Molecular and clinical aspects of borderline ovarian tumours

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The study presented in this thesis was carried out at the departments of Obstetrics & Gynaecology, division of Gynaecological Oncology, Pathology and Clinical Genetics of the VU University Medical Center, Amsterdam and the department of Pathology of the Academic hospital Maastricht, The Netherlands.

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Voor mijn ouders
Voor Ronald en Floor

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Chapter 1

Introduction.

Borderline ovarian tumours, clinical and pathological behaviour, diagnostics and therapy. A review

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Borderline ovarian tumours – a review

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INTRODUCTION

Definition

Borderline ovarian tumours (BOT), also referred to as ovarian tumours of low malignant potential (LMP), were first described by Taylor in 1929 and incorporated in the FIGO and WHO ovarian tumour classification in 1973. Histopathologically, these epithelial tumours display multilayering, nuclear atypia and formation of papillae, usually in cysts, but lack stromal invasion. The main histological subtypes are serous borderline ovarian tumours (SBTs) and mucinous borderline ovarian tumours (MBTs) making up approximately 55% and 40% of all BOT, respectively. Other subtypes are endometrioid, clear cell, transitional and Brenner, together making up 5% of all BOT[1].

Incidence

Borderline ovarian tumours constitute up to 16% of all non-benign epithelial ovarian tumours, resulting in an annual incidence of 9300 in Western Europe [2], and a prevalence of 24.7 per million females in the USA[3].

Clinical behaviour

Unlike ovarian carcinoma, the overall ten-year survival of BOT is excellent, being 99% and 77% for early and advanced disease, respectively [4]. Because even women with advanced stage borderline tumours have a very good prognosis, extraovarian lesions associated with these tumours are classified as "implants" rather than metastases. Even microinvasion, lymph node localisations and invasive implants seem to have limited impact on the clinical behaviour of these tumours.

ETIOLOGY

Genetic changes

Copy number changes
Genomic imbalances are not frequently found in SBT. In 1999 Wolf et al[5] described genomic imbalances in only 3 out of 10 SBTs, mainly gains of chromosomes 5, 7, 8q, 12p, 14q, 15q and 20. Mayr et al[6] found amplifications of the *FGFR1* and *MDM2* genes in 4 out of 5 SBTs and a gain of the *PIK3CA* gene. Subdividing SBT in Atypical Proliferative Serous Tumours (APST) and Micropapillary Serous Carcinoma (MPSC), chromosomal imbalances were found by comparative genomic hybridization (CGH) analysis in 3 of 9 APST and 6 of 10 MPSC[7]. We analysed a total of 41 SBTs by Multiplex Ligation dependent Probe Amplification (MLPA) using a probe set directed to 32 genes and found only 6 amplifications (0.5%): *RNF139* (8q24.13) (2x), *MTSS1* (8q24.13), *MYC* (8q24.21), *MYCBP* (1p34.3) and *CCNE1* (19q12)[8]. No studies on copy number changes in MBT seem to have been performed.

Mutations in p53, KRAS and BRAF
Only few genes have been described to bear mutations in SBT. The *p53* gene was found to be mutated in only 2 out of 25 SBTs whereas 30 out of 59 high-grade ovarian carcinomas showed a mutation in p53[9]. The *KRAS* signalling pathway (*RAS-RAF-MEK-ERK-MAP* kinase) however seems to play an important role in the development of SBTs and MBTs.

KRAS mutations in SBT were first described by Mok et al[10] and activating mutations in *KRAS* and *BRAF* are characteristic findings (60%) in SBTs and low-grade serous carcinomas, whereas in high grade ovarian carcinomas *KRAS* and *BRAF* mutations are never found[8-11]. We have described *BRAF* mutations in 11 of 27 (41%) primary SBTs that had no effect on clinical behaviour of these tumors (Verbruggen *et al*, Int J Gyn Cancer, In press). This makes it unlikely that SBT will progress to high-grade serous ovarian cancer.
KRAS mutations are also found in 79% of mucinous borderline ovarian tumors (MBTs) and in 56% of mucinous ovarian carcinomas (MCA) while in these tumours no BRAF mutations were found. This argues in favour of an mucinous adenoma – borderline – carcinoma sequence in primary ovarian mucinous neoplasms[12, 13], and may explain why MBTs may even coexist with foci of mucinous carcinoma[14].

Hormonal influences

Fertility drugs
The use of fertility drugs to induce ovulation is possibly an independent risk factor for the development of ovarian neoplasia. While no correlation between fertility drug use and invasive ovarian carcinoma has been confirmed in the literature, an association between fertility drugs and BOT has been observed[15-17]. A standardized incidence ratio (SIR) of 3.3 (95% CI: 1.1-7.8) was found for BOT in a cohort of 3837 infertile women[18]. In case-control studies a relative risk for the development of a BOT in infertile women was 3.5 (95% CI 1.2 – 10.1) and after hMG use 9.4 (95% CI: 1.7 – 52.1).
Three studies have specifically examined the risk of BOT after fertility drug use and also found a statistical correlation[19-21]. However, the association between BOT and the use of fertility drugs is still not consistent in the literature[22, 23]. This risk may also be attributed to the increased medical surveillance and younger age of infertile women. The use of infertility drugs after conservative treatment for an early stage BOT appears to be safe in a group of 25 women[24].

Hormone replacement therapy
Women who were receiving combined hormone replacement therapy (HRT) were found to have higher rates of cardiovascular disease, stroke, pulmonary embolism and breast cancer[25], though this absolute risk was minimal and disappeared after discontinuation of medication. Another study also found an elevated ovarian cancer risk associated with long-term use of HRT[26]. However, an association between HRT-use and the incidence of BOT was not specifically studied and remains unclear. A higher percentage of borderline tumours was found in one population-based case-control study of newly diagnosed ovarian cancer patients (n=256). In this group 74 BOTs were found of which 7 after 2-3 years of HRT use. The odds ratio (OR) was 2.57 (95% CI: 1.05 – 6.32), but the trend for ever HRT use and the association with BOT was not statistically significant (p=0.42). The authors conclude that this finding may be due to the younger median age of the group that was analysed[27]. So, in conclusion, no increased risk for BOT can with certainty be attributed to HRT use.

Cigarette smoking and alcohol drinking

There is no clear association between cigarette smoking and invasive ovarian cancer. The risk of BOT, however, is more than twice as high for women who smoke or have smoked before. Smoking for more than 20 years even triples the risk of BOT compared to never smokers[28]. A positive dose-response relation was found for cigarette smoking and serous BOT with an odds

ratio of 1.91 (95% CI: 1.09 – 3.34). It may therefore be concluded that smokers have an increased risk of developing BOT[29].
While alcohol consumption (14 or more drinks a week) was associated with a lower risk of developing invasive ovarian cancer (OR 0.36, 95% CI: 0.19 – 0.70), the use of spirits was associated with an increased risk of serous BOT (OR 2.66, 95% CI: 1.46 – 4.85) and former wine drinkers were shown to have an increased risk of mucinous BOT[30].

A Cystadenoma – Borderline tumor – Carcinoma sequence?

There is an ongoing debate whether cystadenomas progress through borderline tumours towards ovarian carcinoma. SBTs usually show areas of serous cystadenoma or cystadenofibroma, so it is quite likely that SBT derives from serous cystadenoma. Progression from here is less clear. At least, SBTs seem to be able to progress to a more epithelium rich, more architecturally complex, higher proliferative and more atypical subform (still without invasion) denoted micropapillary serous carcinomas (MPSCs) or intraepithelial low-grade serous carcinomas[31-33], that are frequently associated with invasive extraovarian implants. Molecular studies (predominantly focusing on KRAS and BRAF mutations, see above) have provided evidence for a serous cystadenoma – SBT – MPSC – invasive low-grade serous carcinoma pathway[34, 35]. In addition, the pattern of allelic imbalances found with CGH in MPSCs is similar to the pattern found in low-grade serous carcinoma but different from the pattern seen in high-grade serous carcinoma[36]. It has been suggested therefore to subdivide invasive serous ovarian tumours into type I (low grade) and type II (high grade) tumours[37]. This, and the absence of KRAS and BRAF mutations in high-grade serous ovarian carcinoma, makes it unlikely that SBT is its precursor of the most common, high grade, type of ovarian carcinoma. It is therefore concluded that SBTs (especially MPSCs) can be precursor lesions of low-grade serous ovarian carcinoma (Type I) but are unrelated to high-grade serous carcinoma (Type II)[38].Nevertheless, progression of SBT to low grade carcinoma is rare.
In contrast, there is general consensus that mucinous ovarian carcinomas develop from a pre-existing MBT[39] (that by itself derives from mucinous cystadenoma). This transition is even identified microscopically in some cases through a stage of mucinous intra-epithelial carcinoma, although microscopic distinction between borderline and frankly malignant mucinous tumour may be extremely difficult and can therefore hardy be used as an argument/proof of transition . Several genetic changes have been identified common to MBTs and invasive mucinous carcinomas[40] and these alterations can also be found in both invasive and non-invasive components of some tumours[41, 42].

DIAGNOSIS

Pelvic examination

Patients with a BOT may have no specific symptoms and a BOT is mostly found by coincidence. However, some women present with abdominal pain (31%), distension of the abdomen (52%), atypical vaginal blood loss (10%) or infertility (1%)[43]. At pelvic examination a mass can be palpated which is usually not painful and mobile unless an ovarian torsion has occurred.

Ultrasound and other imaging techniques

Transvaginal ultrasound is the imaging technique of first choice in the examination of ovarian masses. Sonographic findings that distinguish BOT from both benign and malignant tumours are not always present or clear. In a study that retrospectively studied 33 BOTs and compared the pre-operative ultrasound findings with 337 benign ovarian tumours and 82 malignant ovarian tumours, the presence of papillae from the cyst wall into the cyst cavity was significantly more frequent in BOT (48% vs 4%). Intracystic solid tissue is more frequently seen in malignant tumours than in BOT (48% vs 18%)[44]. A prospective study of 224 women with an adnexal mass in which a tumour pattern recognition method was used showed a high specificity. Ultrasound findings suggestive of BOT were: unilocular cyst with a positive ovarian crescent sign and extensive papillary projections arising from the inner wall, or a cyst with a well defined multilocular nodule. A correct pre-operative diagnosis of BOT was made in 24/35 cases (68.6%), resulting in a sensitivity of 69% and a specificity of 94%, leaving one-third of cases misdiagnosed, typically as benign lesions[45]. A model including intracystic complexity (either vegetations or septa), pulsatility index of less than 1.0, absence of confluence of vessels, CA125 of less than 150 u/L, in a woman under 60 years of age allowed borderline tumours to be detected with 85% sensitivity, 92% specificity and 91% accuracy[46]. Standardisation of ultrasonographic findings and descriptions could help to distinguish benign or borderline from malignant ovarian cysts[47-49].
Computed tomography (CT) might provide additional information in the examination of ovarian cysts. Especially involvement of lymph nodes, the omentum and extra abdominal disease can be seen on CT. Combined 18F-fluorodeoxyglucose position emission tomography and computed tomography (FDG-PET/CT) has recently been studied in 30 women suspected of ovarian cancer. Sensitivity for ovarian carcinoma was 100% but distinction between a BOT and a benign cyst proved less reliable and therefore of low diagnostic value[50]. Magnetic resonance imaging (MRI) may also be used to examine a pelvic mass, especially when ultrasonography is indeterminate. The accuracy of finding malignancy is 88% - 93% but lower for BOT[51].

CA 125 and CA 19.9

Serum CA 125 can be helpful in discriminating between benign and malignant masses in the pelvis. In BOT, however, this serum marker is much less useful. A serum CA 125 higher than 35 IU/l was found in 16/23 cases (69%), although the histopathological subtype of these BOTs was not stated[52]. In another study among 13 patients with an MBT, CA 125 levels were raised in 7 cases (54%) and in 14/24 SBTs (58%). Preoperative CA 125 was elevated in 92% of all SBTs FIGO stage II-IV in this study[53]. In a study among 101 BOT patients, elevated CA 125 levels were seen in 68% of serous and 52% of mucinous BOTs. A tendency of elevated CA 125 was observed with rising FIGO stage[54]. In 31 BOT patients elevated CA 125 levels were found in 4/11 SBTs and 3/20 MBTs (36% and 15%, respectively)[55].
While serum CA 125 is more frequently elevated in SBTs, serum CA 19.9 is more frequently elevated in MBTs[56]. A study in 44 BOT patients revealed that in 8/14 MBTs a serum CA 19.9 > 37 U/ml was seen (57%) whereas this was found in only 2/9 SBTs (22%). It is concluded that serum CA 125 and CA 19.9 assessments should be included in the follow-up of BOT patients[55].
In conclusion, serum markers are not elevated in the majority of cases of BOT, however especially CA 19.9 may be helpful in diagnosing MBTs or at least in distinguishing it from serous tumours, whereas CA 125 will be elevated in about half of the cases of SBTs.

Histo- and cytopathology

BOTs are usually cystic, lined by one to two layers of epithelium with little atypia and few mitoses. SBTs contain more serous fluid in the cystic spaces, are papillary with slender stromal stalks, lack intracytoplasmic mucus production and may show ciliae. Psammoma bodies are commonly found. MBTs contain more viscous cyst fluid, show intracytoplasmic mucus production in varying degrees, either of the intestinal or the endocervical type. While endocervical type MBT may be admixed with a component of SBT, intestinal type MBT is usually purely mucinous. Both are usually found within the serous cystadenoma (or cystadenofibroma) or mucinous cystadenoma from which they derive. Microinvasion (< 2 mm) may occur but this does not seem to influence prognosis[57].

Within both SBT and MBT, more advanced non-invasive lesions occur. For SBT, these are characterized by the presence of more epithelium (relative to the stroma), more complex architecture with longer finger-like epithelial micropapillary projections or a cribriform pattern, more atypia and more mitoses (although still lacking invasion) denoted micropapillary serous carcinomas (MPSCs) or intraepithelial low-grade serous carcinomas[32, 33, 58]. Prognosis of these lesions was claimed to be worse[59, 60], possibly related to the more frequent presence of invasive implants, but this has not been confirmed by others[61, 62].
For MBT, these lesions show multilayering and presence of severe nuclear atypia and increased mitotic activity (although still lacking invasion), denoted mucinous intraepithelial carcinoma. Prognosis of these lesions seems to be worse[63]. MBT (and to a lesser extent SBT) may also bear circumscribed malignant areas with signet cell or anaplastic adenocarcinoma, carcinosarcoma or sarcoma, usually denoted “mural nodule”[64, 65]. Due to their malignant nature, these mural nodules may negatively influence prognosis.
Cytology of ascites and peritoneal washings of BOT may show papillary groups that may sometimes be indistinguishable from metastatic ovarian carcinoma cells[66].

Flow cytometry and morphometry

Flow and image cytometry have been used to asses DNA-ploidy status in BOTs and has been suggested to be a strong prognostic indicator in BOTs[67-69]. In 1984 it was suggested that DNA aneuploidy in BOTs may indicate that clinical behaviour in these tumours will be worse than in diploid BOTs, which form the majority of BOT cases (42/44)[70]. Another study demonstrated 4 aneuploid BOTs among 50 cases. Two of the 4 patients with aneuploid BOTs died of the tumour, all diploid BOT patients remained disease free[71]. In 15 DNA samples of BOTs no aneuploidy was found by Lodhi et al. The authors conclude that aneuploidy in BOTs is rare and warrants extensive follow-up of the patient[72].
In 1985 Baak et al proposed that also morphometric characteristics may define a subgroup of BOTs with worse prognosis[73]. They concluded that BOTs with a Mitotic Activity Index (MAI) greater than 30 and a Volume Percentage of Epithelium (VPE) greater than 70% would have an unfavourable prognosis. However, these results could not be reproduced in a prospective follow-up study of 93 cases of which the 6 cases with MAI > 30 and VPE > 70% all had a good prognosis while 9 cases with favourable morphometric characteristics showed recurrence and even demise[74].

Extra-ovarian disease

Extra-ovarian disease is common in BOT, especially in SBTs, may present as peritoneal lesions (lacking stromal reaction, with stromal desmoplasia or with local invasion) and within lymph nodes, and is of clinical significance. Stage I tumours, with no accompanying implants, have an almost 100% survival after follow-up of over 6 years. For tumours with non-invasive implants this was 95% whereas tumours with invasive implants appeared to have a survival rate of 66% after a mean follow-up of 7.4 years. Furthermore, the presence of a micropapillary architecture in the primary SBT is a strong predictor of invasive implants and therefore of less favourable prognosis[75]. The clinical significance of lymph node involvement (LNI) has been studied in 74 patients with SBTs[76]. Implants were found in lymph nodes within the true pelvis (58%), omentum (29%), and paraaortic (26%) or supradiaphragmatic lymph nodes (6%). There was no difference in survival for patients with or without LNI. However, the presence of nodular aggregates of SBT in the lymph nodes was associated with decreased disease free survival and with micropapillary architecture of the primary SBT (ref). We described 3 patients with FIGO stage IV SBT with supradiaphragmatic lymph nodes at first presentation of the disease. All remained disease-free during follow-up of 4 to 7 years[77]. Likely, the main pitfall in these patients is that these lymph node lesions may initially be regarded as metastases and thereby the primary tumour as a carcinoma.
In MBTs, extraovarian spread is much less common[78]. Cases with implants in the abdominal cavity, in scar tissue of earlier abdominal surgery[79] and with extensive pseudomyxoma peritonei[80, 81] have been described. There is, however, debate about the primary tumour in these latter cases. It is now generally assumed that the appendiceal mucinous tumours (adenomas or carcinomas) are the origin of the pseudomyxoma and that the ovarian lesions found in these cases are seeded from these as well and are not primary MBT[82].

TREATMENT

Surgery

Surgery is the cornerstone in the treatment of BOT[2, 83]. Aim of surgery is to achieve complete cytoreduction. The recommended primary surgery of BOT used to be similar to that of ovarian carcinomas, being a complete surgical staging including bilateral adnexectomy, omentectomy, lymph node sampling, peritoneal lavage cytology and possibly hysterectomy. In mucinous tumours appendicectomy is advised to exclude colo-rectal origin. However, in recent years these recommendations have been altered. In early stage BOT (stage I/II), extensive surgery results in upstaging of the disease in 16% tot 26% of patients[84-86]. However, the impact of surgical staging on therapeutic management is not defined. Incomplete surgical staging results in increased recurrence rate but does not affect survival[1]. Surgical staging in early stage BOT may be omitted if: 1) the peritoneum is clearly reported as normal during initial surgery, 2) there is no MPST and 3) if the patients agrees to careful follow-up[87].
In advanced stage BOT complete surgical staging resulting in aggressive cytoreduction is warranted. Residual tumour < 2 cm increases survival rate significantly compared to residual tumour after surgery of 2 to 5 cm and >5 cm[88].

Laparoscopic surgery

In the last two decades laparoscopic surgery has become the main approach for treatment of adnexal tumours without clinical signs of malignancy and even became more important in the treatment and staging of adnexal cancer. Laparoscopic treatment of BOT seems feasible since a suspect ovarian cyst may be removed laparoscopically and successive staging can be performed after the definitive pathological anatomical diagnosis has been established. This is of special importance since BOTs tend to occur in young women, who wish to avoid a major laparotomy and preserve fertility. In a series of 62 BOTs of which 30 were operated laparoscopically, 37% of laparoscopically treated patients had persistent or recurrent disease, possibly because of more frequent rupture of the tumour, resulting in stage IC disease. After laparotomy, 22% of patients had relapse of ovarian disease. Follow-up was done for 33-138 months. It is concluded that masses smaller than 5 cm may be treated laparoscopically, preferably by adnexectomy[89]. In a study of 34 BOT patients treated laparoscopically, 6 patients recurred after a median follow-up of 45 months. These recurrences could be treated laparoscopically again in 5 cases. Two port-site metastases were observed[90]. Unlike port-site metastases in other gynaecological malignancies the prognosis of these metastases in BOT is excellent. Treatment is surgical resection[91]. Also in patients with BOT with non- invasive implants (i.e. APSTs, FIGO stage II and III) laparoscopic treatment seems to be safe [92]. It may be concluded that in young patients conservative laparoscopic treatment of BOT fertility, pregnancy outcome and survival remain excellent[93].
BOTs may be treated by (laparoscopic) cystectomy rather than adnexectomy, although recurrence rates are higher in the former. Two studies independently showed a recurrence rate of 30% to 34% after cystectomy, but also emphasised that these patients could easily again be treated surgically without jeopardizing the prognosis[94, 95]. On the other hand, in a study in 62 women with BOT, 22 underwent cystectomy and 40 unilateral adnexectomy. Recurrence of disease was observed in 23% and 27% of cases respectively after follow-up of 88 months. In this group 38 pregnancies occurred[96]. It should be emphasized that patients must be willing to undergo careful and prolonged follow-up, especially after cystectomy.

