORs. Offspring of mothers with the highest DHEAS levels had a significantly decreased risk of TC adjusted for GD, maternal age and maternal smoking status (OR, 0.18; 95% CI, 0.06–0.58). However, offspring of mothers with the highest androstenedione levels had an increased risk of TC adjusted for GD, maternal age and maternal smoking status (OR, 4.05; 95% CI, 1.2–12.0), (Table 5.3). Comparable decreased risk of TC associated with increased DHEAS level (OR, 0.36; 95% CI, 0.17–0.74), and increased risk of TC associated with increased androstenedione level (OR, 3.1; 95% CI, 1.05–9.2) were also observed using log-transformed continuous scales of the hormone levels. With adjustment removed the ORs were the same as when adjusted. The results were similar when the Finnish data was analysed separately.

In a stratified analysis using the continuous scale, the risk of TC in cases diagnosed under 8 years of age (the infantile/prepubertal period) and risk of nonseminoma TC adjusted for GD, maternal age and maternal smoking status tended to be decreased among the offspring of women with increased DHEAS level (OR, 0.10; 95% CI, 0.01–0.80), (OR, 0.11; 95% CI, 0.03–0.51) respectively. The opposite was true for increased androstenedione level (OR, 47; 95% CI, 2.3–970), (OR, 27; 95% CI, 2.7–270) respectively. Other maternal steroid hormone levels and risk of TC in the offspring did not show statistically significant differences in the different histology and age groups (data not shown).

High maternal total oestradiol level showed a very high point estimate for the increased risk of TC in the offspring adjusted for GD, maternal age and maternal smoking status (OR, 32; 95% CI, 0.98–1090) (Table 5.3).

Table 5.3. Odds ratio (95% CI) of TC associated with maternal hormone levels.

Hormone	Quartile categories				<i>p</i> for trend
	1	2	3	4	
DHEAS					
Cut points, µmol/L	≤ 2.5	2.6–3.6	3.7–4.9	>4.9	
Number (cases/controls)	91 (23/68)	92 (19/73)	88 (16/72)	90 (15/75)	
OR (95% CI)*	1.0 (Referent)	0.33 (0.13–0.83)	0.25 (0.09–0.71)	0.18 (0.06–0.58)	0.13
Androstenedione					
Cut points, ng/mL	≤2.9	3.0–3.6	3.7–4.5	>4.5	
Number (cases/controls)	91 (12/79)	90 (21/69)	90 (20/70)	90 (20/70)	
OR (95% CI)*	1.0 (Referent)	3.6 (1.3–9.8)	3.03 (1.02–8.9)	4.05 (1.2–12.0)	0.17
Total testosterone					
Cut points, nmol/L	≤1.8	1.9–2.5	2.6–3.6	>3.6	
Number (cases/controls)	93 (15/78)	90 (21/69)	89 (21/68)	88 (15/73)	
OR (95% CI)*	1.0 (Referent)	2.9 (0.9–9.1)	4.2 (1.04–16.0)	2.02 (0.36–11.0)	0.85
Free testosterone					
Cut points, pmol/L	≤11.3	11.3–18.02	18.03–28.42	>28.42	
Number (cases/controls)	90 (21/69)	90 (17/73)	90 (17/73)	89 (17/72)	0.50
OR (95% CI)*	1.0 (Referent)	0.64 (0.21–2.02)	0.39 (0.08–1.8)	0.49 (0.07–3.3)	
Total oestradiol					
Cut points, pmol/L	≤603	604–984	985–1880	>1880	
Number (cases/controls)	88 (10/78)	87 (23/64)	88 (19/69)	87 (18/69)	
OR (95% CI)*	1.0 (Referent)	4.7 (0.81–27)	13.0 (1.1–180)	32.0 (0.98–1090)	0.22
Free oestradiol					
Cut points, pmol/L	≤7.76	7.77–11.14	11.15–18.08	>18.08	
Number (cases/controls)	87 (15/72)	88 (19/69)	87 (19/68)	87 (17/70)	
OR (95% CI)*	1.0 (Referent)	0.64 (0.17–2.3)	0.39 (0.07–1.9)	0.31 (0.03–3.3)	0.71
Oestrone					
Cut points, pg/mL	≤547	547.01–864	864.01–1700	>1700	
Number (cases/controls)	91 (13/78)	91 (24/67)	90 (18/72)	89 (18/71)	
OR (95% CI)*	1.0 (Referent)	1.1 (0.24–5.1)	0.69 (0.09–5.02)	0.22 (0.01–3.8)	0.54
SHBG					
Cut points, nmol/L	≤139	139.1–222.0	222.1–281	>281	
Number (cases/controls)	92 (16/76)	91 (21/70)	87 (14/73)	90 (22/68)	
OR (95% CI)*	1.0 (Referent)	0.77 (0.23–2.5)	0.28 (0.06–1.4)	0.25 (0.04–1.6)	0.47

* OR (95% CI) from conditional logistic regression adjusted for gestational day (GD), maternal age and maternal smoking status.

6. DISCUSSION

The present studies were conducted to investigate the impact of congenital infections or susceptibility to perinatal infections, maternal smoking, and early pregnancy steroid hormones on risk of TC in the offspring. Over-generation linkage of population-based maternity cohorts and cancer registries enabled a unique nested case-control setting for the study. The risk exposures were associated from blinded, first trimester serum samples of the index and control mothers by state-of-the-art laboratory methods. Furthermore, exposure assessment from the stored sera had been validated.

No consistent pattern of variation of the hormones or binding-proteins with storage time was found. Serum samples can, therefore, be used to study hormone-disease associations, but matching for storage time is necessary for all biobank-based studies. High levels of maternal EBV IgG antibodies were associated with the risk of TC in the offspring, especially with the risk of nonseminomatous TC diagnosed under eight years of age. A statistically significant association was also noted between decreased risk of TC diagnosed under eight years of age and maternal CMV IgG antibodies. Maternal serum cotinine analysis did not provide support for the hypothesis that maternal smoking during pregnancy is a risk factor for TC. As for the steroid hormones, offspring of mothers with high DHEAS levels had a significantly decreased risk of TC, whereas offspring of mothers with high androstenedione level had an increased risk of TC. Below the validity of our approach and observations, and the generated new data are discussed.

6.1. Strengths and limitations of the study

6.1.1. Biobank-based approach in the context of TC aetiology

The large Nordic maternity screening cohort based biobanks used in the present studies offered a unique opportunity to study the impact of early pregnancy exposures on risk of TC in the male offspring. The examination of prospectively collected samples is crucial to establish the temporal relationship required for inferences on

causality. Epidemiological studies investigating the biological effect of pregnancy and maternal risk factors associated with the offspring's cancer risk have, however, rarely relied on early pregnancy hormone measurements (van de Beek et al. 2004, Troisi et al. 2003b). Indeed, during the first trimester of pregnancy the foetus is particularly vulnerable to infections or metabolically active substances with a potential to deteriorate cellular growth and differentiation. This period of pregnancy is also critical for urogenital differentiation. The first trimester of pregnancy differs endocrinologically from the second and third trimesters. At best moderate degrees of correlation in steroid hormone levels between either maternal serum from the second/third trimester of pregnancy and amniotic fluid (van de Beek et al. 2004), or umbilical cord samples have not encouraged the use of maternal samples for biobank studies (Troisi et al. 2003b).

In biobank-based studies the process from the decision to take a sample from an individual to the moment the sample is safely placed in the biobank consists of several phases including collection of samples, handling and storage of samples. Failure in one of these standardized procedures may have significant consequences as the quality of the material stored in the biobank as well as conclusions and recommendations based on the analysis of such material may be severely affected.

Overall, the stability of steroid and other pregnancy-related hormones and associated binding-proteins or causes of their degradation in stored serum/plasma samples are not well known. The effect of storage time on the measurement of specific hormones may vary according to the assay method. Different antibodies may bind to different parts of peptide hormones, and this could affect the performance in old samples if, for example, the terminal amino acids are lost. There is also the potential of hormone measurement variability by BLT (Hankinson et al. 1989, Key et al. 1996, Masse et al. 2000, Kristal et al. 2005).

Our validation study investigated the impact of long-term storage of serum samples obtained from pregnant women, on the measurement of steroid and other pregnancy related hormones and associated proteins. The study did not show consistent variations in steroid hormone levels by storage time from 2 to 22 years. Some hormone and the associated binding-protein levels decreased and some increased with

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increasing storage time. However, the changes did not exceed the inter-assay variations of the enzyme-immunological tests, and the levels of variation remained within the clinically normal ranges. Furthermore, no substantial effect of the length of BLT on the steroid and pregnancy-specific hormone and binding-protein levels could be found.