Adjuvant therapy

A number of retrospective studies did not indicate any effect of adjuvant chemotherapy, hormonal therapy or radiotherapy in BOT[1, 97, 98]. Response rates to platinum based chemotherapy and/or paclitaxel in patients with progressive or recurrent SBT are around 13% for complete or partial response. Hormonal therapy with tamoxifen or leuprolide did not show response nor did whole pelvic radiotherapy[98]. Although, two case reports have described response of BOT to tamoxifen[99] and leuprolide[100].

RECOMMENDATIONS

BOTs are typically found in young women (in the fertile age group) presenting with abdominal distension or pain, atypical vaginal bleeding and/or infertility. The diagnosis is made by pelvic examination, transvaginal ultrasound and serum tumour markers (CA 125 and CA 19.9). When a BOT is suspected, laparoscopic adnexectomy or cystectomy is the first choice operation for histopathological diagnosis and treatment. During laparoscopy the contralateral ovary should be inspected, the abdominal cavity should be examined for implants which should be removed if possible (but at least biopsied), and abdominal fluid may be obtained for cytologic examination. Enlarged lymph nodes should be removed but no routine lymph node sampling is recommended.
BRAF mutation analysis may be helpful in distinguishing a serous borderline ovarian tumour (SBT) from high grade serous carcinoma. If a serous borderline ovarian tumour (SBT) is diagnosed, one should inform the patient about this diagnosis and the chance of recurrence (i.e. around 20% within 5 years[101]). Clinical follow-up should be performed every six months for at least 5 years including pelvic examination, ultrasound and serum CA 125. The patient should also be encouraged to present with any abnormal abdominal complaints or swelling. If recurrence is seen, therapy should be surgical, preferably again laparoscopically. In postmenopausal women with an SBT, bilateral adnexectomy can be considered because of the probability of bilateral disease (10%-15%). After that, follow-up is still warranted because of the probability of extraovarian spread of the disease. There is no evidence that this policy should be altered in case of microinvasion, (invasive) implants, lymph node localisations or tumour cells in the peritoneal washing or ascites. Chemotherapy is not indicated.
If a mucinous borderline ovarian tumour (MBT) is diagnosed, the patient should be informed about the (very small) chance of recurrence and malignant transformation of these tumours. In addition, the pathologist should confirm thorough examination of the specimen to exclude mucinous adenocarcinoma or mural nodules in the same cyst. Clinical follow-up should be performed every six months for at least 5 years including pelvic examination, ultrasound and serum CA 125 and CA 19.9. When recurrence is observed, laparoscopic or laparotomic staging should be performed in order to treat the relapse and exclude mucinous carcinoma. In the latter case adjuvant chemotherapy is warranted.
In case of pseudomyxoma peritonei, the appendix should be removed and carefully examined by the pathologist for the mucinous primary. The following treatment depends on the primary tumour that is found.
If a serous or mucinous adenocarcinoma or any other kind of ovarian tumour is diagnosed, usual carcinoma guidelines should be followed.

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Chapter 2

The prognostic and clinical value of morphometry and DNA cytometry in borderline ovarian tumors: a prospective study

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Abstract

Our aim was to evaluate if morphometric features (Mitotic Activity Index, Volume Percentage of Epithelium and DNA-ploidy) are prognostic markers in borderline ovarian tumours (BOT).
Ninety-three serous and mucinous consecutive BOTs diagnosed between 1989 and 2002 were studied. In all tumours mitotic activity index (MAI), volume percentage of epithelium (VPE) and DNA ploidy were determined prospectively. Consecutively, age at diagnosis, calculated tumour volume, FIGO stage, and treatment by extensive staging were evaluated after a median follow-up of 52 months.
Serous BOTs presented at a younger age ($p<0.05$), with smaller volume ($p< 0.001$), with higher FIGO stage ($p<0.001$) and were more frequently bilateral ($p<0.001$) than mucinous BOTs. Patients with serous BOT ($p<0.05$) and beyond stage IA ($p<0.01$) showed worse recurrence free survival. No prognostic significance could be established for DNA ploidy or morphometry.
The previously claimed prognostic power of DNA ploidy and morphometry could not be corroborated in this prospective study and can therefore not be recommended to direct clinical management in BOTs. In contrast, histologic subtype and FIGO stage seem to be stable prognosticators in BOTs.

Introduction

Borderline ovarian tumours (BOT), also referred to as ovarian tumours of low malignant potential (LMP), were first identified by Taylor in 1929 and incorporated in the FIGO and WHO ovarian tumour classification in 1973[1,2]. These tumours constitute 9-16% of all non-benign epithelial ovarian tumours , resulting in an annual incidence of 9300 in Western Europe [1]. Unlike ovarian carcinoma, the overall ten-year survival of BOT is excellent, being 99% and 77% for early and advanced disease, respectively [2]. It has been suggested that these tumours display a unique combination of clinical and pathological features that is unparalleled in clinical oncology [3].
BOT are defined as tumours which display epithelial cellular proliferation, stratification of the epithelial lining of the papillae, multilayer mitotic activity and nuclear atypia as is seen in ovarian carcinoma, but lack significant stromal invasion [4]. In addition to these diagnostic criteria, quantitative microscopy to determine Mitotic Activity Index (MAI) and Volume percentage of epithelium (VPE), has been advocated [5]. These indices have been considered to be of important predictive value for prognosis in BOT [6]. A MAI greater than 30 per 4 mm^2 and a VPE higher than 65% have been considered to be associated with an unfavourable prognosis in a small retrospective study [7]. Furthermore, DNA ploidy, assessed by flow cytometry, was considered to be a strong prognostic factor in several BOT studies [8-10]. Conservative surgical treatment of patients with diploid stage I borderline ovarian tumours with favourable prognostic features is generally accepted [11, 12]. It has been a matter of dispute whether unfavourable morphometrical characteristics or DNA aneuploidy of BOT would warrant a more aggressive approach or even full surgical staging, including taking of biopsies, bilateral adnexectomy, omentectomy, peritoneal washing and optional hysterectomy [13]. This study aimed to evaluate the prognostic value of clinical features and prospectively assessed morphometric features and DNA ploidy in a group of serous and mucinous BOT with long term follow up.

Materials and methods

We analyzed a consecutive series of 93 cases of serous and mucinous BOT of all stages (excluding micropapillary serous carcinoma and mucinous intraepithelal carcinomas) diagnosed in the Amsterdam area at the VU University Medical Center, and Sint Lucas / Andreas Hospital in Amsterdam, the Kennemer Gasthuis in Haarlem and the Zuiderzee Hospital in Lelystad between 1989 and 2002. Pathological slides were reviewed in every case and morphometric analysis had prospectively been performed according to criteria described by Baak *et al*. [6]. In short, the MAI was counted in 4 mm^2 at a x40 magnification, and the VPE was assessed by stereology in an area of approximately 0.5x0.5 cm in size in the most epithelium rich areas of the tumours. Cases with a MAI $\geq$ 30 and VPE $\geq$ 65% were considered to be at high morphometric risk. DNA ploidy had also been prospectively been assessed by flow cytometry as before[14].
Clinical data and follow-up of all patients were retrieved from patient files at the hospitals, including original operation notes and original pathology reports. Patients were staged according to revised FIGO criteria (1988) based on all available clinical information. In patients with mucinous BOTs that were surgically staged the appendix was always removed to assure no primary gastrointestinal tumour was present. When involvement of the appendix was found the case was excluded from this study. Tumour volume (cm^3) was calculated from measurements by pathological examination in combination with findings on ultrasound and/or CT imaging.

Cross tables were statistically tested with the χ-square test. For univariate recurrence free survival analysis, patients were grouped according to logical classes, or for continuous variables according to the median values or previously established thresholds. Kaplan-Meier curves were plotted, and differences between the curves were analyzed with the Log-rank test. For multivariate recurrence free survival analysis, Cox regression was done. P-values < 0.05 were considered significant.

Results

Histologic subtypes
Thirty-six tumours were of the serous histotype (39%) of which 11 (31%) were bilateral and 57 tumours were of the mucinous type (61%) of which only 1 (2%) was bilateral (p=0.00005).

Age at diagnosis
The mean age at diagnosis of a serous borderline ovarian tumour was 42.9 years (Median: 38.5 years, range: 28-74 years), whereas the mean age at presentation of a mucinous BOT was 48.7 years (median: 52 years, range: 14-83 years) (p<0.05).

Tumour volume
Mean calculated tumour volume in the serous group (n=29, 581 cm^3) differed significantly (p<0.001) from that in the mucinous group (n=53, 4013 cm^3).

FIGO stage
In the group of serous tumours, 21 (58%) presented with stage I disease, 8 (22%) with stage II disease, 5 (14%) with stage III disease and 2 (6%) with stage IV disease. Extra-ovarian implants were found in 14 (39%) patients with a serous BOT but none of the implants were invasive. In the group of mucinous tumours 55 (96%) presented with stage I disease and 2 (4%) with stage III disease (p<0.0001), based on non-invasive implants in the peritoneum.

Follow-up
The median follow-up time was 57 months (range: 17-163) or 213 woman-years in the serous tumour group and 47 months (range: 1-130) or 233 woman-years in the mucinous group.

Quantitative characteristics
Unfavourable morphometric characteristics were seen in 1 (3%) of serous BOT, whereas 5 (9%) of mucinous BOT were morphometrically unfavourable. Ten cases of serous BOT (28%) were aneuploid and 11 cases of mucinous BOT (21%). Figures 1 and 2 show the quantitative features for the individual serous respectively mucinous BOT patients in relation to prognosis.

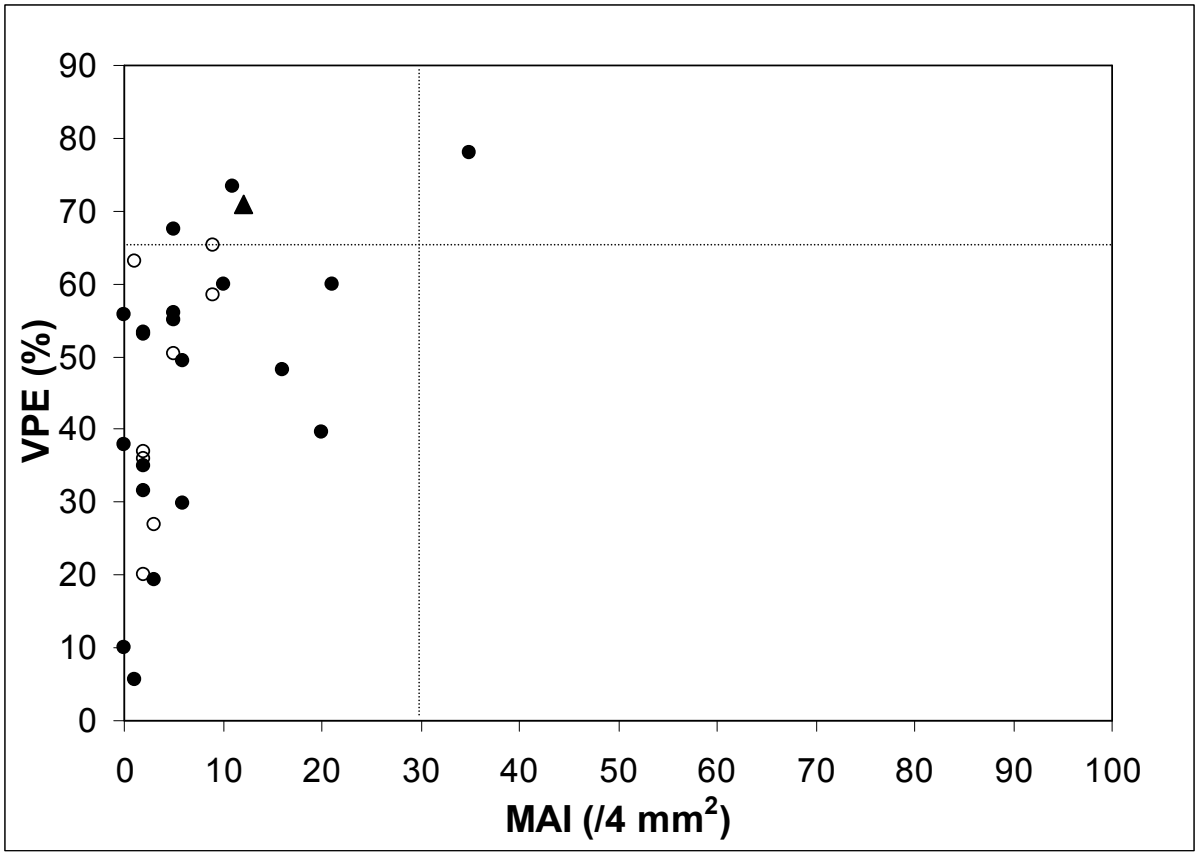

Figure 1. Quantitative features for the individual serous BOT patients in relation to prognosis. ●=diploid, ○=aneuploid, ■=Progression to invasive carcinoma, ▲=Death of disease

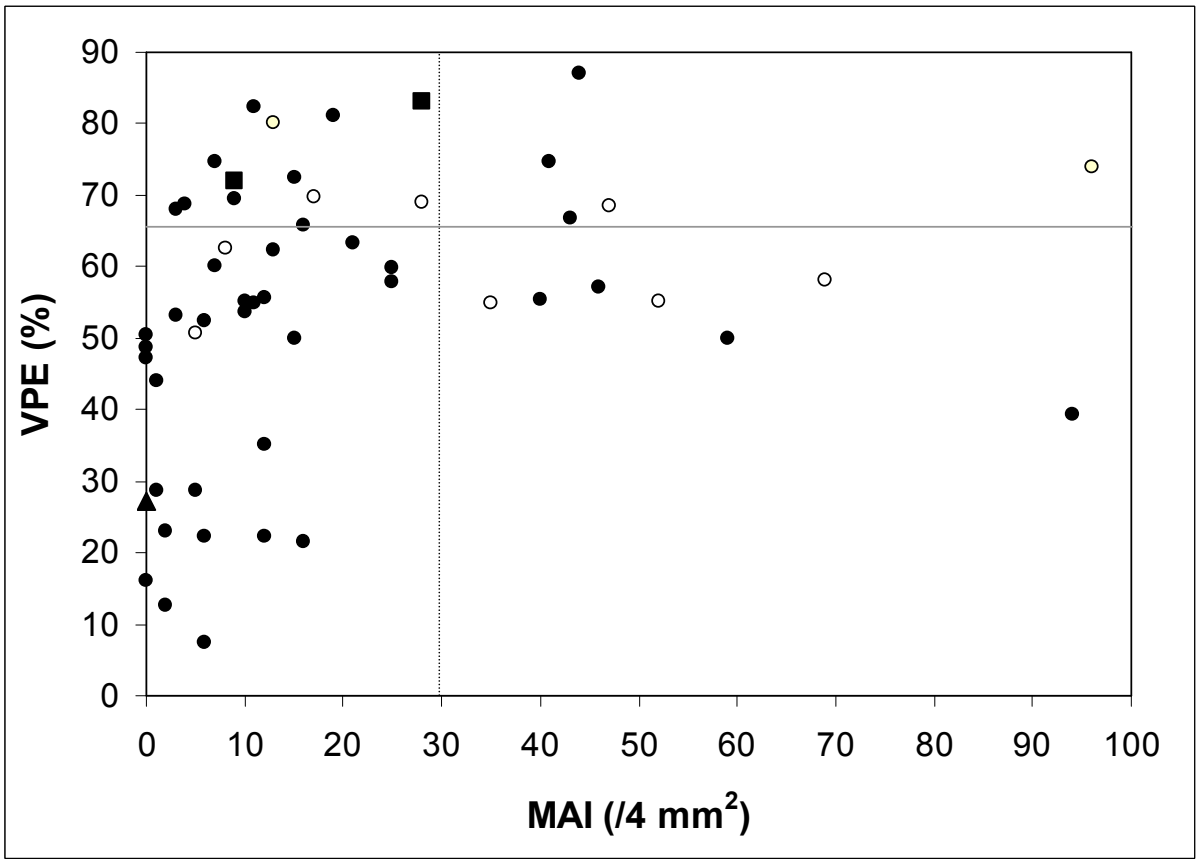

Figure 2. Quantitative features for the individual mucinous BOT patients in relation to prognosis. ●=diploid, ○=aneuploid, ■=Progression to invasive carcinoma, ▲=Death of disease

Survival analysis

Six patients had morphometrically unfavourable tumours. Five of these were FIGO stage Ia and the sixth was FIGO stage Ic. One of these patients was not surgically staged because of very young age at diagnosis (21 years). She was free of disease after a follow-up of 126 months. The other five patients were surgically staged, where no peritoneal implants or enlarged lymph nodes were found, and had an uneventful mean disease free survival of 51 months (range: 18-113 months). Outcome of follow-up of all patients is shown in Tables 1 and 2.

Table 1: Outcome of patients with borderline tumours of the ovary (n=93) according to morphometric risk status.

		Disease status		**Survival status**		
Morphometry	**N**	**NED**	**AWD**	**DOD**	**DoC**	**LFU**
Favourable	87	71	5	2	4	5
(serous/mucinous)	(35/52)	(30/41)	(5/0)	(1/1)	(0/4)	(0/5)
Unfavourable	6	6	0	0	0	0
(serous/mucinous)	(1/5)	(1/5)				
Total	93	77	5	2	4	5
(serous/mucinous)	(36/57)	(31/46)	(5/0)	(1/1)	(0/4)	(0/5)

NED=No Evidence of Disease, AWD=Alive With Disease, DOD=Death of Disease, DoC=Death of other Cause, LFU=Lost to follow-up.

Table 2: Outcome of patients with borderline tumours of the ovary (n=93) according to DNA ploidy.

		Disease status		**Survival status**		
DNA ploidy	**N**	**NED**	**AWD**	**DOD**	**DoC**	**LFU**
Diploid	70	55	4	2	4	5
(serous/mucinous)	(26/44)	(21/34)	(4/0)	(1/1)	(0/4)	(0/5)
Aneuploid	21	20	1	0	0	0
(serous/mucinous)	(10/11)	(9/11)	(1/0)			
Total	91	75	5	2	4	5
(serous/mucinous)	(36/55)	(30/45)	(5/0)	(1/1)	(0/4)	(0/5)

NED=No Evidence of Disease, AWD=Alive With Disease, DOD=Death of Disease, DoC=Death of other Cause, LFU=Lost to follow-up.

Progression or relapse of disease was seen in 8 patients (8.6%), 5 with serous BOT and 3 with mucinous BOT. All tumours had favourable morphometric characteristics, and all but one were DNA diploid. Mean time to relapse was 28 months (Range: 7-63 months). One patient with a serous BOT died of disease after developing invasive implants 10 years after diagnosis of the primary tumour. This patient had low MAI, high VPE and was DNA diploid. One patient with a mucinous BOT died of disease 5,5 years after diagnosis of the primary tumour. Her tumour also was DNA diploid and had low MAI and low VPE. Two patients with mucinous BOT progressed to high grade mucinous ovarian cancer. They had both low MAI end high VPE, and were diploid. Characteristics of all patients with relapse of disease are shown in Table 3.

Table 3: Main characteristics of patients with borderline tumours of the ovary with progression of disease.

No	Age at diagnosis (years)	Stage, type and location	MAI (4 mm^2), VPE (%), DNA-Ploidy	Primary treatment	Interval to progression (months)	Site of progression	Treatment	Outcome follow-up (months)
1	31	Ic, serous, ovary	12, 71, Diploid	Cystectomy	7	Contralateral ovary	Cystectomy biopsies	DOD (139)
2	84	Ib, mucinous, ovary	0, 27, Diploid	Bilateral adnectomy and hysterectomy	Not known	Lower abdomen	None, patient refused	DOD (75)
3	29	Ia, serous, ovary	21, 60, Diploid	Adnectomy during pregnancy	63	Contralateral ovary	Full surgical staging	NED (128)
4	29	Ia, serous, ovary	2, 36, Aneuploid	Adnectomy	36	Contralateral ovary	Partial staging	NED (82)
5	30	IIb, serous, peritoneum	2, 53, Diploid	Cystectomy, peritoneal biopsies	36	Left ovary	Partial staging	AWD (40)
6	60	Ic, serous, bilateral	5, 55, Diploid	Surgical staging	12	Axillary lymph node	Chemotherapy	AWD (17)
7	42	Ic, mucinous, ovary	28, 83, Diploid	Surgical staging	26	Peritoneum	Debulking	AWD, (OC,29)
8	54	Ic, mucinous, ovary	9, 72, Diploid	Adnectomy	20	Contralateral ovary	Full surgical staging	AWD, (OC, 56)

Abbreviations: MAI=Mitotic Activity Index, VPE=Volume percentage of Epithelium, S=Serous, M=Mucinous, NED=No Evidence of Disease, AWD=Alive With Disease, OC=Ovarian Carcinoma.

In univariate recurrence free survival analysis (Table 4), mucinous cases had better recurrence free survival than serous cases (p=0.0245). Also stage IA cases had better survival than cases of higher stage (p=0.0044). In contrast, there were no significant differences in recurrence free survival for MAI and VPE as individual features, morphometric risk and DNA ploidy. There was also no recurrence free survival difference between patients undergoing staging-or-not, also not in the subgroup of DNA aneuploid cases (p=0.636).
In Cox regression for the whole group of patients, only stage (IA vs others) was selected and no other feature had additional prognostic value, even staging.

Table 4. Recurrence free survival results of patients with borderline tumours of the ovary (n=93) according to clinicopathological and quantitative features.

Feature	grouping	N	# with recurrence	% recurrence free	p-value
Type	serous mucinous	36 57	7 2	80% 96%	**0.0245**
VPE	< 65% > 65%	79 14	6 3	92% 89%	0.607
MAI	<30 >30	87 6	9 0	89% 100%	0.192
Morphometric risk	low high	87 6	9 0	89% 100%	0.419
Ploidy	diploid aneuploid	72 21	6 3	81% 86%	0.533
Stage	Ia others	57 34	2 7	96% 79%	**0.0044**
Extensive staging	no yes	60 31	4 5	93% 84%	0.182
Age	< 44 > 44	49 44	7 2	85% 95%	0.163

Discussion

The aim of the present study was to evaluate the clinical behaviour of BOT in relation to histology and morphometric characteristics and DNA ploidy. Women with serous BOT presented at a younger age, with smaller tumour volume, at higher FIGO stage, and more often bilaterally compared to mucinous BOT. These findings are similar to those found in previous studies[15]. In addition, recurrence or progression was mainly seen in the group of serous tumours as before.
In this study, six patients had morphometrically unfavourable tumours. Five of the 6 morphometrically unfavourable patients were treated by extensive staging, which is theoretically a confounder here as it may have improved survival in these patients up to the level of low risk patients, leading to lack of difference in survival between these groups of patients in the present study. However, full surgical staging did not result in upstaging these patients to a higher FIGO stage, and surgical staging has also been described to have no influence on survival and recurrence rate in BOT[16]. Further, the one unfavourable patient that was not staged remained recurrence free, whereas 7 patients with favourable morphometric features showed recurrence. Therefore, it is unlikely that morphometry is prognostically useful in BOT patients as in the single positive study by Baak et al [6], despite the possible confounder of extensive staging. In contrast, morphometrical variables do have an important value in predicting prognosis in advanced invasive ovarian cancer[17].
DNA ploidy has been suggested to be an important prognostic factor in BOT[8, 10] although this is being argued by others[8, 18, 19]. In the present study we could not find a difference in prognosis between diploid and aneuploid BOT, whether surgically staged or not. A total of 21 tumours (23%) appeared to be aneuploid. One (5%) of these patients relapsed (see table 3) while 6 (8%) patients with diploid tumours relapsed. The two patients that died of disease had diploid primary tumours.
It is known that after a follow-up of 10 years survival rate among women with higher stage (i.e. stage II-IV) BOT is around 90%. The deaths are suggested to be caused by extraovarian implants secondary to the BOT, that undergo malignant transformation, sometimes after years of dormancy[20]. It has been proposed that such implants and the primary BOT share a common origin[21]. Thus far, only FIGO stage and implant status proved to be of prognostic significance[22].
Subclassification of serous BOT into *micropapillary serous carcinoma* (MPSC) and *atypical proliferative serous tumour* (APST) has been suggested to describe prognostic less favourable and more benign behaving BOT, respectively[23-25]. However, further investigations failed to demonstrate that overall survival differed between both entities[26-28]. MPSC were excluded in the present study, as were mucinous intraepithelial carcinomas.
Mutational analysis of KRAS and BRAF have led to the conclusion that progression from serous BOT to high grade carcinoma does not take place, but mucinous BOT may progress to invasive high grade carcinoma[29, 30]. In our study population the latter is confirmed, although this occurred in only 2 cases.

In conclusion, we recommend conservative surgery in low stage BOT and intensive follow-up for five years (including ultrasound and serum tumour markers CA 125 and CA 19.9), as has been accepted worldwide already. For higher stage BOT (including stage Ic with spill during operation or suspicious cells in ascites or peritoneal washing) surgical removal of all tumour visible is recommended. This patient group may also benefit from a ten-year follow-up, because of a high probability of late recurrence of disease. In mucinous BOT recurrence may lead even to invasive high grade carcinoma.

Neither assessment of morphometric characteristics nor DNA-aneuploidy appears to have relevant additional prognostic value in BOT. Other, genomic or proteomic features may be investigated in order to predict prognosis in BOT.

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Chapter 3

Serous borderline tumour of the ovary presenting with cervical lymph node involvement
A report of 3 cases

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Abstract.

Supradiaphragmatic lymhadenopathy is extremely rare in patients with a serous borderline ovarian tumour, and clinically difficult to recognize.
We describe three cases of serous borderline ovarian tumour that primarily presented with arm thrombosis due to supradiaphragmatic lymphadenopathy. In all three cases fine needle aspiration cytology initially indicated metastatic adenocarcinoma. The primary tumour was not immediately apparent, and multiple diagnostic examinations had to be done before the definitive diagnosis of serous borderline ovarian tumour, FIGO stage IV could be made. In the meanwhile, erroneous therapies had been given in one case. After surgical removal of the adnexal masses and full surgical staging, all three patients remained free of disease after a follow-up period of 48 to 84 months.
In conclusion, supradiaphragmatic lymph node involvement can be present in patients with serous borderline ovarian tumours, and can even be the presenting symptom. When fine needle aspiration cytology of such a lymph node is compatible with adenocarcinoma of unknown primary (ACUP), serous borderline ovarian tumour should be included in the differential diagnosis and pelvic examination should be performed.

Introduction

Borderline ovarian tumours (BOT), also referred to as ovarian tumours of low malignant potential (LMP), constitute 9-16% of all ovarian tumours.[1] Unlike ovarian carcinoma, the overall ten-year survival of BOT is excellent, being 80-99%, even in advanced stages.[2] Usually, patients with BOT present with abdominal complaints and a pelvic mass on physical examination, ultrasound or computed tomography (CT). Spread is usually confined to the abdominal cavity with occasional spread to regional lymph nodes.[3, 4] Supradiaphragmatic lymph node involvement is very rare, and is usually described in recurrent BOT several years after primary diagnosis.[5-8]
We now describe three cases of serous BOT that initially presented with cervical lymphadenopathy resulting in arm thrombosis. Furthermore, immunohistochemical stainings of these serous BOT and lymph node localizations are being described.

Case reports

The first case presented with thrombosis of the left subclavian vein at the age of 35 years. Further evaluation revealed supra-clavicular lymphadenopathy on the left side. Fine needle aspiration cytology of this lymph node was consistent with adenocarcinoma of unknown primary (ACUP). Chest X-ray, thoracic or abdominal CT, cervical ultrasound, mammogram and gastroscopy revealed no apparent primary tumour. However, abdominal ultrasound revealed a multilocular cystic left ovary measuring 10x7 cm. The CA 125 serum level was elevated at 120 U/ml.
At exploratory laparotomy two weeks later, a total hysterectomy and bilateral salpingo-oophorectomy with infracolic omentectomy and periaortic lymphadenectomy was performed under the clinical diagnosis of ovarian carcinoma FIGO stage IV. However, histologic examination of the ovaries revealed a bilateral serous BOT with diffuse localizations in the para-aortic lymph nodes. On histology, the ovarian tumour had partly the appearance of cystadenofibroma, partly with a more complex papillary nature qualifying as serous BOT (figure 1). There was no frank atypia and no invasion. DNA flow cytometry revealed DNA diploidy. Para-aortic lymph nodes contained localizations of serous BOT, whereas omentum and ovarian adhesions showed endosalpingiosis. In the sinuses of the cervical lymph node, extensive deposits were seen of partly tubule forming, partly papillary serous cells, but also partly rounded single cells with abundant cytoplasm (figure 2). No atypia, no invasion and no mitoses were seen. There were less than 1% proliferating cells (MIB-1).
Immunohistochemical staining revealed positivity for CA 125, CAM 5.2 and Cytokeratin 7, and negativity for thrombomodulin and calretinin in both ovarian tumour and lymph node. Progesterone and estrogen receptor stainings were positive in the ovarian tumour but negative in the cervical lymph node localizations, staining for vimentin was positive in the tumour and only focally positive in the lymph node localizations, Cytokeratin 20 and Carcinoembryonic antigen (CEA) were negative in the primary tumour but positive in the cervical lymph node localizations (table 1).
Five months after surgery CA 125 was normalized to 8 U/ml. No additional therapy was given. After 4 years of follow-up the patient is still free of disease.

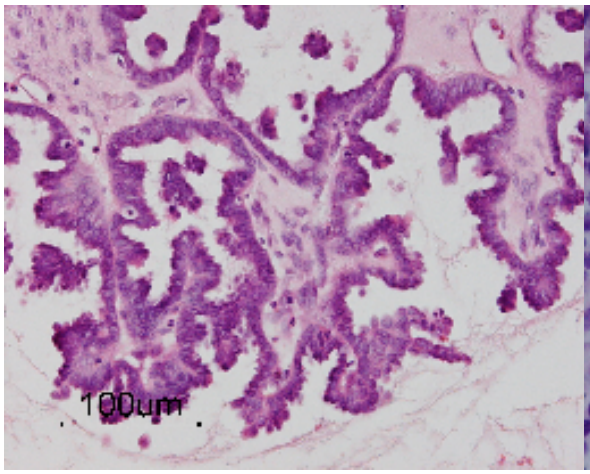

Figure 1: Serous borderline ovarian tumour in case 1. (Hematoxylin and Eosin staining)

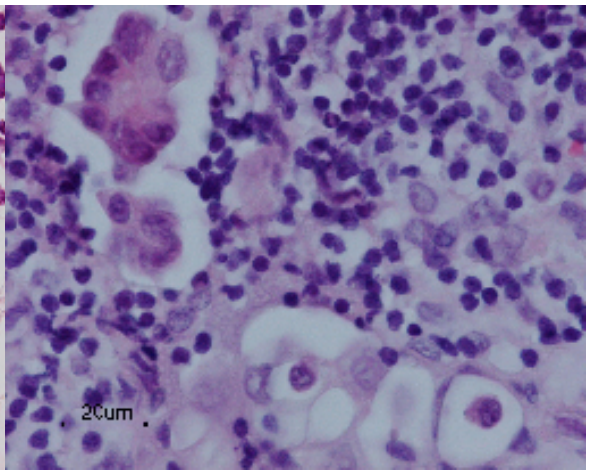

Figure 2: Implant in cervical lymph node in case 1. (Hematoxylin and Eosin staining)

The second case presented with deep venous thrombosis of the left arm at the age of 32, and one year after that with thrombosis of the right arm. Physical examination showed bilateral cervical lymphadenopathy. Fine needle aspiration cytology was consistent with metastatic adenocarcinoma, likely of ovarian origin in view of the immunocytochemical profile (Cytokeratin 7 and CA-125 positive, Cytokeratin 20 and CEA negative).[9] Abdominal CT showed a pelvic cystic mass with a diameter of 5.5 cm and ascites. Mammography and thyroid-ultrasound were normal. Serum CA 125 was not determined. The patient received chemotherapy with cisplatinum, adriamycin and cyclophosphamide for what was assumed to be a FIGO stage IV ovarian carcinoma. After five courses of chemotherapy only a partial response was seen and the patient was referred to our clinic for further evaluation and treatment. Abdominal CT revealed a cystic and solid pelvic mass measuring 6x7 cm and ascites, but no abdominal lymphadenopathy, liver metastasis or pleural effusion. CA 125 was raised at 266 U/ml. Second line chemotherapy containing paclitaxel, etoposide and cisplatinum was given during several months and again neither remission or progression was seen. Chemotherapy was discontinued. After two years of a wait and see policy, progression of the pelvic tumour was detected on CT and explorative laparotomy was performed. Histologic examination of the removed ovaries revealed a bilateral serous borderline ovarian tumour with no frank atypia and no invasion (figure 3). DNA flow cytometry showed DNA aneuploidy. A paratubal lymph vessel showed localization of serous BOT. Endosalpingiosis was found focally on the peritoneum. The cervical lymph node was histologically quite similar to that of the first patient with no atypia, no invasion and no mitoses (figure 4). Immunohistochemical stainings were similar to the results seen in the first patient, with the exception of Cytokeratin 20 and CEA that were negative in both ovarian tumour and cervical lymph node cells in this case (table 1).
A macroscopic complete debulking was performed and CA 125 reduced to normal levels at 12 U/ml. After a follow-up of 4.5 years no evidence of recurrent disease is present.

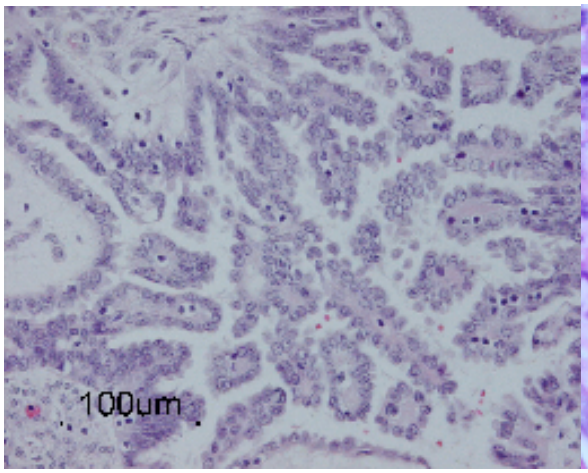

Figure 3: Serous borderline ovarian tumour in case 2. (Hematoxylin and Eosin staining)

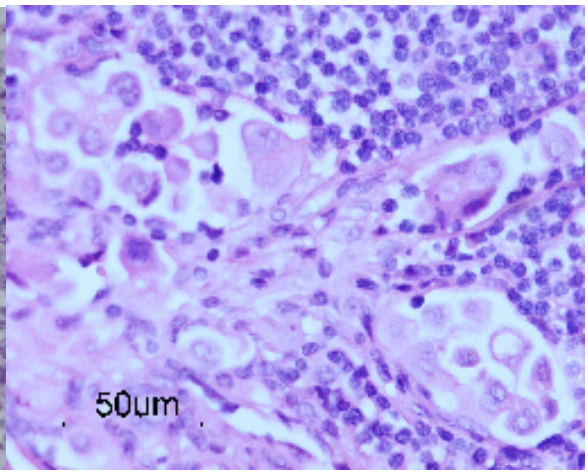

Figure 4: Implant in cervical lymph node in case 2. (Hematoxylin and Eosin staining)

The third case presented with deep venous thrombosis of the right arm at the age of 19, which was caused by enlarged supraclavicular lymph nodes at the right side. Further physical examination was unremarkable. Fine needle aspiration cytology of the supraclavicular lymph nodes revealed undifferentiated carcinoma of unknown primary. Additional immunohistochemical stainings denied a digestive tract tumour (CEA negative), ovarian tumour (β-HCG and α-fetoprotein negative), thyroid tumour (thyroglobulin and calcitonin negative) or melanoma (HMB-45 negative). Because of the localization of the enlarged lymph nodes a breast carcinoma was considered, but clinically this could not be confirmed and estrogen receptor staining was negative. A primary tumour could not be located with either cervical, thoracic or abdominal CT, chest X-ray, cervical, abdominal and breast ultrasound/mammography. Blood coagulation parameters were normal and after acenocoumarol therapy during three months she remained free of any complaints.
After five years of follow-up she developed complaints of abdominal pain and deep dyspareunia. Gynaecologic examination, ultrasound and abdominal CT scan revealed bilateral ovarian cysts, with a maximum diameter of 7 cm. Serum CA 125 was raised at 70 U/ml. At diagnostic laparoscopy a bilateral ovarian tumour was found and a biopsy of the surface of the cyst revealed serous BOT. Subsequently, a bilateral cystectomy was performed and histology showed no invasion and no clear atypia (figure 5). Immunohistochemical staining revealed positivity for CA 125, CAM 5.2, Cytokeratin 7 and Vimentin, while Cytokeratin 20, p53, Calretinin and CEA were negative (table 1). The tumour cells were DNA diploid. The subsequent surgical staging revealed non-invasive implants in omentum and pouch of Douglas, but para-aortic lymph nodes and further staging biopsies were free of disease. Retrospectively, the supraclavicular lymph node (figure 6) appeared to be positive for CA 125 and could therefore be attributed to localization of the borderline ovarian tumour. This patient remained in complete remission during the following seven years.

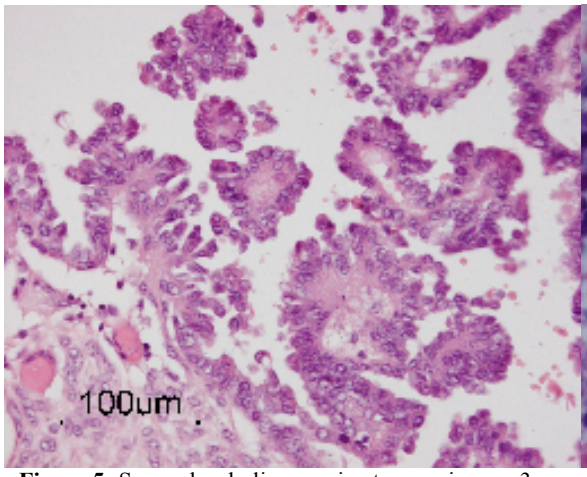

Figure 5: Serous borderline ovarian tumour in case 3. (Hematoxylin and Eosin staining)

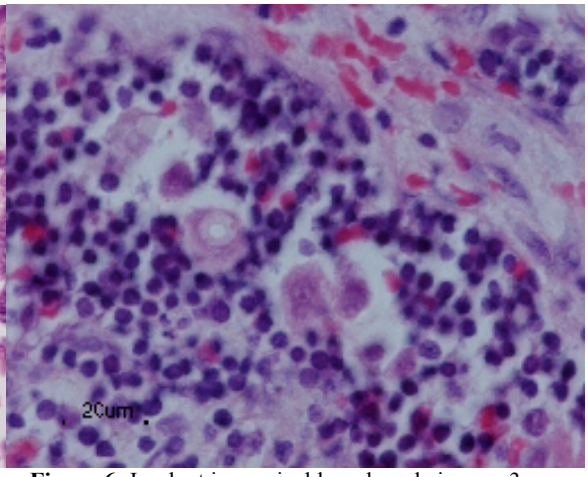

Figure 6: Implant in cervical lymph node in case 3. (Hematoxylin and Eosin staining)

Table 1: Immunohistochemical stainings of supradiaphragmatic lymph node inclusions and primary tumours in three patients with borderline ovarian tumours.