During pregnancy the different steroid hormone levels undergo considerable variations. In our validation study the variation of hormone levels by GD followed the dynamics of the pregnancy and was in agreement with what was expected according to available clinical data (Speroff 1994). However, trimester-specific reference intervals for several hormones would have been difficult to establish due to lack of specificity of the enzyme-immunoassays for a given steroid hormone (Soldin et al. 2005). Thus, in nested case-control studies considering pregnancy-related hormones the data on GD at sample withdrawal must be taken into account in matching.

Although the reliability of the cotinine measurements and measurements of antibody levels to EBV and CMV in serum samples stored for a long time have not been evaluated in this study, there is some evidence in the literature that these markers are relatively stable over storage time. For instance, in one study cotinine levels retained their discriminant value even after ten years of storage (Riboli et al. 1995). The stored samples (up to 25 years) were shown to be reliable for the diagnosis of congenital CMV using assays for specific antibodies and pathogen nucleic acids (Nørgaard-Pedersen and Simonsen 1999). The stability of antibodies to EBV in biological samples with storage time was also shown in one study (Stevens et al. 2007).

6.1.2. Representativeness of the study population

Testicular tumours are a heterogeneous group of neoplasms traditionally separated by histology (seminoma and nonseminoma) and age at diagnosis (infantile/prepubertal TC, postpubertal TC and spermatocytic seminoma). Each of these tumours has its own epidemiological, clinical, pathologic and genetic characteristics. They all originate from germ cells at different stages of development. The most common TCs (seminomas) arise in postpubertal men and are characterized genetically by having

one or more copies of an isochromosome of the short arm of chromosome 12 [i(12p)] or other forms of 12p amplification. Tumours arising in infantile/prepubertal gonads are either teratomas or yolk sac tumours, tend to be diploid and are not associated with i(12p). Furthermore, the worldwide incidence of infantile/prepubertal TC has no apparent evidence on trends over time (Walsh et al. 2008) whereas the incidence of postpubertal TC has dramatically increased over the last 40 years (Huyghe et al. 2003, Huyghe et al. 2007).

Due to the young age of the present subjects (aged between 0 and 25 years) the infantile/prepubertal TC cases dominate in our study population compared to normal population, where these tumours are very rare (0.12–2 per 100,000 men). Especially low numbers of seminomas must be noted in our study population. Therefore, our results, probably, cannot be extrapolated to the normal population of TC cases aged between 20 to 40 years. However, given time the biobank based approach will also be valid for aetiological studies in these cancer cases, i.e., the bulk of TC.

The study populations, i.e. the Nordic maternity cohorts, stem from population-based screening for congenital infections (e.g., syphilis), which for decades has been a constant component of health care in the affluent Nordic countries. Various studies have shown that participation in screening is virtually complete (98% of the population participates) (Koskela et al. 2000). This representativeness of the maternity cohorts has also been shown when comparing the incidences of chronic diseases in the maternity cohorts to those of the female population in general, with the exception of some cancers, e.g. ovarian cancer, where pregnancy has a significant impact (Pukkala et al. 2007). This, however, is not a relevant issue for studies on the aetiology of cancer in the offspring.

The ASR of TC in Finland, Sweden and Iceland does not vary widely (3.7 per 100,000 men in Finland; 5.3 per 100,000 men in Sweden; 5.4 per 100,000 men in Iceland). The majority of the cases were from Finland, and in the different analyses we gave both country-specific results and pooled results (with country-matched cases and controls) to obtain by far the largest longitudinal, over-generation linked study population.

A possible limitation of our study was also that we did not control for some pregnancy related characteristics not available in the maternity cohorts (e.g., cryptorchidism, maternal weight, birth weight, nausea etc.), which might have confounded the disease-exposure association. Furthermore, the confidence intervals for the point estimates in this study are relatively large due to the small number of cases. Also, the small numbers of cases does not enable statistical interaction analyses. Of particular interest is the interaction between pregnancy-related characteristics (maternal age, maternal smoking etc.) and levels of steroid hormones and their risk to TC development. Therefore, studies with longer follow-up time and larger sample size are needed.

6.1.3. Validity of the assays and analyte measurements

Primary EBV infection during pregnancy seems to be rare, occurring in approximately 1.5% of all pregnancies (Eskild et al. 2005). Pregnant women may, however, have higher incidence of EBV reactivation due to immune suppression during pregnancy (Purtilo and Sakamoto 1982, Nakamura et al. 1993, Obel et al. 1996, Bruu et al. 2000). EBV may cross the placenta causing foetal infections (Avgil and Ornoy 2006). However, serology is an indirect and non-specific measure of exposure to an infectious agent. High EBV VCA IgG antibodies are present following infectious mononucleosis and EBV reactivation, but also in asymptomatic or past infections. EBV VCA IgM antibodies indicate primary infection (Okano et al. 1988) but in EBV reactivations, the sensitivity and specificity of the VCA IgG and IgM antibody tests are not good (Bruu et al. 2000). Thus, other EBV tests are needed to confirm the reactivation of EBV infection in the index mothers during pregnancy.

Nicotine addiction is strong, and indication of smoking at any time during pregnancy is likely to be representative of the whole pregnancy (Kullander and Källén 1971, Raatikainen et al. 2007). It has, however, been noted that due to the common knowledge of the adverse effects of smoking on the health and development of the foetus pregnant women tend to deny or under-report their smoking habit (Cope et al. 2001). This is why objective indicators of current smoking are ideal indicators of smoking in pregnant women. It is important to note that, our cotinine results, a biochemically determined metabolite of nicotine, based smoking prevalence were

consistent with self-reported smoking prevalence among young women in Finland (26%) (Raatikainen et al. 2007) and in Sweden (21%) (Högberg and Cnattingius 2007).

The sensitivity (>95%) of the novel HPLC/MS/MS method we used to analyse cotinine was adequate. The limit for active smoking was set between 10 and 20 ng of cotinine per one ml. Variation of the cotinine levels in the serum samples analysed was wide, from less than 2 to 538 ng/ml. The criterion set for active smoking exceeding 15 ng/ml was represented in 20% of our study population, while 11% exceeded 100 ng/ml, which was considered an indicator for heavy smoking among females.

In general, the steroid hormone levels we report remained within the clinically normal range of early pregnancy and were consistent with our validation study and earlier study, evaluating serum levels of steroid hormones during the first trimester of pregnancy (Soldin et al. 2005). The measured hormone levels did not exceed the inter-assay variations determined by the enzyme-immunological tests. Furthermore, the cross-reactivity of the assays was very low, even to similar steroid hormones. However, the ranges of the oestrogens may be regarded as high with some outliers included. Indeed, GD may have had an effect on the hormone ranges. However, in this study matching for GD was taken into account to avoid its effect on true exposure-disease association. Furthermore, an appropriate descriptive way of expressing the variation of skewed hormone distribution was to give the median hormone levels, and the hormone levels at the 25th and 75th percentiles, which excludes the outliers.

6.2. Comparison of the study results with findings from other studies

The histological spectrum of TC and the typical incidence peak at very young age suggest that its risk factors operate early in life, possibly already *in utero*. The first trimester of pregnancy is the critical period for urogenital differentiation. Exposures acting during foetal life can disrupt the normal development of the primordial germ cells and/or gonocytes, generating CIS lesions (Skakkebaek et al. 1987, Rajpert-De

Meyts et al. 2003). This eventually gives rise to germ cell tumours of a variety of histological types depending on their particular stage of “developmental arrest” (Rajpert-De Meyts 2006).

6.2.1. Association of maternal infections and risk of TC in the offspring

In adulthood the prevalence of EBV and CMV infections, depending on age, race and socioeconomic status, accounts for about 80–90% and 40–90% of the total population respectively. Interestingly, primary infections with EBV and CMV occur in approximately 1.5% and 0.7–4.1% of pregnancies respectively (Eskild et al. 2005, Pusztai et al. 2004). Indeed, in our study population EBV IgM and CMV IgM antibodies were practically missing indicating absence of primary infection with EBV and CMV. However, EBV and CMV can pass through the placenta even if the infection and immune response have occurred prior to conception and are reactivated during the pregnancy (Avgil and Ornoy 2006, Pass et al. 2006). This may, in principle, contribute to testicular carcinogenesis (Lang and Kummer 1972, Mueller et al. 1988). In our study population, however, we could not confirm the reactivation of EBV and CMV infections in the index and control mothers.