	Case 1		Case 2		Case 3	
	Lymph node	BOT	Lymph node	BOT	Lymph node	BOT
CA 125	+	+	+	+	+	+
CAM 5.2	+	+	+	+	+	+
Keratin 7	+	+	+	+	+	+
Vimentin	+/-	+	+/-	+	-	+
Keratin 20	+	-	-	-	-	-
P53	-	-	-	-	-	-
Thrombomodulin	-	-	-	-	ND	ND
Calretinin	-	-	-	-	ND	-
CEA	+	-	-	-	-	-
PR	-	+/-	+/-	+	ND	ND
ER	-	+	+/-	+	-	ND

BOT = Borderline Ovarian Tumour, + = Positive staining, - = negative staining, +/- = Focal staining, ND = Not Done.

Discussion

Lymph node involvement in women with serous BOT is rare. Moreover, supradiaphragmatic lymph node involvement in BOT is extremely rare. To our knowledge, supradiaphragmatic lymph node involvement in serous BOT has only been described in the literature in a few case reports, years after the primary tumour had been diagnosed[5-8, 10, 11], although stage IV BOT cases have been described recently with implants in the mediastinum and in pleural effusions.[11, 12] This is the first report of 3 cases in which the supradiaphragmatic lymph node involvement was seen at the same time as the BOT, or even years before.
Presentation with supradiaphragmatic lymphadenopathy in patients with serous adenocarcinoma of the ovary as well as at relapse of serous ovarian carcinoma after several years of remission is well known and has been described in the literature.[13, 14] However, initial presentation in serous BOT with supradiaphragmatic lymphadenopathy accompanied by a pelvic (cystic) mass can lead to an inaccurate clinical diagnosis of metastatic ovarian cancer. Because the histo/cytological and immunohistochemical[9] presentation of cells derived from lymph nodes containing serous BOT localization is usually indistinguishable from that of adenocarcinoma cells, this can even lead to the erroneous diagnosis of metastatic ovarian carcinoma or ACUP and hence to ineffective treatment with chemotherapy or radiotherapy as

in our second case. Moreover, the prognosis of serous BOT (even stage IV) differs significantly and positively from that of ovarian carcinoma and therefore provides the patient with a totally different perspective. This is especially important in young women, in whom these tumours tend to occur, as fertility sparing treatment is warranted for BOT.[15]
Consequently, it is important to obtain a histological diagnosis of the ovarian tumour if BOT or ovarian carcinoma is considered on the basis of distant (lymphatic) "metastases".
Molecular biology techniques might also be useful in distinguishing serous BOT from invasive serous ovarian carcinoma. Mutations in the Braf gene, for example, have been found to be present in approximately 40% of serous BOT, but not in invasive serous carcinoma.[16] When lymph nodes containing cells compatible with adenocarcinoma (of unknown primary) are found, the finding of a mutation in Braf could thus point in the direction of a primary serous BOT. Unfortunately, analysis of two of the present cases revealed a Braf mutation in neither BOT or lymph node. It can be expected that the further development of DNA-test, but than based on multiple DNA markers, will provide valuable tools to discriminate these entities more unequivocally.
Inclusions are frequently seen in (enlarged) lymph nodes that accompany ovarian malignancies (29%), with a predominance in serous BOT (85%).[17] In general, lymph node involvement in BOT is not clearly associated with worse prognosis in BOT and routine lymphadenectomy is not recommended.[18]
Lymph node inclusions can present in glandular or papillary epithelial structures with sometimes psammoma bodies, but may also present as dissociated cells resembling hyperplastic mesothelial cells[19]. In the absence of an accompanying ovarian neoplasm, they are usually referred to as endosalpingiosis or Mullerian inclusion cysts (MIC). In the presence of a serous BOT, however, they are usually called "implants", suggesting that they originate from the BOT. Whether this is actually the case, is not proven.
In conclusion, supradiaphragmatic lymph node inclusions can rarely be found in patients with serous BOTs, and cervical lymphadenopathy with arm thrombosis may even be the presenting symptom. When fine needle aspiration cytology of such a lymph node is compatible with adenocarcinoma, it is advised to include serous BOT in the differential diagnosis and histological diagnosis of the ovarian primary should ideally be obtained before deciding on systemic therapy.

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Chapter 4

BRAF mutation status in serous borderline ovarian tumours and the effect on clinical behaviour.

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Abstract

Aims: To determine the incidence of activating BRAF mutations in 30 Serous borderline tumours (SBTs) of the ovary and the accompanying implants and to link BRAF mutation status to clinical behaviour of these tumours.
Methods and results: SBTs and non-invasive implants of 30 patients were analysed for the presence of the *BRAF* V599E mutation, and mutation status was correlated to 70 months of clinical follow up. Mutation status could be assessed in 27 SBTs. Eleven (41%) showed a *BRAF* mulation. Four of 5 (80 %) patients with bilateral SBT showed a *BRAF* mutation in both ovaries. From the 8 implants that were analysed for *BRAF*, 2 (25%) were mutated together with their primary tumour. *BRAF* mutation positive SBTs tend to present with a lower FIGO-stage, a higher tumourvolume and are less frequently aneuploid. Seventy months follow up indicated no significant recurrence free survival difference between these groups.
Conclusions: BRAF mutations are common in ovarian SBT, are strongly associated with bilateral tumours and are also found in implants. A larger number of tumours should be investigated to assess clinical importance of *BRAF* mutation status in SBTs.

Introduction

Activating mutations in the *BRAF* gene coding for a protein kinase play an important role in the development of human cancers, especially in melanomas, colorectal carcinomas and thyroid and ovarian tumours[1]. Mutations in the *BRAF* gene are predominantly concentrated in exon 15 (98% of all mutations) and 92% of these contain a V599E mutation at the protein level, replacing a valine by a glutamic acid which alters the substrate binding site. It has been suggested, based on the detection of mutations in the *BRAF* and also *KRAS* genes, that the RAS-RAF-MEK-ERK-MAP kinase pathway plays an important role in the formation of ovarian serous borderline tumours (SBTs)[2-4].
SBTs are an intermediate form, both clinically and morphologically, of ovarian neoplasm's, placed between benign cystadenomas and malignant high-grade serous carcinomas (SCA). In around 20 % of cases, SBTs present bilaterally and in 30% of cases, peritoneal lesions of similar morphology, usually referred to as "implants", can be found both intra- and retroperitonally[5]. In one study mutations in the *BRAF* gene have been found in 36% of SBTs, whereas no *BRAF* mutations were found in high grade serous carcinoma of the ovary (SCA).[6]
It has been suggested that bilateral SBTs are from a monoclonal origin, pointing to a metastatic potential of cells from the primary tumour which can than subsequently seed on the peritoneal surfaces. However, little is known on the relationship between the peritoneal lesions and the SBT. The name "implants" suggests that they indeed derive from the primary SBTs, but they may equally constitute primary lesions reflecting a field change that can affect the lining of the ovaries and peritoneum, and may lead to lymph node localisations. Furthermore, the clinical importance of *BRAF* mutations in SBTs is not known.

To determine whether clinical behaviour of SBTs is altered by the presence of a *BRAF* mutation the *BRAF* mutation status was linked to clinical parameters and follow-up.

In addition, to asses monoclonality, contralateral tumours and peritoneal non-invasive implants accompanying SBTs were investigated, to determine whether these carry the same *BRAF* mutation as their primary SBTs.

Materials and methods

Patient characteristics

Clinical files and tumour material of 30 SBT patients diagnosed in the VU University Medical Center between 1989 and 2002 were retrieved. Age at diagnosis, FIGO stage, tumour volume (calculated from ultrasound measurements and expressed in cm^3), DNA ploidy of tumour cells, CA 125 (U/l) at diagnosis and recurrence of disease were assessed. Slides were reviewed by two experienced gynaecopathologists (PvD and NS). Six SBTs were bilateral and nine were associated with non-invasive implants.

DNA extraction

Tumour cells were microdissected from 10μm slides taken from representative paraffin blocks after deparaffinization and staining with hematoxylin, using laser microdissection (Leica, Rijswijk, The Netherlands) if necessary because of a reletively low percentage of epithelium (7 cases). DNA extraction was performed using a standard proteinase K digestion followed by heat inactivation of the enzyme[7].
DNA concentrations were measured by spectrophotometry using the GeneQuant (Biochrom Ltd, Cambridge, UK). DNA concentrations ranged between 55 and 400 μg/ml.

Mutation analysis

Samples were analysed for the *BRAF* mutation V599E, located in exon 15 of the *BRAF* gene, by performing a semi-nested PCR followed by restriction fragment length polymorfism (RFLP). Firstly, a larger product was formed covering the complete exon 15 of the *BRAF* gene (255 base pairs) using forward primer 5'-TCA TAA TGC TTG CTC TGA TAG GA-3' and reverse primer 5'-ATG ACT TTC TAG TAA CTC AGC AGC-3'. Secondly, this product was used as a template for amplification of a 224 base pair fragment using the same forward primer as in the first PCR and reverse primer 5'-GGC CAA AAA TTT AAT CAG TGG A-3'. Subsequently, an RFLP was performed using restriction enzyme TspR I (New England BioLabs Inc., Hitchin, UK) which cuts wild type *BRAF* exon 15 into two fragments and leaves mutated *BRAF* exon 15 intact. The fragments were subsequently analysed on a 6% polyacrylamide gel. Positive and negative controls for this analysis were DNA isolated from the HT-29 cell line (harbouring the V599E mutation) and the CaCo2 cell line (wild type *BRAF*), respectively. All analysis were performed in duplicate.

Statistical analysis

Possible associations between *BRAF* mutation status and clinicopathological features were evaluated with the T-test for continuous features and by X^2 -test for the categorical features. Prognostic value of *BRAF* mutation status was evaluated by plotting Kaplan-Meyer curves and comparing the curves with the logrank test. Significance level was set at $P<0.05$.

Results

BRAF mutation status

In 27 primary SBTs, 11 (41%) were found to have the V599E *BRAF* mutation in exon 15. In 5 bilateral SBTs, 4 (80%) had this mutation in both ovaries and the fifth was negative for the *BRAF* mutation on both sides. In 3 cases DNA quality was not sufficient for analysis.
Of the 8 SBTs with non-invasive implants 2 had the mutation in both primary tumour and implant and 1 primary SBT had the mutation while this was not seen in the non-invasive implant. Five had no mutation in either the primary tumour or implants. Results are summarised in Table 1.

Table 1: *BRAF* mutation analysis in 27 SBT patients.

No.	Patient No.	Primary SBT	Contralateral ovary	Non-invasive implant
1	14	Mut	Mut	Mut
2	29	Mut	Mut	Wt
3	20	Mut	Mut	
4	26	Mut	Mut	
5	30	WT	WT	
6	4	Mut		Mut
7	11	WT		WT
8	16	WT		WT
9	18	WT		WT
10	19	WT		WT
11	22	WT		WT
12	1	WT		
13	2	Mut		
14	3	Mut		
15	5	WT		
16	6	WT		
17	7	Mut		
18	9	WT		
19	12	Mut		
20	13	Mut		
21	15	Mut		
22	17	WT		
23	21	WT		
24	23	WT		
25	24	WT		
26	25	WT		
27	28	WT		
	Total	**27**	**5**	**8**

WT = Wild Type *BRAF*, Mut = Mutated *BRAF*, No. = Number.

Clinical parameters linked to BRAF mutation status

SBTs harbouring a *BRAF* mutation presented more frequently in FIGO stage Ia, had a higher tumour volume, were less frequently aneuploid and lower CA125 levels were found compared to *BRAF*- mutation-negative cases, although none of these differences reached statistical significance (Table 2). No differences between both groups could be determined for mean age at diagnosis and recurrence rate of disease.
Seventy months follow up of *BRAF* mutation-positive and negative cases indicated no significant recurrence free survival difference between these groups.

Table 2: Clinical parameters linked to *BRAF* mutation status in 27 primary SBT.

	No. (%)	**Mean age at diagnosis** year (± SD)	**FIGO stage** Ia	≥ Ib	**Mean Tumour volume** cm^3 (± SD)	**DNA Aneuploidy** No. (%)	**Mean CA 125** (U/l)	**Mean Follow-up** Months (Range)	**Recurrence** No. (%)	**Interval to recurrence** Months (Range)	**Death of disease** No. (%)
BRAF Mutation	11 (41%)	43.7 (± 12.0)	55%	45%	1591 (± 2559)	2 (18%)	139	70 (17-128)	3 (27%)	37 (13-63)	0
No *BRAF* mutation	16 (59%)	41.4 (± 9.4)	25%	75%	219 (± 272)	5 (31%)	306	70 (20-163)	4 (25%)	12.5 (7-21)	1 (6%)
P-value		0.42*	0.11**		0.07*	0.45**	0.22*				

* T-test,** X^2-test.

Discussion

This report describes the analysis of *BRAF* mutations in monolateral and bilateral SBTs and accompanying extra-ovarian non-invasive implants. Our study confirms earlier findings on the high incidence of *BRAF* mutations in SBTs of around 40%[6].
The finding that the *BRAF* status of 7 out of 8 non-invasive implants was identical with their corresponding primary SBTs supports the hypothesis that primary SBTs and their associated non-invasive implants are monoclonal[8]. This is also in line with reports on the findings of identical *KRAS* mutations in both ovarian and extra-ovarian lesions of SBT[9].
However, the finding that in one case the *BRAF* mutation was seen in the primary SBT but not in the non-invasive implant may favour the theory that extra-ovarian lesions found with SBTs might also arise due to a field change rather than are caused by metastatic behaviour of the primary SBT cells. Mutations in the *BRAF* and *KRAS* gene are thought to be equivalent in their tumorigenic effects[10] and mutations in either *BRAF* or *KRAS* have been found in approximately 65% of SBTs[2, 6]. All *BRAF* mutations, however, are thusfar exclusively found in SBTs and not in serous cystadenomas[11], serous carcinomas[12] (SCA) nor in mucinous ovarian tumours[6, 13]. Therefore this mutation can be used to discriminate between SBT and other ovarian neoplasms.

No significant correlation between *BRAF* mutation status in SBTs and clinical course was found, although there was a tendency of SBTs with *BRAF* mutations to display more favourable clinical parameters. SBTs with a *BRAF* mutation did present more frequently in FIGO stage Ia (tumour confined to one ovary and no extraovarian lesions) compared to SBT without *BRAF* mutation. However, this was not statistically significant ($p=0.11$, X^2 test). In addition, the calculated tumour volume of *BRAF* mutation carrying SBTs tends to be higher compared to SBTs without a *BRAF* mutation ($p=0.07$, T-test). The median tumour volume for *BRAF* mutation positive an negative SBTs was 512 cm^3 and 130 cm^3 respectively (range: 24-8000 and 1.5-1000 cm^3). This may explain why *BRAF* mutation carrying tumours were more often found in a lower FIGO stage as these earlier cause clinical complaints and therefore demand additional examination of the patient.
Although *BRAF* mutation positive tumours were less often aneuploid and accompanying CA 125 levels tended to be lower, this did not reach statistical significance. No differences between both groups could be determined for mean age at diagnosis and recurrence rate of disease after a mean of 70 months follow-up in both groups.

We conclude that *BRAF* mutations were found in approximately 40% of ovarian SBTs, were strongly associated with bilateral tumours, and were also (although less frequently) found in implants. This favours the theory that the contralateral and the peritoneal non-invasive implants accompanying SBTs are of monoclonal origin. Presence of a *BRAF* mutation does not seem to have an effect on prognosis in SBT patients.
Because *BRAF* mutations are exclusively found in SBTs this can be used to differentiate between SBT and invasive SCA or between serous and mucinous borderline ovarian tumours.

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Chapter 5

Array comparative genomic hybridization demonstrates increasing genomic imbalances during long term follow-up of a progressing serous borderline tumour of the ovary

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Abstract

Objective
Serous borderline ovarian tumors (SBTs) have morphologic features of both benign and malignant tumors, but usually follow a benign clinical course. Some lesions however progress over time into a locally aggressive tumor behaving like low-grade carcinoma. However, this progression is not associated with the acquisition of morphological characteristics of overt carcinoma. We studied genome-wide alterations by array comparative genomic hybridization (array-CGH) in a progressive SBT in search of genetic changes associated with local aggressive behavior.

Methods
From a woman diagnosed at the age of 30 years with an SBT that locally progressed until death followed 12 years later, we studied tumor samples by array CGH at four different time points during progression.

Results
Genome-wide, only 2% of the studied loci revealed gains or losses with no high-level amplifications. In the initial sample, gains were found in 2q32-33, 4q31-35, 5q34, 7q11, 7q33-35, 8p21.3, 8q21.1, 10p11.2, 12p12-13, 12q12-24, 14q24, 15q21-22, 15q26, 16p12-13, 17q21, 17q25, 19q13.2 and 22q13.1. Of these, especially the gains on chromosomes 12q12-24 were consistently found in the later samples. Gains at 1q31-41 emerged in the second sample (1995) and remained detectable in the follow-up samples. Losses in the 1p12-36 region emerged in the second sample and remained detectable.

Conclusions
Even progressive SBTs do not seem to harbor many genomic imbalances. However, gains at 1q31-41 and 12q12-24 and losses at 1p12-36 may play a role in clinical progression of SBT. Since genetic changes do not match those found in carcinoma, although clinical progression in this particular patient suggests otherwise, CGH-analysis does not support transgression from borderline to malignant tumor.

Introduction

Serous borderline ovarian tumors (SBTs) are pathologically defined by cysts with complex papillary stromal proliferations lined by single- or multilayered serous epithelium with limited mitotic activity and low nuclear atypia, but no stromal invasion[1]. They have a low potential to invade and/or metastasize ("low malignant potential"). Although some cases may exhibit local progression with peritoneal lesions that spread, invade and over many years lead to bowel obstruction and death of the patient, the prognosis is significantly better than for overt epithelial ovarian cancer[2]. Pathologists have attempted to identify progressive SBTs by morphometric[3] and morphological criteria [4], but follow-up studies have failed to confirm the value of these approaches[5] . However, identification of high risk SBTs remains important since an extensive staging procedure is warranted in these patients.
Accumulation of genetic events frequently occurs at tumor progression. Little is known about the genetic make-up of SBTs apart from the frequent occurrence of BRAF mutations[6, 7] and no studies have looked at genetic events associated with progression of SBTs. Long-term longitudinal follow-up of progressive SBT cases monitoring genetic events could potentially indicate the genetic progression pathways. Here, we report a case of SBT that was well documented over the course of more than ten years, during which the tumor progressed from a local SBT to widespread intra-abdominal disease with invasive implants causing bowel obstruction and eventually demise. Array CGH was used to determine progressive genomic imbalances in the tumor cells at four different time-points during progression.

Materials and methods

Patient
A 26 years old para 0 was first admitted to the department of gynaecology of the VU University Medical Centre, Amsterdam, in 1988 because of primary infertility after adnexitis and she underwent bilateral neostomia. In 1991 IVF-treatment resulted in an ectopic pregnancy. In 1992 a right ovarian cyst was seen on ultrasound and cystectomy was performed. Pathological examination in our own institute as well as by external experts (Professors Fox, Manchester, UK, and Scully, Boston, USA) confirmed an SBT with a diameter of 6.5 cm (Fig. 1A and 1B). There was no disease outside the right ovary at that time.
During the years after this diagnosis the patient was checked regularly by physical examination, ultrasound and serum CA 125 determination and she underwent multiple operations because of recurrent disease (Fig. 2). In total, she was operated seven times between 1992 and 2002 without change in pathological diagnosis except for development of invasive implants with micropapillary formations in 1992. Finally, the progressive tumor was inoperable and in view of the aggressiveness of the tumor it was decided, in spite of the pathological diagnosis of SBT, to treat her with chemotherapy (carboplatin/taxol followed by cyclophosphamide/celecoxib). As expected, there was no response and in 2004 she succumbed to progressive bowel obstruction at the age of 42.

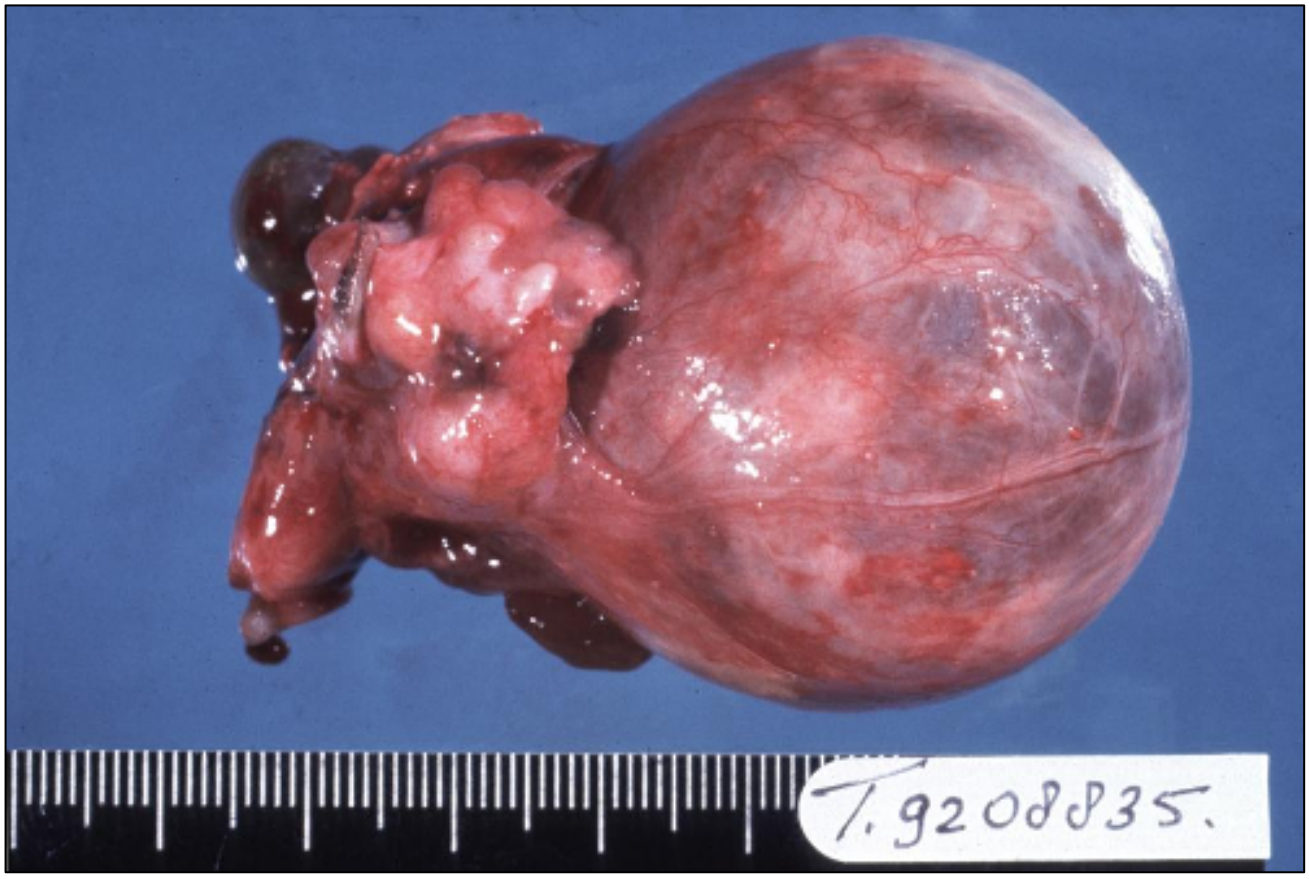

Figure 1A. Cyst from the right ovary and right Fallopian tube. Pathological examination confirmed a serous borderline ovarian tumor (SBT).

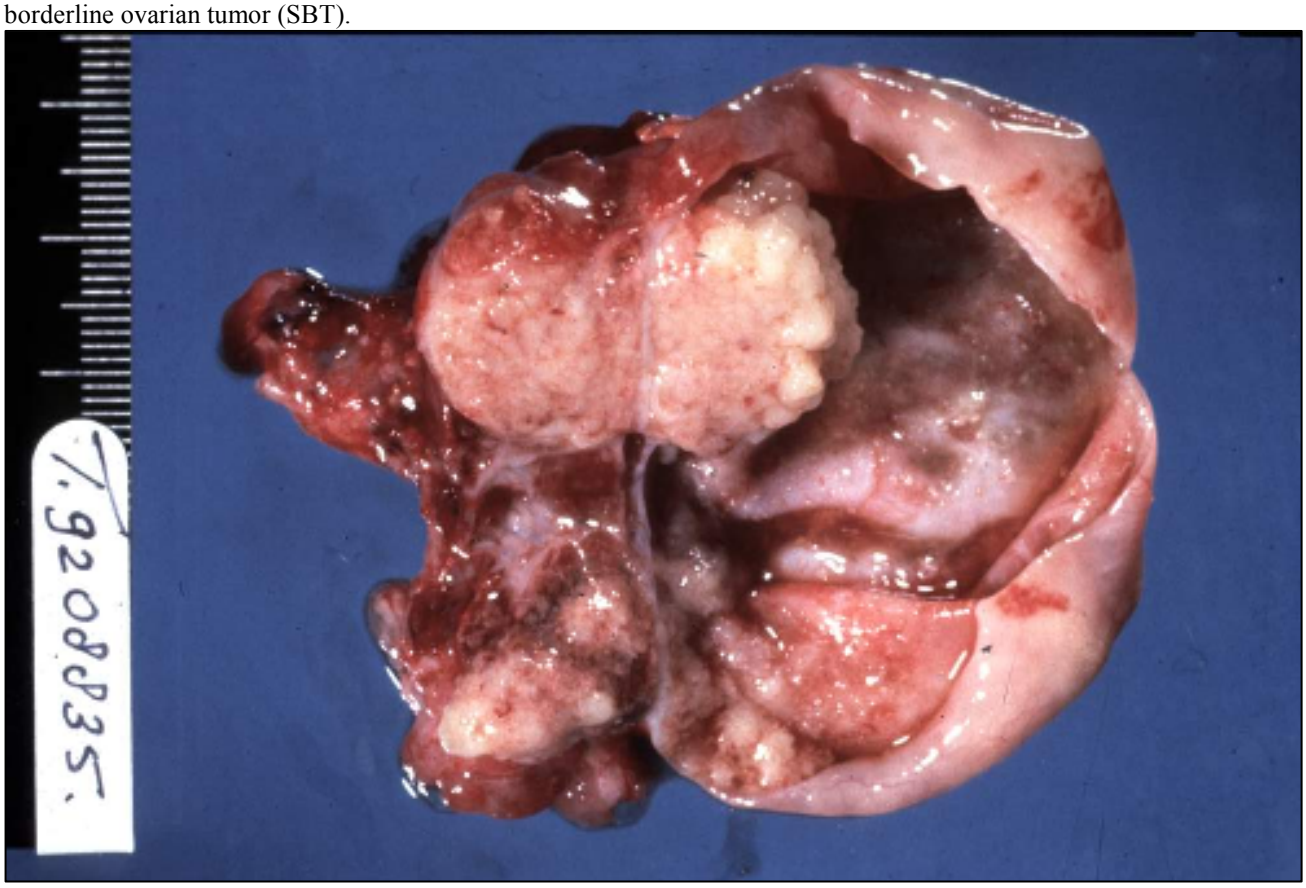

Figure 1B. Sample as shown in figure 1A, after opening of the cyst.

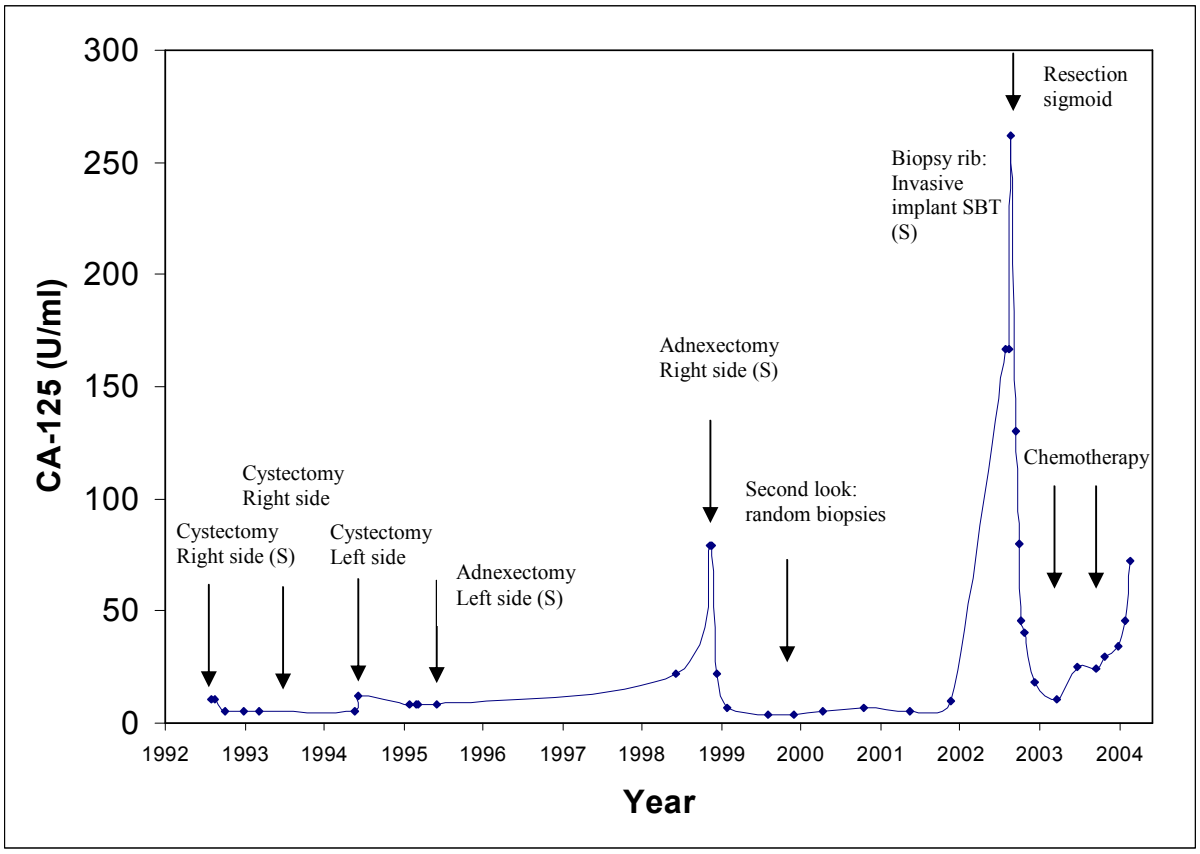

Figure 2. CA 125 in time in relation to therapy. (S) = Sample analysed by array-CGH.

DNA extraction

Tumor tissue was obtained at each operation and embedded in paraffin. Representative tumor areas were microdissected from 10μm slides after deparaffinization and staining with hematoxylin using a laser microdissector (Leica, Rijswijk, The Netherlands). DNA extraction was performed using a standard proteinase K digestion followed by heat inactivation of the enzyme[8]. DNA concentrations were measured by spectrophotometry using GeneQuant (Biochrom Ltd, Cambridge, UK). DNA concentrations ranged between 300 and 2790 μg/ml.

Array Comparative Genomic Hybridization (CGH) and BRAF mutation analysis

Array CGH was performed as described before using arrays of 2464 BACs provided by the UCSF Comprehensive Cancer Center Microarray Shared Resource [9, 10]. A gain was defined as a $\log_2$ratio > 0.45, high-level amplifications as a $\log_2$ratio > 1.0, and a loss as a $\log_2$ratio < -0.45[11]. We searched for known genes via the UCSC genome browser (http://www.genome.ucsc.edu; March 2006 assembly).

Samples were analyzed for the *BRAF* mutation V599E, located in exon 15 of the *BRAF* gene, by performing a semi-nested polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) as described before[6].

Results

No mutation in the *BRAF* gene was found in any of the lesions analyzed by array CGH. DNA quality of four out of the seven samples was sufficient to perform array CGH analysis and included samples obtained in 1992, 1995, 1998 and 2002. Overall, only 2% of the loci showed a gain or loss and no high-level amplifications were seen in any of the samples.
In the initial sample from 1992 gains were found at 2q32-33, 4q31-35, 5q34, 7q11, 7q33-35, 8p21.3, 8q21.1, 10p11.2, 12p12-13, 12q12-24, 14q24, 15q21-22, 15q26, 16p12-13, 17q21, 17q25, 19q13.2 and 22q13.1 (Table 1). Not all of these gains remained detectable in the later samples, but especially the gains on chromosome 12q12-24 were consistently found in the later samples (1995, 1998 and 2002). Gains in the 1q31-41 region emerged in the second sample (1995), which remained detectable through the 2002 sample.
Loss of a single clone was found on 6q26, a region known to contain a copy number polymorphism, in all samples (Table 2). Losses at the 1p12-36 region emerged in the 1995 sample and remained detectable throughout 2002.

Table 1: Gains found by array CGH in samples from a serous borderline tumor at different time-points during clinical progression.

Gains:				**Putative**	
1992	**1995**	**1998**	**2002**	**target gene**	**Function**
	1q25				
	1q31	1q31	1q31		
	1q32.1	1q32.1			
1q32-41	1q32-41	1q32-41	1q32-41		
	1q41	1q41	1q41	*DUSP10*	Cell proliferation
	1q43-44				
	1 q tel				
2q32.3-2q33				*STAT1*	Transcription activator
3q26.3				*PIK3CA*	Oncogenic gene
4q31.1					
4q33-4q34		4q33-4q34			
4q35		4q35			
5 p tel					
		5p13-5p14			
		5q31			
5q34		5q34			
7p13d-p13e					
7 q11.23		7 q11.23			
7q33-35		7q33-35	7q33-35	*BRAF*	Cell proliferation
8p21.3		8p21.3			
			8p22-8p23		
8p23.1					
8q21.1		8q21.1			
9q21.3					
10p11.2					
11p11.12					
11p15.2					
11q22					
		11q23			
	12 p tel				
	12p11.2				
12p12	12p12-12p13	12p12		*KRAS*	Cell proliferation
12p13	12p13				
	12p13.3	12p13.3	12p13.3		
12q12-12q13		12q12-12q13			
	12q13	12q13	12q13		
12q13.2-q13.3	12q13.2-q13.3	12q13.2-q13.3	12q13.2-q13.3	*TSPAN9*	regulation of cell development, activation, growth
	12q13.3				
12q14	12q14	12q14			
12q14.1					
	12q14-12q15	12q14-12q15		*MDM2*	Increases tumorigenic potential
	12q21.3-12q22				
12q22-12q23					
12q23-12q24.1					

12q24.1 12q24.2	12q24.1 12q24.2 12q24.33	12q24.1	12q24.33		
13q14					
14q24 14q24.2-14q24.3 14q32.2					
15q21.3 15q22.1 15q26.1		15q26.1		*PML* *IGF1R*	Contribute to senescence and tumor suppression Anti-apoptotic activity
16p12-16p13.1 16p13.3					
17 p13 17p13.3 17q21 17q24 17q25.3		17 p13 17q21 17q21.2 17q25 17q25.3		*TP53*	Cell cycle regulator
18p11.31-.32					
19p12-19p13.1 19p13.2 19p13.3 19q13.2 19q13.4	19q13.2	19p12-19p13.1 19p13.2 19p13.3 19q13.1 19q13.2 19q13.4	19q13.2	*CCNE1* *AKT2*	Oncogene, found in OVCA
20q12 20q13.3 22q13.1		20q13.3			
X/Y p tel Xp11.3 Xp22.2 Xq12 Xq13		X/Y p tel			

Table 2: Losses found by array CGH in samples from a serous borderline tumor at different time-points during clinical progression.

Losses:					
1992	**1995**	**1998**	**2002**	**Gene**	**Function**
	1p12-13				
	1p13	1p13	1p13		
	1p13.2	1p13.2	1p13.2	*RAP1A*	Counteracts mitogenic function of RAS
	1p13-21	1p13-21			
	1p21	1p21	1p21		
1p22	1p22	1p22	1p22	*BRDT*	Transcription regulator
	1p22-31	1p22-31			
	1p31	1p31	1p31		
	1p31.1	1p31.1	1p31.1		
	1p31.1-1p31.2	1p31.1-1p31.2			
	1p31.2-1p31.3	1p31.2-1p31.3			
	1p32	1p32	1p32		
	1p32.1	1p32.1	1p32.1		
	1p32-33				
	1p33				
	1p33-1p34.1	1p33-1p34.1	1p33-1p34.1		
	1p34.2				
	1p35				
	1p36		1p36		
	1p36.2-.3	1p36.2-.3	1p36.2-.3	*FGR*	
	1p36.3	1p36.3			
	1q31.3	1q31.3			
3p14					
3p14.1					
	3p21.2		3p21.2		
			4p13-4p14		
	4q35		4q35		
		5q33.2			
6p12					
6q26	6q26	6q26	6q26		Known copy number polymorphism
7q21.11f-q21.11g		7q21.11f-q21.11g			
			7q31		
			7q35		
8p23.2		8p23.2			
	8q21.1	8q21.1	8q21.1	*PK1A*	Protein kinase inhibitor
			9p21-9p22		
	10p11.2		10p11.2		
	15q22.1		15q22.1		
			15q23-q25		
			16q24		
20q13.1					

Comment

We studied an SBT by genome-wide array CGH analysis during clinical progression over a period of ten years until death of the patient. Interestingly, overall only 2% of the studied loci revealed genomic imbalances, the majority being low-level gains, while high-level amplifications were lacking. This emphasises that, in contrast to malignant serous ovarian tumors[8], SBTs harbor only few genomic imbalances and that the carcinogenetic pathway of SBT is evidently different from that of overt high-grade ovarian cancer. Since there are also few known mutations in oncogenes and tumor suppressor genes in SBTs, it is possible that other genetic changes such as epigenetic changes may play a role in the development and progression of these tumors.

The gained regions harbor only a few known genes that could, in view of their biological function, be implicated in SBT-genesis (Table 1): the transcription activator STAT1 at 2q32.3-2q33 (http://bioinf.uta.fi/STAT1base/), IGF-1R at 15q26.1 which binds IGF and acts

as an anti-apoptotic tyrosine kinase enhancing cell survival and the AKT2 oncogene at 19q13.2 known to be implicated in ovarian cancer[12]. Although the gained regions at 12p12 and 7q33-35 contain respectively the KRAS and BRAF genes which have been implicated in SBT-genesis[6], we cannot propose these genes as drivers of SBT genesis as the accepted mechanism for activation of these oncogenes are activating (point-) mutations and not gene amplifications.
Overall, there were few losses but a consistent one over time was found at 6q26. This region is known to contain a copy number polymorphism. Losses in the 1p12-36 region were consistently found in the second, third and fourth sample suggesting that this region contains genes that are implicated in tumor progression in SBTs.
There are few other studies published on CGH of SBT. Helou *et al.* also found losses in 1p and 6q and additionally in 2q, 4q and 5q in 8 borderline tumors (6 serous and 2 mucinous). Gains were seen in 6p and chromosome 12. That study showed similar patterns of gains and losses to benign ovarian cyst cell DNA and also in their study the SBTs clearly showed fewer gains and losses than overt ovarian carcinomas[13].
There were few gains or losses that remained constantly detectable through time. Moreover, although gains were relatively frequently found in the primary tumor, they tended to disappear over time, whereas losses were very rare initially, but seemed to appear and remain in subsequent lesions. The rare constant events might be related to clinical progression."
These concern gains in regions 1q31-41 and 12q12-24, and losses at 1p12-36. These relatively low numbers of additional events indicate that also during clinical progression, there is only a modest increase in genomic imbalance. The 1p13.2 locus harbors the RAP1A gene that counteracts mitogenic function of RAS and the 1p22 locus the transcription regulator BRDT that may act as tumor suppressor.
Despite these additional changes, the morphology of the progressive lesions detected over time remained similar, underlining that genetic events driving progression do not necessarily lead to morphological changes towards overt carcinoma.
As to other genes potentially involved in SBT genesis and progression, Bonome *et al.* identified genes that contribute to the typical behaviour of SBTs by Affymetrix microarrays in 20 SBTs[14]. Part of the described profile was found in low-grade serous cancers as well, but differed entirely from the genetic profiles seen in high-grade serous carcinomas. This finding implies that some SBTs may progress to low-grade serous carcinomas (type I) although probably rarely, but not to high-grade ovarian carcinomas (type II)[15]. The progression model as proposed by Shih *et al* is probably seen in this patient[16].
In conclusion, even progressive SBTs do not seem to harbor many genomic imbalances. Based on this study, gains at 1q31-41 and 12q12-24, and losses at 1p12-36 may play a role in clinical progression of SBT.