EBV has been considered as a possible cause of TC due to its high transforming potential and its association with various human neoplasms (Shibata et al. 1991, Iezioni et al. 1995, Lehtinen et al. 2003, Rezk and Weiss 2007) most notably Hodgkin’s lymphoma (Herbst et al. 1990, Brousset et al. 1993). Indeed, Hodgkin’s lymphoma shows epidemiological similarities to TC with a peak age in adolescents and young adults (Newell et al. 1984). It has been suggested that susceptibility to both TC and EBV orchitis may differ before and after the completion of testicular differentiation (Shimakage et al. 1996). Furthermore, women with significant EBV reactivation tend to have shorter pregnancies and offspring with lower birth weight (Eskild et al. 2005).

Most normal testicular deoxyribonucleic acid (DNA) samples from adolescents or young adults as well as testicular tumour DNA samples contain the EBV genome, but

the expression of viral RNA has been detected in embryonal testicular tumours (Shimakage et al. 1993, Shimakage et al. 1996). EBNA 2 and latent membrane protein (LMP) were also found in the testicular tumours (Shimakage et al. 1996). The fact that expression of early EBV RNA has been reported in embryonal carcinomas but not in seminomas (Shimakage et al. 1993) is in line with our results. To rule out the possibility that EBV reactivation is a marker of a pathogenetic process rather than the causal factor (Akre et al. 1999, Sundström et al. 2004, Eskild et al. 2005) further studies are needed.

Protection against childhood TC was associated with the presence of maternal CMV IgG antibodies. This finding may not have an interpretation. It suggests that CMV infection of the infant may be associated with the risk of TC. Indeed, testicle is a site exposed to CMV (Lang and Kummer 1972). Hence, CMV infection could from the early years of life induce carcinogenic alterations in the testicular tissue. Further studies, however, are needed to confirm the role of CMV infection in TC.

6.2.2. Association of maternal smoking and risk of TC in the offspring

An examination of parallel trends in rates of TC and female lung and bladder cancers led author to hypothesise that maternal smoking is the risk factor for TC (Clemmensen 1997). In addition, one ecological study showed a highly significant association between prevalence of female smoking and incidence of TC in the Nordic countries. This was, however, not true for Finland. Both the prevalence of smoking among females and TC incidence were highest in Denmark and lowest in Finland (Pettersson et al. 2004). On the other hand, several questionnaire-based studies with sufficient information on exposure, outcome and possible confounding factors have not shown an association between maternal smoking and risk of TC (Brown et al. 1986, Swerdlow et al. 1987, Weir et al. 2000, Coupland et al. 2004, McGlynn et al. 2006, Pettersson et al. 2007a, Sonke et al. 2007). The source of the data in these studies, however, may be a concern due to possibly lower participation rate of mothers who smoked in pregnancy. The present study also provides no evidence for an association between maternal smoking and risk of TC, when smoking status was

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based on a biological measurement, serum cotinine levels. To the best of our knowledge this is the first study on TC risk in the offspring and maternal serum cotinine levels.

An association between maternal smoking and TC may be affected by another potential common risk factor such as alcohol consumption. However, no association was found between childhood germ cell tumours and maternal alcohol intake in several studies (Weir et al. 2000, Chen et al. 2005a). Moreover, there are no reliable methods to determine alcohol consumption from the stored serum samples.

Although pregnancy smoking cannot be entirely discarded as a minor risk factor for TC, we believe that our biobank-based study together with previous questionnaire-based studies included in our meta-analysis provide strong evidence to rule out smoking as the cause of the epidemic rise in TC observed in many populations.

6.2.3. Association of maternal steroid hormones and risk of TC in the offspring

According to the leading hypothesis on TC aetiology, exposure to a specific pattern of steroid hormones *in utero* is the major determinant of TC risk. In particular, high oestrogen and low androgen levels are believed to be aetiologically important (Henderson et al. 1979, Henderson et al. 1988a, Henderson et al. 1988b, Sharpe and Skakkebaek 1993, Sharpe 2003). The present study is the first to evaluate the association of early pregnancy steroid hormone levels with the risk of TC in the offspring. Indeed, DHEAS is a precursor of steroid hormones and a major source for oestrogen formation in the foeto-placental unit. About one half of total oestradiol produced in the placenta originates from maternal DHEAS (Tagawa et al. 2004). During pregnancy, the maternal adrenal production rate of DHEAS is doubled, but the maternal serum level of DHEAS is reduced down to one-third of the non-pregnancy level (Braunstein 2002, p799). The decrease of DHEAS is associated with increasing oestrogen levels due to enhanced oestrogen biosynthesis by the placenta (Tagawa et al. 2004). As gestation advances, increasing quantities of 17β -oestradiol are synthesized from the conversion of circulating maternal and foetal DHEAS. At term,

approximately equal amounts of oestrogens are produced from circulating maternal and foetal DHEAS (Tulchinsky and Hobel 1973).