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Chapter 6

Discrimination between high grade serous ovarian carcinoma and serous borderline ovarian tumours by multiplex ligation dependent probe amplification

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Abstract

Introduction
We studied DNA copy number profiles of serous borderline tumours (SBTs) and high-grade serous ovarian carcinomas (OVCAs) by multiplex ligation dependent probe amplification (MLPA). From these profiles two models were derived that could discriminate between SBT and OVCA DNA.

Material and methods
DNA samples of 20 high-grade OVCAs and 18 SBTs were collected and MLPA was done using 2 probesets that cover 32 genes. From the observed gain and loss patterns mathematic models were derived by logistic regression analysis. These models were then blindly tested on an independent validation set of 27 OVCAs and 23 SBTs.

Results
Of the OVCA, 42% showed gains and 6% losses, compared to 11% and 4% in the SBT group, respectively. Two classification models were composed containing genes *MYC*, *CCNE1* (model 1), and*, EVI1, and NFKBIE*, *BCL2L1, PSMB4* and *MDM2 (model 2)*, respectively. When tested on the validation set, b. oth models could discriminate well discriminate OVCAs and SBTsa SBT (model 1: 85% and 65% of cases (p=0.00025, Chi-square-test); model 2: 67% and 78% (p=0.0015)).

Conclusion
Genetic alterations are not frequently seen in SBTs compared to high-grade OVCAs and copy number changes that are found in SBT differ from those in OVCAs. These results underline the theory that SBTs are not precursors of high-grade serous OVCAs but are a separate entity in the ovarian tumour spectrum. The described MLPA probe-sets can provide a practical tool to distinguish high grade OVCAs and SBTs in difficult cases.

Introduction

Ovarian carcinoma (OVCA) is a common gynaecological malignancy and a leading cause of death from gynaecological cancer in the Western world[1]. In contrast, serous borderline ovarian tumours (SBTs) have an excellent overall 10-year survival of up to 99%, even in advanced stages[2]. Recently, different molecular pathways have been proposed in the development of low-grade serous (borderline malignant) tumours and high-grade serous OVCAs [3, 4] concerning genes like *KRAS*, *BRAF*, *TP53*, *HER-2 and AKT* [5, 6].
We recently reported on array comparative genomic hybridization (array CGH) analysis of serous OVCAs and serous Fallopian tube carcinomas (FTCs) and a pilot multiplex ligation-dependent probe amplification (MLPA) study of a number of selected genes, that were based on the array CGH analysis[7]. MLPA is a genomic technique that can be used for copy number estimation of up to 50 different target genes in an easy and rapidly to perform polymerase chain reaction (PCR)-based test. Furthermore, MLPA is inexpensive and needs a relatively low amount of DNA[8] that can well be derived from paraffin-embedded tissue. The OCVA/FTC array CGH study revealed differences in the genomic profiles between these two tumour types but also important shared features, and identified various genes possibly involved in the carcinogenesis of high-grade serous OVCA and FTC. In a second study, we expanded the pilot MLPA set and we developed two gain probe-sets, specifically tailored to high-grade serous OVCAs and FTCs (submitted).
In the present study, these two MLPA gain probe-sets were used to study DNA copy number profiles of 18 SBTs and 20 high-grade serous OVCAs. Based on current ideas, we hypothesized that with these MLPA sets based on aberrations in high-grade serous OVCAs and FTCs, SBTs could be discriminated from high-grade serous OVCAs and differences in their molecular pathways could possibly be substantiated. Logistic regression resulted in mathematic models, displaying sets of genes that discriminated between serous OVCAs and SBTs. The proposed models were then validated on an independent and blinded set of 50 high-grade serous OVCAs and SBTs.

Material and methods

Formalin-fixed, paraffin-embedded samples of 20 high-grade (i.e. grade 2 and 3, according to WHO criteria[9]) serous OVCAs and 18 SBTs, all diagnosed in the VU University Medical Center, Amsterdam, The Netherlands, were collected. All samples were histologically reviewed by one pathologist (PvD). The age range of OVCA patients was 40-79 years, with a mean age of 60 years. The age range of SBT patients was 29-60 years, with a mean age of 39 years. The main characteristics are shown in Table 1.
Tumour DNA was extracted from 20 microdissected 10 μm paraffin-embedded sections per specimen. DNA was prepared using a QIAmp® DNA mini kit (Qiagen GmbH, Hilden, Germany) following a modification of the manufacturer's protocol[10]. DNA concentrations were determined spectrophotometrically (GeneQuant, Amersham Biosciences, Upsala, Sweden; NanoDrop, Nanodrop technologies, Wilmington, USA).
MLPA probes were designed according to manufacturer's protocols (MRC Holland, Amsterdam, The Netherlands, www.mrc-holland.com). Each probe consists of two hemiprobes, one synthetic and one M13-derived oligonucleotide, which contain target-specific sequences. The probe sequences are available on request.

Table 1: Main characteristics of the study population

Serous Borderline Tumours			Ovarian Carcinomas		
Case	FIGO stage	Age	Case	FIGO stage	Age
1	IA	39	1	IIIC	52
2	IC	31	2	IIIC	48
3	IA	29	3	IIIC	62
4	IIA	31	4	IIIC	45
5	IIC	39	5	IIIC	62
6	IIC	33	6	IIIC	52
7	IA	56	7	IIIC	68
8	IV	35	8	IIIC	60
9	IV	35	9	IIIC	50
10	IA	29	10	IA	43
11	IA	36	11	IV	58
12	IB	54	12	IIC	53
13	IA	48	13	IV	79
14	IC	60	14	IA	77
15	IIIC	35	15	IV	68
16	IIB	30	16	IIIC	73
17	IIIC	41	17	IB	68
18	IIC	39	18	IIIC	68
			19	IIIC	65
			20	IIIC	40

Abbreviations: FIGO, International Federation of Gynaecology and Obstetrics.

The probe sets were designed primarily based on our previous array CGH and pilot MLPA results in high-grade serous FTCs and OVCAs[7], and on genes of interest from the literature (Table 2). The first probe set (P134B, MRC Holland) contains probes for 16 genes, of which 12 were covered by 2 probes at different exons. The second probe set (P135B. MRC Holland) targets 16 genes of interest of which 15 are covered by two probes. The probes target regions on 1p, 1q, 3q, 6p, 7q, 8p, 8q, 12p, 12q, 16p, 17q, 19p, 19q, 20q, and 22q. Furthermore, 8 and 9 control probes, respectively, were included for normalisation purposes. These control probes have been validated earlier[7].

All samples were analysed in triplicate. Furthermore, each MLPA experiment included five reference runs performed on normal human female DNA from multiple donors (Promega, Madison, Wisconsin, USA) that were spread through the sample plate for normalisation. All MLPA reactions were performed using a Biometra thermocycler (Biometra, Goettingen, Germany) and MLPA products were separated and quantified on a Beckman CEQ8000 capillary system (Beckman Coulter, Fullerton, USA). Fragment analysis was performed as described before (Nowee et al., in press). Ratios less than 0.7 were considered a deletion (loss), ratios higher than 1.3 a gain and ratios higher than 2.0 were considered an amplification.

From the observed gain and loss patterns in the 20 high grade serous OVCAs and 18 SBTs, mathematic models were derived using logistic regression (SPSS for Windows, release 11.0.1) to predict the probability of a tumour being an OVCA or an SBT. These models were then tested on a blind independent set of 50 serous high-grade OVCA and SBT DNA samples, provided by the Department of Pathology of the Maastricht University Medical Center, Maastricht, The Netherlands. The predicted and histomorphologically defined tumour types were compared, summarised in a 2x2 table and the percentage of correctly classified cancers was calculated. Statistical significance was calculated by Chi square-tests and defined by $p<0.05$.

Table 2A. Multiplex ligation-dependent probe amplification gene probemix 1 (P134B)				
Gene symbol	Number of probes	Chromosome position‡	Array CGH‖	Literature§
PIK3CA	2	03q26.32		amp+overexpression OVCA
GLI3	2	07p14.1	no change	
CCM2	1	07p13.0	no change	
LIMK1	2	07q11.23	recurrent gain OVCA	
FGFR1	2	08p12		amp OVCA
MYC	2	08q24.21	recurrent gain OVCA; amp FTC+OVCA	
PTK2	2	08q24.3	recurrent gain OVCA; amp FTC+OVCA	
EXT2	1	11p11.2	no change	
VMD2	1	11q12.3	no change	
JARID1A	2	12p13.33	amp FTC+OVCA	
TSPAN9 (NET5)	1	12p13.33-p13.32	recurrent gain OVCA; amp FTC+OVCA	
MDM2	2	12q15		amp+overexpression OVCA
SOCS1 (JAB)	1	16p13.13		hypermethylation OVCA
OMG	1	17q11.2	no change	
ERBB2	2	17q12	amp FTC+OVCA	
SMARCA4 (BRG1)	2	19p13.2	recurrent gain FTC+OVCA; amp FTC	
AKT2	2	19q13.2		amp+overexpression OVCA
ZNF337	1	20p11.21	no change	
PYGB	1	20p11.21	no change	
BCL2L1	2	20q11.21	recurrent gain OVCA; amp OVCA	
NCOA3	1	20q13.12	recurrent gain OVCA; amp FTC	
PTPN1	1	20q13.13	recurrent gain OVCA; amp FTC+OVCA	
KCNQ2	2	20q13.33	recurrent gain OVCA; amp FTC+OVCA	

‡ The chromosome positions were determined with the March 2006 Assembly of the UCSC Genome Browser.

‖ Most of the genes in the two multiplex ligation-dependent probe amplification (MLPA) probe sets were chosen based on our previous array comparative genomic hybridization (array CGH) and pilot MLPA data (either recurrently gained or showing high-level amplifications; Nowee *et al*., J Pathol, 2007 (213) 46-55).
Reference probes were chosen based on the chromosome loci in our previous array CGH analysis that did not contain DNA aberrations in any of the analyzed high-grade serous FTCs and OVCAs.

§ A small subset of the genes was chosen based on genes of interest from data mining.

Note. Abbreviations: FTC, Fallopian tube carcinoma; OVCA, ovarian carcinoma; amp, high-level amplification.

Table 2B. Multiplex ligation-dependent probe amplification gene probemix 2 (P135B)				
Gene symbol	Number of probes	Chromosome position‡	Array CGH‖	Literature§
MYCBP	2	01p34.3		stimulates transcription by *MYC*
PSMB4	2	01q21.3	recurrent gain OVCA; amp OVCA	
COL3A1	1	02q32.2	no change	
EVI1	2	03q26.2	amp FTC+OVCA	
NFKBIE	2	06p21.1	amp OVCA	
GLI3	3	07p14.1	no change	
MTSS1	2	08q24.13	recurrent gain OVCA; amp FTC+OVCA	
RNF139	2	08q24.13	amp FTC+OVCA	
EIF2C2	2	08q24.3	recurrent gain OVCA; amp FTC+OVCA	
ALX4	2	11p11.2	no change	
EXT2	1	11p11.2	no change	
VMD2	1	11q12.3	no change	
TEAD4	2	12p13.33	recurrent gain OVCA; amp FTC+OVCA	
GPRC5A (RAI3)	2	12p13.1	recurrent gain OVCA; amp FTC+OVCA	
OMG	1	17q11.2	no change	
CCNE1	1	19q12	amp FTC+OVCA	
DPF1	2	19q13.13-q13.2	recurrent gain FTC; amp FTC	
ACTN4	2	19q13.2	recurrent gain FTC; amp FTC	
SPINT2	2	19q13.2	recurrent gain FTC; amp FTC	
BCAS4	2	20q13.13	amp FTC+OVCA	
NFATC2	2	20q13.2		overexpression breast cancer
PDGFB	2	22q13.1		amp in OVCA

‡, ‖, § Notes and abbreviations as in Table 2A.

Results

In our dataset of 20 high-grade serous OVCAs and 18 SBT a total of 332 gains was found of which 66 (20%) were amplifications, and a total of 61 losses was found. In the group of OVCAs, 42% and 6% of tumours showed gains and losses, respectively, while in the SBT group only 11% showed gains and 4% losses.
Known genes in ovarian tumorigenesis, e.g. *PIK3CA* [11], *AKT2* [12], *FGFR1* [13] and *PDGFB* [14] were gained in 5% to 55% of OVCAs compared to 0% to 28% in SBTs. Amplification of these genes was only seen in the OVCAs (Table 3).

Table 3. Summary MLPA results of serous borderline ovarian tumours (18) and ovarian carcinomas (20)							
		serous borderline tumours			ovarian carcinomas		
Gene symbols	Chromosome position	Gain (%)	Amp (n)	Loss (%)	Gain (%)	Amp (n)	Loss (%)
MYCBP	01p34.3	11%	1	6%	5%	0	15%
PSMB4	01q21.3	0%	0	0%	50%	3	0%
EVI1	03q26.2	0%	0	11%	35%	1	10%
PIK3CA	03q26.32	6%	0	6%	10%	0	10%
NFKBIE	06p21.1	0%	0	0%	70%	2	5%
LIMK1	07q11.23	6%	0	0%	30%	0	5%
FGFR1	08p12	28%	0	0%	55%	3	10%
RNF139	08q24.13	50%	1	0%	80%	4	0%
MTSS1	08q24.13	11%	0	0%	55%	1	0%
MYC	08q24.21	39%	0	0%	85%	7	5%
EIF2C2	08q24.3	17%	0	0%	65%	4	0%
PTK2	08q24.3	44%	0	0%	50%	2	0%
JARID1A	12p13.33	6%	0	0%	40%	2	0%
TEAD4	12p13.33	28%	0	0%	70%	6	0%
TSPAN9	12p13.33-p13.32	0%	0	0%	45%	1	5%
GPRC5A	12p13.1	22%	0	0%	45%	4	0%
MDM2	12q15	0%	0	17%	0%	0	80%
SOCS1	16p13.13	6%	0	0%	45%	1	5%
ERBB2	17q12	11%	0	0%	20%	2	15%
SMARCA4	19p13.2	6%	0	0%	15%	0	10%
CCNE1	19q12	6%	0	11%	40%	2	0%
DPF1	19q13.13-q13.2	28%	0	0%	70%	3	0%
SPINT2	19q13.2	6%	0	6%	35%	2	0%
ACTN4	19q13.2	0%	0	0%	45%	1	0%
AKT2	19q13.2	28%	0	28%	25%	1	0%
BCL2L1	20q11.21	0%	0	0%	60%	3	0%
NCOA3	20q13.12	0%	0	11%	10%	0	5%
PTPN1	20q13.13	0%	0	22%	35%	1	0%
BCAS4	20q13.13	0%	0	6%	40%	2	0%
NFATC2	20q13.2	0%	0	6%	45%	2	0%
KCNQ2	20q13.33	11%	0	0%	50%	3	0%
PDGFB	22q13.1	0%	0	0%	5%	1	10%

Logistic regression analysis resulted in a mathematic model (Table 4, model 1) containing *MYC*, *CCNE1* and *EVI1*, all found to be gained or amplified in our preliminary array CGH[7] in high-grade OVCA and FTCs. This model correctly predicted 95% of OVCAs and 94% of SBTs. Subsequently, this model was blindly tested on a validation set of 50 high-grade serous OVCAs and SBTs, in random order, with our MLPA probe sets. We found that the model differentiated well between OVCAs and SBTs in the second independent set of tumours (Table 5), correctly predicting OVCAs and SBTs in 85% and 65% of cases, respectively (p=0.00025, Chi-square-test).

Since gain of *NFKBIE*, *BCL2L1* and *PSMB4* and loss of MDM2 were seen in 70%, 60%, 50% and 80% respectively of the original OVCA group while the first three genes never showed gains and only 17% losses in the SBTs (Table 3), we forced these genes into a second logistic regression model that correctly predicted 19 of the 20 OVCAs and 15 of 18 SBTs (Table 4, model 2). When we tested this second model blindly on the second independent set of 50 OVCAs and SBTs, 18 of 27 OVCAs (67%) and 18 of 23 SBT (78%) were predicted correctly (p=0.0015, Chi-square-test)(Table 5).

Table 4. Mathematic models derived by logistic regression analysis from MPLA results in a learning set of 20 ovarian carcinomas and 18 serous borderline tumours.		
Genes	**Correctly predicted ovarian carcinomas (20)**	**Correctly predicted Serous borderline tumours (18)**
Model 1 *MYC, CCNE1, EVI1*	**19** (95%)	**17** (94%)
Model 2 *MDM2, NFKBIE, BCL1L2, PSMB4*	**19** (95%)	**18** (100%)

Table 5. Mathematic models used to predict tumour type in the validation set of 50 ovarian carcinomas and serous borderline tumours.			
	Correctly predicted OVCA (27)	**Correctly predicted SBT (23)**	**p-value** X^2 test
Model 1 *MYC, CCNE1, EVI1*	**23 (85%)**	**15 (65%)**	0.00025
Model 2 *MDM2, NFKBIE, BCL2L1, PSMB4*	**18 (67%)**	**18 (78%)**	0.0015

Discussion

In the present study a DNA-change profile was created of 20 high-grade serous OVCAs and 18 SBTs (all FIGO stages) by MLPA, with probe-sets tailored to high-grade serous OVCAs and FTCs, as described earlier. As could be expected, many more genomic alterations were found in the high-grade serous OVCAs than in the SBTs. In this dataset 80% of gains (266/332) and 62% of losses (38/61) were found in the OVCAs and only 3% of amplifications (2/66) of studied genes were seen in the SBT group. This information was used to derive mathematic models to predict whether tumors were likely high grade serous OVCA or SBT. Subsequently, the same MLPA probe-sets were used in a validation set of 50 high grade serous OVCAs and SBTs. Different mathematic models yielded high percentages of correct classifications of high grade serous OVCAs or SBTs in this validation set, the best model containing the *MYC*, *CCNE1* and *EVI1* genes that yielded 85% and 65% correctly classified OVCA and SBT, respectively.
MYC (8q24.21) has been found to be recurrently gained in high-grade serous OVCAs and was found to be amplified in OVCAs and FTCs by array CGH[7]. We found 17 gains, of which 7 were amplifications, in our 20 high grade serous OVCAs (30%) and 7 gains but no amplifications in the 18 SBTs. In the validation set 14 gains of *MYC* of which 6 were amplifications, were seen in 27 OVCAs (22%) and 7 gains (1 amplification) were found in the 23 SBTs (30%). The *CCNE1* gene (19q12) has been found to be amplified in high-grade serous OVCAs and FTCs by array CGH[15]. A gain of this gene was found 8 times (2 amplifications, 10%) in our dataset of 20 high grade serous OVCAs, and one gain and two losses of this gene were seen in the group of 18 SBTs. The validation set revealed 6 gains (3 amplifications, 11%) and one loss of this gene among 27 high grade serous OVCAs and one amplification in an SBT (4%). *EVI1* (3q26.2) has also previously been found amplified in high-grade serous OVCAs and FTCs by array CGH[7]. We found 7 gains (1 amplification) and one loss of *EVI1* in 20 high grade serous OVCAs and two losses of the gene in 18 SBTs. A total of 12 gains (3 amplifications, 11%) were found in the validation set of 27 OVCAs while no alterations of the gene were found in 23 SBTs.