Androstenedione produced either from DHEAS or 17-hydroxyprogesterone is a precursor of testosterone and oestrogens. Because DHEAS and androstenedione share the same metabolic pathways it is natural that their serum levels correlate positively with one another. However, maternal serum DHEAS and androstenedione level associated point estimates of TC were opposite, and we do not have a good explanation for this.

Decrease of androgen levels with increasing age at pregnancy is probably a risk factor for TC in the offspring. Moreover, serum DHEAS and androstenedione levels decline after the third decade of life, and in our study both DHEAS and androstenedione levels were inversely correlated with maternal age. Various studies have, however, found increased maternal age to be associated with an increased risk of TC (Moller and Skakkebaek 1997, Sabroe and Olsen 1998), a decreased risk of TC (Cook et al. 2008b, Dusek et al. 2008) or no risk of TC (Swerdlow et al. 1982, Weir et al. 2000). Crude and maternal age adjusted statistical analyses of our results showed comparable opposite associations for DHEAS and androstenedione with the risk of TC. Although in our study maternal smoking status was not associated with a risk of TC, it was still positively correlated with DHEAS and androstenedione but not with other hormones.

A better knowledge on the impact of populations' lifestyle and environment on endogenous hormone metabolism during pregnancy and its role in TC development is needed. TC associates with cryptorchidism, hypospadias and low sperm counts, and may also be considered as a disorder of TDS. Causes of TC including environmental chemical exposure can lead to deficiency in androgen, in particular testosterone production/action or malfunctions of the Leydig and/or Sertoli cells during foetal testis development (Skakkebaek et al. 2001, Sharpe and Skakkebaek 2008). In addition, the oestrogens hypothesis postulates that the increasing incidence of reproductive abnormalities in the human male may also be related to increased oestrogen exposure *in utero* (Henderson et al. 1979, Henderson et al. 1988a, Henderson et al. 1988b, Sharpe and Skakkebaek 1993, Sharpe 2003). Recently the endocrine-disrupting chemicals hypothesis emerged, suggesting that some of the

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increase in TC incidence in recent decades could be due to the accumulation of these chemicals in the environment (Hardell et al. 2006, McGlynn et al. 2008). Therefore, further studies are needed to confirm the role of endocrine-disrupting chemicals in endogenous hormone metabolism during pregnancy and risk of TC in the offspring.

7. CONCLUSIONS

TC is the most common cancer in young men (aged between 20 and 40 years), however, compared to other human cancers, it is a rare disease. In populations considered to have high frequency of the disease, its lifetime incidence is approximately 0.5%. Thus, the present study on the epidemiology and aetiology of TC was motivated predominantly by biological and clinical interest.

The findings of this study permit the following conclusions to be drawn:

1. The serum samples of the FMC, preserved for up to 22 years at -25°C can be used to study hormone-disease associations. However, matching for storage time, a common design component of nested case-control studies, is needed to control for the observed variations in the hormones.
2. To the best of our knowledge this was the first study exploiting early pregnancy serum samples for the evaluation of the association of congenital infection with EBV and CMV and the risk of TC in the offspring. We found significantly more frequently elevated EBV IgG antibody levels in the mothers of nonseminoma TC cases than in the control mothers. This indicates that activation of EBV infection during pregnancy may have a role in the development of nonseminomatous TC, as has been suggested by a number of studies showing genetic material of the virus in the carcinoma cells.
3. There was no association between maternal smoking as measured by cotinine level in serum samples from the first trimester of pregnancy and risk of TC in the offspring. This is in agreement with a meta-analysis of seven epidemiological studies using questionnaire-based data on maternal smoking.
4. Determination of early pregnancy serum steroid hormone levels in the index and control mothers with the risk of TC generated new support for the intriguing role of high oestrogen and low androgen levels in risk of TC. We presented evidence that endogenous steroid hormones, especially DHEAS and

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androstenedione, in early pregnancy play an important role in the aetiology of TC in the offspring. However, further studies with longer follow up time and larger sample size are needed. A better understanding of the impact of populations' lifestyle and environment on endogenous hormone metabolism during pregnancy and its role in TC is needed.

8. ACKNOWLEDGEMENTS

The present work was carried out at the Tampere School of Public Health, University of Tampere and at the National Institute for Health and Welfare, during the period 2005-2009. The work was supported by the Cancer Control using Population Based Registries and Biobanks (CCPRB) European Union (EU) Network which is sincerely appreciated.

First I would like to express my deepest gratitude to my experienced and knowledgeable supervisor, Prof. Matti Lehtinen, for giving me the opportunity to carry out my doctoral work under his excellent guidance and within CCPRB EU Network, Spreading of Excellence (SoE) Doctoral Program. I would also like to place on record his constant encouragement, support and guidance which helped me to complete my doctoral dissertation successfully. His obsession with research has been a source of great inspiration to me. I always found solace in his humour.

I sincerely express my thanks to my other supervisor, Dr. Annekatrin Lukanova for her encouragement and valuable critical comments which contributed to the successful development of the dissertation and the final exit to graduation.

I am indebted to my official reviewers, Prof. Toivo T. Salmi and Docent Arto Leminen, for their constructive criticism, useful comments and the valuable time they rendered in my support by reviewing the dissertation. I am sincerely grateful to Prof. Eero Pukkala and Dr. Heljä-Marja Surcel for their time, endless support and valuable suggestions.

I wish to express my deepest thanks to all members of CCPRB network for their constant encouragement and tremendous contribution to my research especially to Prof. Kjell Grankvist, Prof. Joakim Dillner, Prof. Helga M. Ögmundsdottir, Prof. Göran Hallmans, Prof. Pentti Koskela, Prof. Göran Wadell, Dr. Eva Lundin, Prof. Timo Hakulinen and Prof. Pär Stattin. I am very grateful to my colleagues at the National Institute for Health and Welfare in Oulu especially to Mrs. Marjo Kaasila and Mrs. Hanna Öhman for their endless help they have been giving throughout my research work. I wish to express my sincere thanks to Prof. Jouko Tuomisto, Dr. Panu

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Rantakokko and Ms. Heini Huhtala for their valuable advices and effective collaboration. I am always thankful to Ms. Gudridur H. Olafsdottir, Ms. Ása Ágren, Ms. Lena Selbrand, Mr. Hubert Sjódin, Ms. Annika Uimanen, Ms. Pirjo Kontiokari, Ms. Mari Päätaalo for their excellent assistance throughout my research, and to each and every person who helped directly or indirectly during my doctoral work.

I am sincerely grateful to all lecturers of Tampere School of Public Health who gave me valuable knowledge. My special thanks go to Prof. Pekka Rissanen, Director of Tampere School of Public Health, for giving me opportunity to study here. I would like to express my sincere gratitude to Ms. Anna-Mari Nykånen, Ms. Tuula Joof, Ms. Taimi Auno, Ms. Raili Voittoinen and Ms. Catarina Ståhle-Nieminen for their continual assistance in all administrative issues every time I needed it. I wish to express my sincere thanks to Ms. Virginia Mattila for her quick and precise checking of the language fluency of my manuscript, Ms. Leena Nikkari for her excellent assistance in official affairs, Ms. Mari Elisa Kuusniemi and Mr. Tapani Ihalainen for their prompt help in printing issues of my dissertation.

I am extremely indebted to my Mum Hanna for her love, optimism, endless support and valuable advices. I am sincerely grateful to my Dad Uladzimir for his love, care and constant encouragement. Also many thanks go to my grandpa Nikolay and brother Alexander for making me laugh with their stories about every day life. My special thanks and love goes to my husband Roland who makes me smile and happy in this life.

Finally many thanks to my SoE and IPPE colleagues especially to Richard Muwonge, Felipe Castro, Arundhati Char, Mangesh Pednenkar, Calypse Agborsangaya, John Phuka, Aleksei Baburin and David Doku. I had a great time with you all.

Tampere, Finland, March, 2009

Katsiaryna Holl

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