Model 2 was intuitively derived from the observation that *NFKBIE*, *BCL2L1* and *PSMB4* were often gained in OVCAs but never in SBTs, and that *MDM2* was lost in 80% of the OVCAs but in only 17% of SBT.
Two genes were more frequently gained in SBTs: *MYCBP* (11% and 1 amplification) and *AKT2* (28% and no amlification), and these might play a direct role in borderline ovarian tumorigenesis. *MYCBP* stimulates transcription by *MYC.* Interestingly, the *MYC* oncogene (8q24.21) was gained in 39% of SBTs and 85% of OVCAs (7 amplifications, Table 3). This may reflect the fact that in OVCAs cell proliferation is generally higher than in SBT. Other genes may be involved in invasion of the OVCA cells. *AKT2* is a known oncogene encoding a protein kinase that is frequently amplified in human ovarian carcinomas[16]. We found *AKT2* to be gained in 28% of SBTs and 25% of the OVCAs (1 amplification).

Altogether, genetic alterations were not frequently seen in the SBTs in this study. This may partly be due to the probe set being tailored to study high-grade serous OVCAs and FTCs but it is also well documented that SBTs usually do not show extensive genetic alterations[17-19]. Furthermore, genetic imbalances seen in the SBTs are different from those seen in serous OVCAs. Even high FIGO stage SBTs do not harbour many genetic alterations and do especially not mimic the gain patterns found in low and higher-stage OVCAs.

In conclusion, SBTs show a much lower degree of genomic instability than OVCAs. While OVCA frequently show copy number changes in *NFKBIE*, *BCL2L1* and *PSMB4* and MDM2, SBT show particularly copy number changes in *MYCBP* and *AKT2*. These results underline the theory that SBTs are not precursors of high-grade serous ovarian carcinomas but are a separate entity in the ovarian tumour spectrum. The described MLPA probe-sets can provide a practical tool to distinguish high grade OVCAs and SBTs in difficult cases. Our results also suggest that other probesets than used in this study will be needed to compare SBTs and low-grade OVCAs.

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Chapter 7

A case of LOH in the BRCA2 gene of a borderline ovarian tumour; Case report and review of literature.

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Abstract

Germline *BRCA1* and *BRCA2* mutations highly increase the risk of breast and female adnexal cancer. The role of these genes in the tumourigenesis of other malignancies is still under debate. Borderline ovarian tumours (BOT) are occasionally found in families with a strong history of breast and/or female adnexal cancer with or without proven germline mutations. We investigated whether a BOT arising in a germline *BRCA2* mutation carrier could be attributed to this mutation, in which case BOT should be added to the *BRCA2* related tumour spectrum.
Tumour DNA of a serous borderline ovarian tumour (sBOT) of a 55-year-old female carrier of a pathogenic *BRCA2* mutation (6085G>T) was analysed for loss of heterozygosity (LOH) of *BRCA2*. The sBOT cells, unexpectedly, revealed loss of the mutant allele of BRCA2. While ovarian stroma cells and peripheral blood lymphocytes contained both wild-type and mutant allele of BRCA2. The finding that no loss of the wild-type *BRCA2* allele was found in the tumour tissue but loss of the mutant allele was seen, suggests that sBOT are not part of the *BRCA2* related tumour spectrum.
In the literature BOT's in germline *BRCA1* and *BRCA2* mutation carriers are described incidentally, while in patients with a BOT a germline *BRCA1* or *BRCA2* mutation is rarely found. Therefore, we conclude that borderline ovarian tumours are neither part of the *BRCA1*- nor the *BRCA2* related tumour spectrum.

Introduction

Borderline ovarian tumours (BOT), also called ovarian tumours of low malignant potential (LMP) constitute approximately 10 to 15% of all epithelial ovarian carcinomas. Their clinical behaviour is fairly benign, with an overall 10-year survival of 77-99%.[1] Histopathologically, these tumours display epithelial multilayering, nuclear atypia and formation of papillae, but lack stromal invasion.

Questions have been raised whether BOT, like tumours of prostate, cervix, colon, male breast and ureter[2], are also part of the BRCA-related tumour spectrum, as breast, ovarian and Fallopian tube carcinomas are. Epidemiological studies show that a positive family history for breast- and/or female adnexal cancer is not more frequently seen in patients with BOT[3;4] and family members of BOT patients are not at increased risk for developing ovarian tumours.[5;6] Additionally, in a group of familial/hereditary ovarian tumours the proportion BOT was about 6-fold lower as seen in a group of sporadic ovarian tumours[7]. Germline DNA testing of patients with BOT indicated that BRCA mutations are found in 0%-5.6% of cases[8;9]. Conversely, in germline BRCA mutated women ovarian tumours appear to be a BOT in 0%-5.7% of cases.[10-12] Recently, 10% of ovarian tumours in germline BRCA2 mutated women were described to be BOT[13], compared to 20% in a group of sporadic tumours. Nevertheless, a causal relationship between germline BRCA mutations and BOT has been suggested.[14] In the present study, loss of wild type allele in BOT tumour cells of a germline *BRCA2* mutation carrier was examined in order to clarify whether this mutation plays a role in BOT tumorigenesis.

Case

A 55-year-old woman was seen for ovarian screening at our family cancer clinic because of a positive family history for breast and female adnexal cancer. She carries a pathogenic germline *BRCA2* (c.6085G>T; p.E1953X) mutation and has a history of breast cancer at the age of 35 which was treated with mastectomy and local radiotherapy with an uneventful follow-up.

She presented without any abdominal or other complaints and the CA 125 level was 10 U/ml. A screening transvaginal ultrasonography revealed an ovarian cyst with papillary structures and a diameter of approximately 6 centimeters. A bilateral salpingo-oophorectomy was performed and microscopic examination and DNA flow cytometry showed a DNA aneuploid serous BOT limited to the right ovary with negative peritoneal lavage cytology (FIGO stage IA). In order to investigate whether this tumour could be considered to be attributable to the germline *BRCA2* mutation in this patient, we performed additional molecular genetic tests.

Materials and methods

LOH analysis

Paraffin-embedded tumour cells were microdissected and DNA was isolated using standard proteinase K digestion overnight at 56°C followed by boiling the samples for 8 minutes, in order to inactivate the enzyme[15]. DNA from ovarian stroma cells and blood lymphocytes of the same patient was used as a control. PCR was performed using primers surrounding the

mutation, resulting in a fragment of 156 base-pairs. The fragments were directly sequenced to detect loss of heterozygosity at this locus.

Review of the literature

A Pubmed search (www.pubmed.com) was performed using search terms: "Borderline ovarian tumour", "Ovarian tumour of Low Malignant Potential", "*BRCA1*", "*BRCA2*" and "Loss of heterozygosity (LOH)". Studies investigating the incidence of germline *BRCA1* or *BRCA2* mutations in patients with BOT were included and are summarised in table 1.

Results

Tumour cells were analysed to find possible loss of wild type *BRCA2* allele, in order to confirm a causative role of a *BRCA2* mutation in the pathogenesis of BOT. Interestingly, this sequence revealed loss of the mutant *BRCA2* allele in the tumour cells, whereas both wild type and mutant allele were present in the control cells (Figure 1).

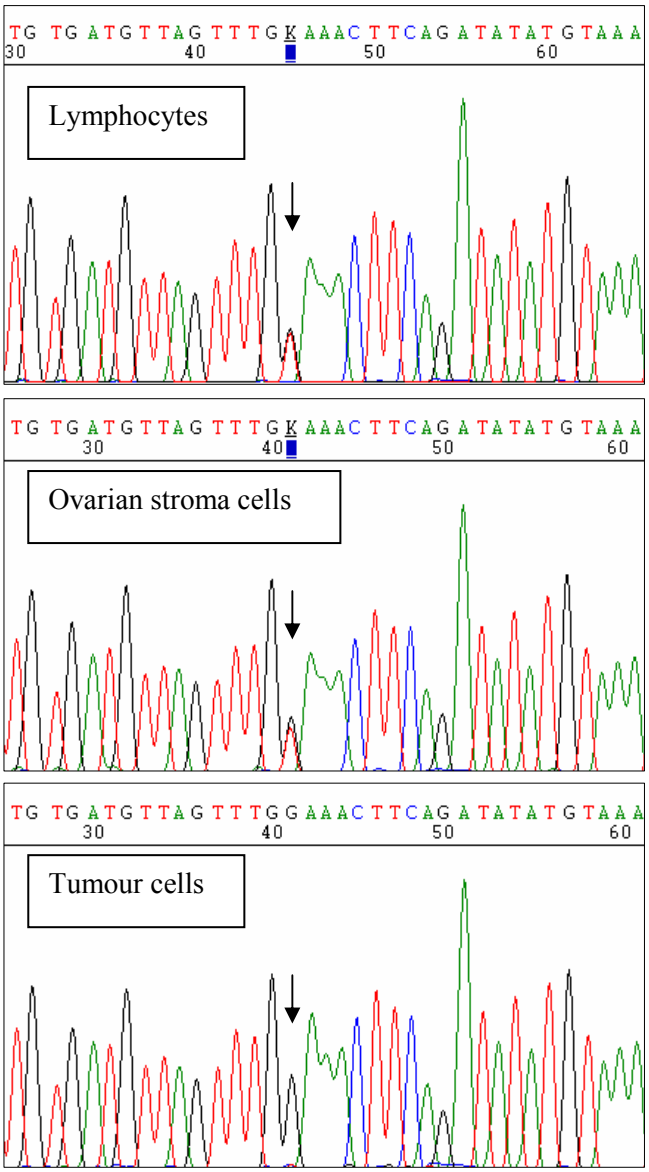

Figure 1: Sequence of PCR products derived from exon 11 of *BRCA2* in lymphocytes and ovarian stromal cells of a germline BRCA2 mutated woman revealing both the wild-type (6085G) and the mutant (6085T) allele, whereas in the DNA from serous borderline ovarian tumour cells only the wild-type allele is seen.

Review of the literature reveals 748 cases of BOT in 14 studies published between 1995 and 2005[8;9;14;16-25]. Of these, ten (1.3%) patients were found to harbour a *BRCA1* germline mutation. In seven studies *BRCA2* mutations were also studied and one (0,2 %) BRCA 2 mutation was detected in 485 BOT patients.
Seven BOT in patients with a *BRCA1* or *BRCA2* germline mutations were of serous histology, one was a mucinous tumour. In 3 cases, the histological subtype was not stated.

Table 1: Review of literature on BRCA1/2 mutations in 748 borderline ovarian tumour patients.

Author	Year	No.BOT	Population	Histotype of BOT	Mutation found	Investigated mutations
Takahash[16]	1995	1	USA	not stated	BRCA1 3489delAA	BRCA1 sequensed
Modan[17]	1996	16	Jewish	not stated		BRCA1 185delAG
Stratton[8]	1997	19	England	12 ser, 7 muc	BRCA1 4287delGT	BRCA1 sequensed
Shushan[18]	1997	7	Jewish	not stated		BRCA1 185delAG and 5382insC BRCA2 6174delT
Modan[28]	1997	36	Jewish	not stated	BRCA1 185delAG (2x)	BRCA1 185delAG
Gotlieb[9]	1998	46	Jewish	36 ser, 10 muc	BRCA1 185delAG	BRCA1 185delAG BRCA2 6174delT
Lu[19]	1999	16	Jewish	8 ser, 6 muc, 2 other		BRCA1 185delAG and 5382insC and BRCA2 6174delT
Tonin[20]	1999	14	Canada	not stated		BRCA1 C4446T, 2953del3+C and 3768insA BRCA2 2816insA, G6085T and 8765delAG
Stratton[21]	1999	77	England	31 ser, 43 muc, 3 other		BRCA1 sequensed BRCA2 nt 3139-7069 sequensed
Risch[22]	2001	134	USA	not stated		7 BRCA1 mutations 4 BRCA2 mutations
Piura[14]	2001	1	Jewish	1 ser	BRCA1 185delAG	case report BRCA1 185delAG mutation carrier with BOT
Bjorge[23]	2004	190	Norway	96 ser, 86 muc, 8 other		BRCA1 1675delAG, 1135insA, 816delGT and 3347delAG
Rafnar[24]	2004	74	Iceland	37 ser, 29 muc, 8 other		BRCA1 g5193a BRCA2 999del5
Gotlieb[25]	2005	117	Jewish	80 ser, 37 other	BRCA1 185delAG (3x) BRCA1 5382insC (1x) BRCA2 6174delT (1x)	BRCA1 185delAG and 5382insC BRCA2 6174delT
Total		**748**		**301 ser, 181 muc, 58 other, 208 not stated**		

No. = Number, Ser = Serous histotype, Muc = Mucinous histotype, BOT = Borderline Ovarian Tumour.

Discussion

The *BRCA2* gene consists of 27 exons encoding 3418 amino acids. A *BRCA2* (6085G>T) mutation in exon 11 results in truncation at amino acid 1962 and therefore non-function of the *BRCA2* tumour suppressor protein. The role of loss of wild-type allele of *BRCA1* and *BRCA2* has been demonstrated in the carcinogenesis of breast-, ovarian- and Fallopian tube cancers[15;26]. Therefore, we hypothesized that if BOT were part of the BRCA related tumour spectrum, loss of wild type allele should be seen in BOT cells of a germline BRCA mutation carrier. We did not detect loss of wild type *BRCA2* allele in the BOT cells of this germline *BRCA2* mutation carrier. This suggests that this BOT is not part of the *BRCA2* related tumour spectrum.
In contrast, we found loss of the mutant *BRCA2* allele in the tumour DNA of our case. The meaning of this finding is not clear. Loss of mutant allele in a tumour of a germline *BRCA2* mutation carrier has previously been described in a lung adenocarcinoma[27]. The authors conclude that this finding argues against a causative role of the mutation in the development of this tumour. However, the possibility remains that an other (point) mutation in the wild type BRCA 2 has occurred. This possibility must be taken into account in our case as well.

In addition we were not able to identify any other germline BRCA 1 or BRCA2 mutation carriers that had developed a BOT in the databases of family cancer clinics in The Netherlands (Amsterdam, Leiden, Groningen and Rotterdam) or England (Manchester, Southampton and London), which underlines the infrequency of these tumours among BRCA 1 and BRCA2 mutation carriers.
In the literature a very low incidence of germline BRCA1 or BRCA2 mutations is described in BOT patients (1.3% and 0.2% respectively, as stated above), whereas the incidence of germline *BRCA1* or *BRCA2* mutation in invasive ovarian carcinoma patients is around 5-10%, depending on the investigated population.[22;28]

Conclusion.

Borderline ovarian tumours do not seem to be part of the germline *BRCA1/2* mutation related tumour spectrum, and BOT should therefore not be considered as an indicator for the hereditary *BRCA1/2* cancer syndrome.

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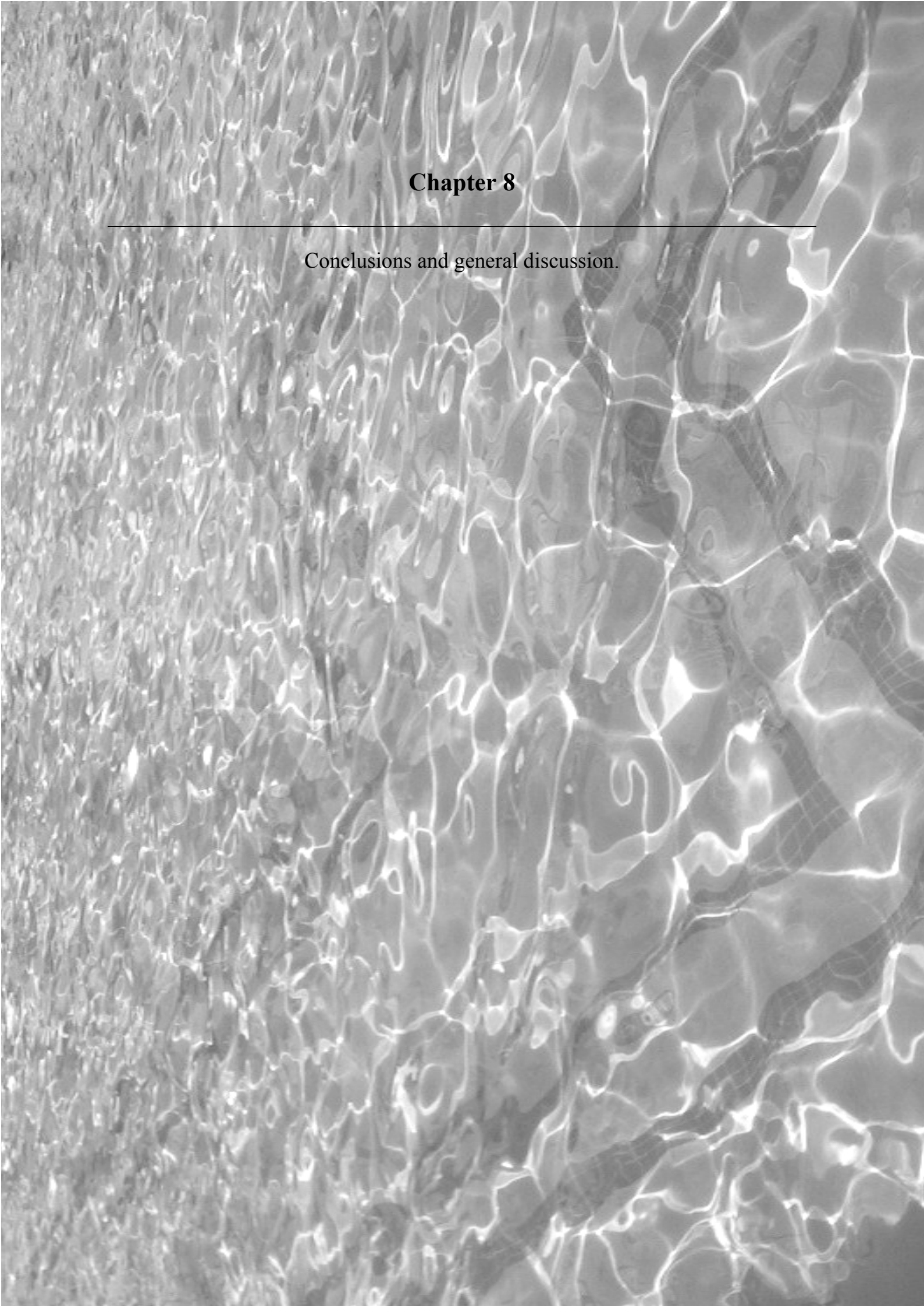

Chapter 8

Conclusions and general discussion.

Conclusions

Borderline ovarian tumours (BOT) or ovarian tumours of low malignant potential (LMP) constitute up to 16% of all non-benign epithelial ovarian tumours[1]. The main histopathologic subtypes are serous BOT (SBTs) and mucinous BOT (MBTs) accounting for 55% and 40% of all BOT, respectively[2]. In this thesis especially SBTs are studied.

Despite their excellent clinical behaviour (overall 10-year survival of 99% for early disease to 77% for advanced disease[3]) there is a small subgroup of BOT that progresses to invasive disease. In SBTs this progression seems to be rare and evolves through atypical proliferative serous tumour (APST) via micropapillary serous carcinoma (MPSC) to low-grade serous ovarian carcinoma (Type I), but never to high-grade serous ovarian carcinoma (Type II) (Figure 1)[4-6]. On the contrary, MBTs do display a continuum from mucinous cystadenoma through MBT progressing to invasive mucinous ovarian carcinoma[7-10].

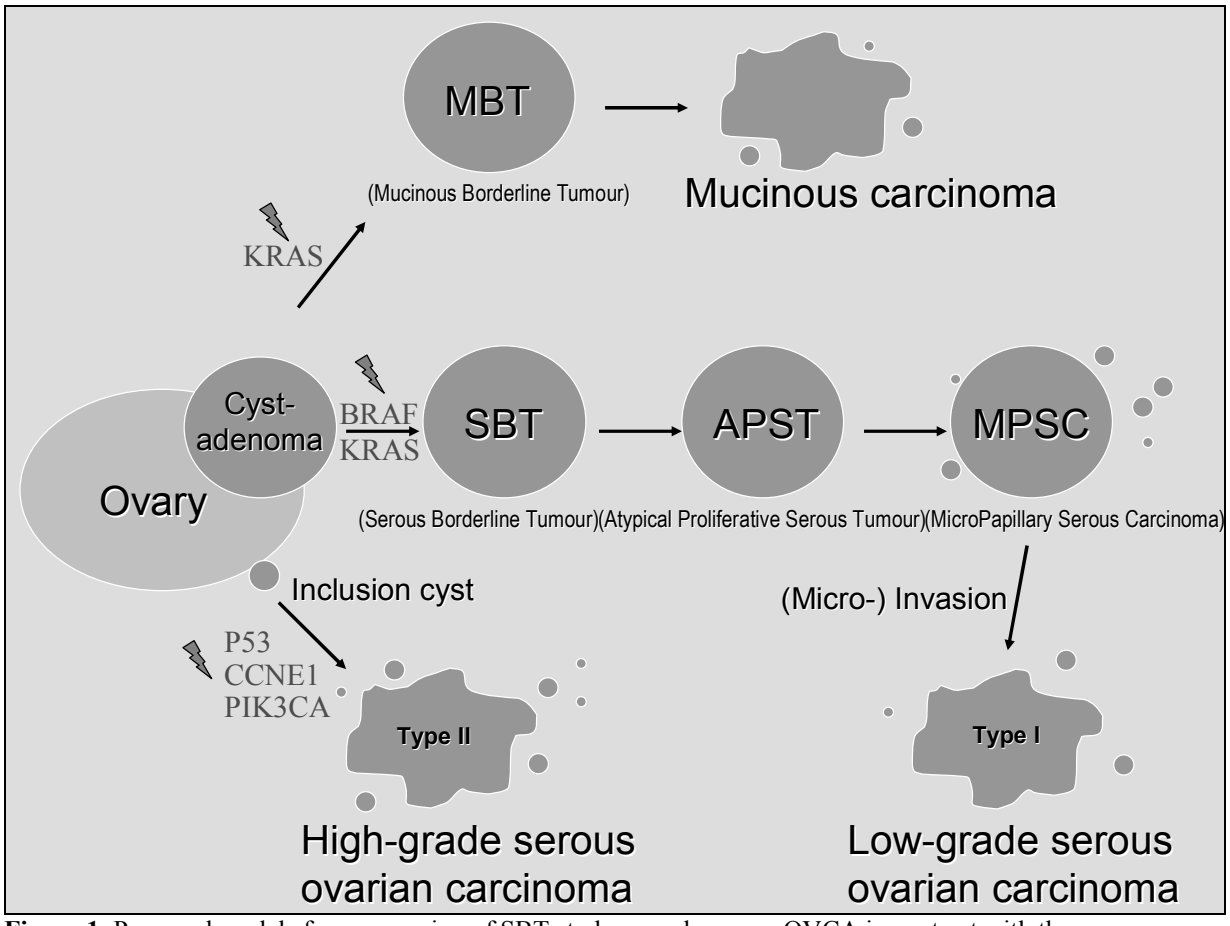

Figure 1. Proposed models for progression of SBTs to low-grade serous OVCA in contrast with the development of high-grade serous OVCA (Modified by MB Verbruggen after Shih and Kurman)

The first objective of this thesis was to find a tool to predict clinical behaviour (i.e. recurrence and progression of disease) in BOT, especially in SBTs.
In order to investigate which tumours do progress a number of studies is carried out. It was found that the use of morphometry (light microscopically assessment of the mitotic activity index (MAI) and volume percentage of epithelium (VPE)) and DNA cytometry is of no additional value for prediction of clinical behaviour[11]. The FIGO stage at first diagnosis of the tumour and the histologic subtype however, are very useful in predicting clinical behaviour.

In addition, the *BRAF* mutation status – in itself unique to SBTs and not found in MBT or carcinomas - was found to influence clinical behaviour. *BRAF* mutation positive tumours tend to present with a lower FIGO stage, but a higher tumour volume, rendering them earlier detecable. They are also less frequently aneuploid, though non of these findings was statistically significant (Verbruggen et al. *Int J Gyn Cancer*, In Press).

The second objective of this thesis was to examine genomic instability in SBTs and to compare this with high-grade serous ovarian carcinoma (OVCA) in order to confirm that SBTs are not precursors of high-grade OVCA. We found that only 2% of SBT DNA contains genomic imbalances and that genomic profiles of SBTs and serous OVCAs differ substantially. It is now generally accepted that SBTs do rarely progress into micropapillary serous carcinomas (MPSCs) and even into low-grade serous ovarian carcinomas (Type I), but never into high-grade serous ovarian carcinoma (Type II)[6].

It is concluded that genetic instability is quite rare in SBTs. When comparing genetic profiles of SBTs and invasive ovarian carcinomas (OVCAs) by multiplex ligation dependent probe amplification (MLPA) only a limited amount of gains and losses is seen in the SBT group. Furthermore, genetic instability during progression of an SBT to low grade invasive carcinoma has been found to be limited as well. However, mutations in the *KRAS* and *BRAF* gene are commonly seen in SBTs, thus underlining the important role of the *RAS-RAF-MEK-ERK-MAP* kinase pathway in these tumours. In contrast, mutation of the *BRCA1* and *BRCA2* tumour suppressor genes does not seem to play a role in the development of SBTs.

General discussion

Because of the low incidence of BOT the diagnostic examination by a pathologist should be centralised to a limited number of (academic) hospitals in the Netherlands. Furthermore, information and treatment should be given by specialised gynaecologists. By centralisation, the possibility of clinical and molecular research will increase.

In this respect it would be interesting to study a group of early (i.e. low FIGO-stage) high-grade invasive serous ovarian carcinomas and compare genomic imbalances with a group of MPSCs or even low-grade ovarian carcinomas by comparative genomic hybridization (CGH) or multiplex ligation dependent probe amplification (MLPA). This might raise more support for the theory that SBTs do not progress to high-grade serous ovarian carcinomas and reveal more genes that are specifically implicated in the formation and progression of SBTs and OVCAs.
Moreover, when a larger amount of SBT DNA would be available, the relationship between *BRAF* mutation status and clinical behaviour might be enlightened. In our study a trend toward more benign clinical behaviour was seen in SBTs with a *BRAF* mutation.

In this thesis the treatment of BOT is not studied. Though surgical treatments have extensively been studied and especially the laparoscopic treatment of BOT is rising[12], a large prospective clinical study with long term follow-up is still lacking. In this proposed study the surgical techniques (i.e. cystectomy, adnexectomy and laparoscopic staging) could be evaluated with respect to morbidity, mortality, recurrence rate and quality of life. Follow-up should be at least 10 years in order to investigate recurrence of disease.

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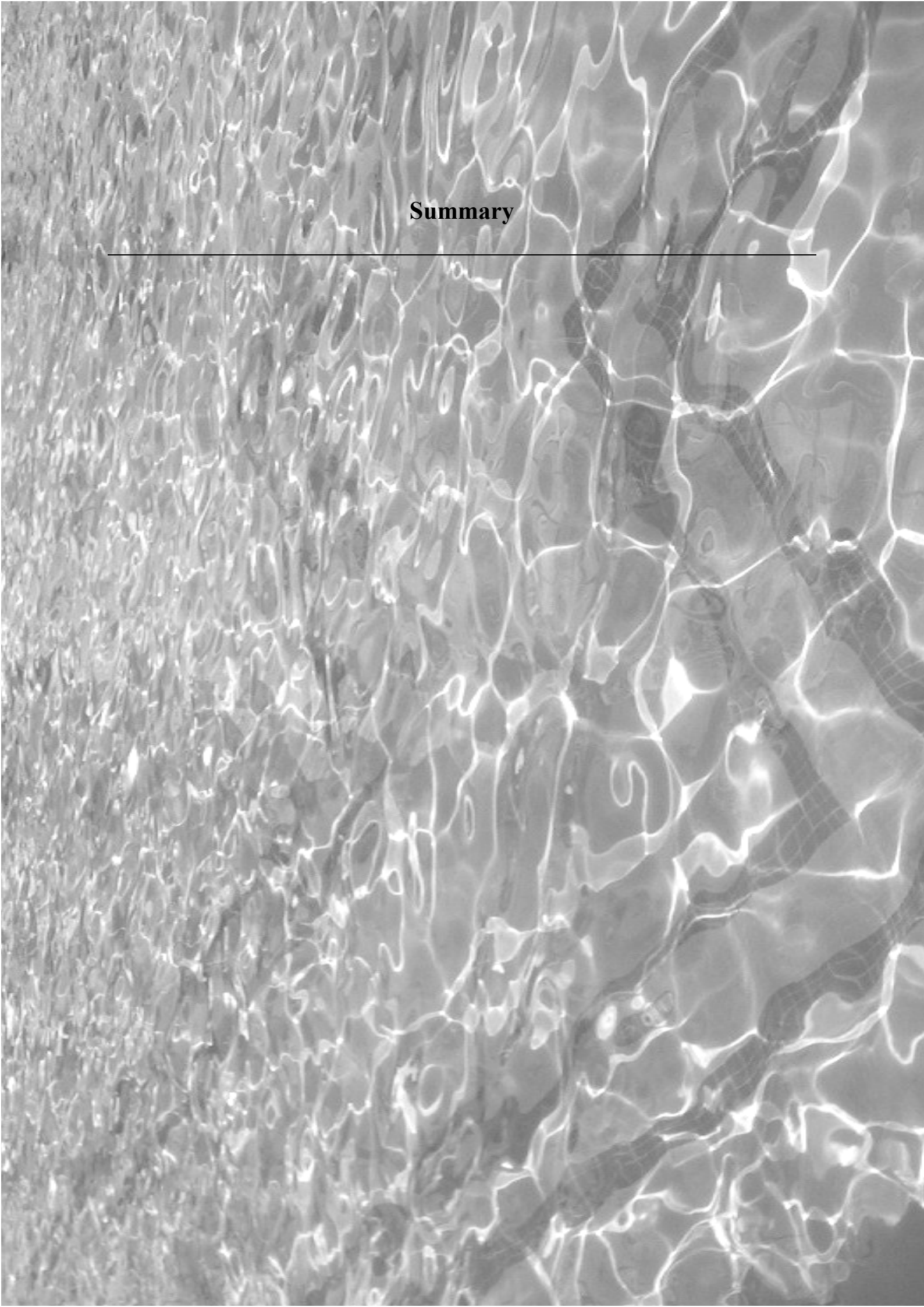


Summary

Summary

In this thesis the molecular and clinical aspects of borderline ovarian tumours are discussed. In **chapter 2** the prognostic and clinical value of morphometry and DNA cytology is studied in 93 borderline ovarian tumours (BOTs) after previous research that claimed prognostic power for these indicators. In this prospective study the prognostic power of morphometry and DNA cytology could not be confirmed. It is concluded that morphometry and DNA cytology are not useful in direct clinical management of BOTs, while histologic subtype and FIGO stage are stable prognostic indicators.

In **chapter 3** a report is presented of three cases of serous borderline ovarian tumour (SBT) with supradiaphragmatic lymph node involvement. In all 3 cases the first symptoms included cervical lympadenomas that after fine needle aspiration showed adenocarcinoma of unknown primary (ACUP). Subsequent extensive examination revealed a SBT in all 3 cases that could be treated curatively by surgery. A panel of histologic stainings of both primary tumour and cervical lymph nodes confirmed FIGO stage IV BOT. It is concluded that extra abdominal spread of implants accompanying an SBT is possible and when ACUP is found and SBT should be in the differential diagnosis.

In **chapter 4** mutations in the *BRAF* gene were determined in 30 SBTs and their accompanying implants and the effect on clinical behaviour is established. A *BRAF* V599E mutation was found in 41% of SBTs. *BRAF* mutation positive SBTs were frequently bilateral (36%) tend to present with lower FIGO stage, higher tumourvolume and are less frequently aneuploid. We hypothesize that *BRAF* mutation positive SBTs might form a subgroup with a relatively benign clinically behaviour.

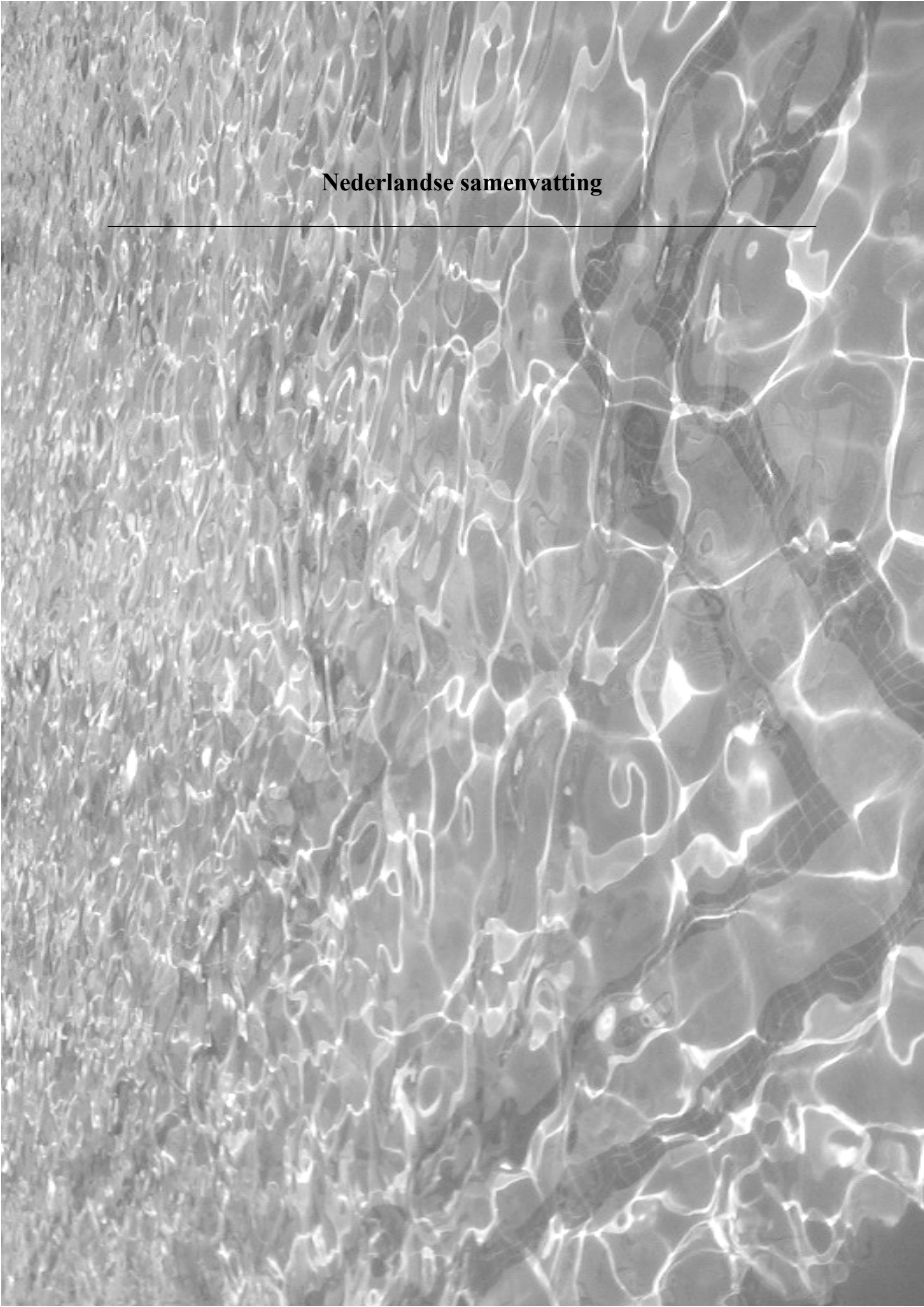
In **chapter 5** we describe array-comparative genomic hybridization of DNA material from tumour cells of a progressive SBT in time. Tumour samples at four different time points over 10 years were studied in order to find genomic imbalances in the tumour DNA during progression en invasion of the tumour cells. It is concluded that even progressive SBTs do not seem to harbor many genomic imbalances and based on this study we conclude that gains at 1q31-41 and 12q12-24 and losses at 1p12-36 may play a role in clinical progression of SBTs.

In **chapter 6** the genomic differences between SBTs and high grade serous ovarian carcinomas (OVCAs) were studied by multiplex ligation dependent probe amplification (MLPA). We found that SBTs harbour much less genomic imbalances compared to serous OVCAs. From the dataset (18 SBTs and 20 OVCAs) six mathematic models were derived to predict whether tumour cell DNA is derived from an SBT or an OVCA. These models were then tested on a second set of 23 SBTs and 27 OVCAs. Four models predicted the tumour type correctly and may possibly be used for clinical purposes. We conclude that these results subscribe the theory that SBTs are no precursor lesions of high-grade serous ovarian carcinomas but are a separate entity in the ovarian tumour spectrum.

In **chapter 7** a case of LOH in the *BRCA2* gene is described in a patients with a SBT. This patient was a known germ-line *BRCA2* mutation carrier and developed an SBT. In order to investigate whether this SBT could be attributed to the *BRCA2* mutation status and therefore could be added to the known *BRCA2*-related tumour spectrum, tumour DNA was tested to find if the wild-type *BRCA2* gene was lost. Instead we found loss of the mutated *BRCA2* gene.

In addition a literature search was conducted for a correlation between SBTs and *BRCA 1/2* mutations which could not be confirmed. It is concluded that SBTs are not part of the *BRCA 1/2* related tumour spectrum.

In **chapter 8** the results of the studies in this thesis are discussed. It is concluded that SBTs are not likely to be precursors of high grade serous ovarian carcinoma but can rarely progress via an entity called micropapillary serous carcinoma (MPSC) to low grade serous ovarian carcinoma. Furthermore, morphometry and DNA cytology are not good prognostic indicators in BOT, BRAF mutations are commonly seen in bilateral SBTs and their accompanying implants and may constitute a prognostic favourable subgroup, SBTs can present with extra-abdominal implants and SBTs are not part of the BRCA 1/2 related tumour spectrum. Directions for future research are given, such as a comparison of SBT DNA material with DNA from low FIGO stage high-grade ovarian carcinomas by CGH or MLPA.



Nederlandse samenvatting

Samenvatting

In dit proefschrift worden de moleculaire en klinische aspecten van borderline ovarium tumoren bestudeerd. In **hoofdstuk 2** wordt de prognostische en klinische waarde van morfometrie en DNA cytologie onderzocht in 93 borderline ovarium tumoren (BOTs) nadat eerder onderzoek aanwijzingen had gegeven dat deze parameters prognostische waarde hebben bij deze tumoren. In deze prospectieve studie kon deze waarde niet worden bevestigd. Wij concluderen dat morfometrie en DNA cytologie niet bruikbaar zijn bij de klinische evaluatie van BOTs, terwijl het histologisch subtype en het FIGO stadium wel van belang zijn.

In **hoofdstuk 3** worden drie casus gepresenteerd met lymfadenopathie boven het diafragma bij een sereuze BOT (SBT). In alle drie deze casus liet de dunne naald biopsie een adenocarcinoom met onbekende oorprong zien. Pas na uitgebreid verder onderzoek kwam een SBT aan het licht. Een panel van histologische kleuringen bevestigde dat de cellen in de lymfklieren afkomstig waren vanuit de SBT en dus dat er sprake was van een FIGO-stadium IV SBT. Er wordt geconcludeerd dat extra-abdominale implants mogelijk zijn bij SBT en dat bij het vinden van adenocarcinoomcellen met onbekende oorprong een SBT in de differentiaal diagnose moet staan.

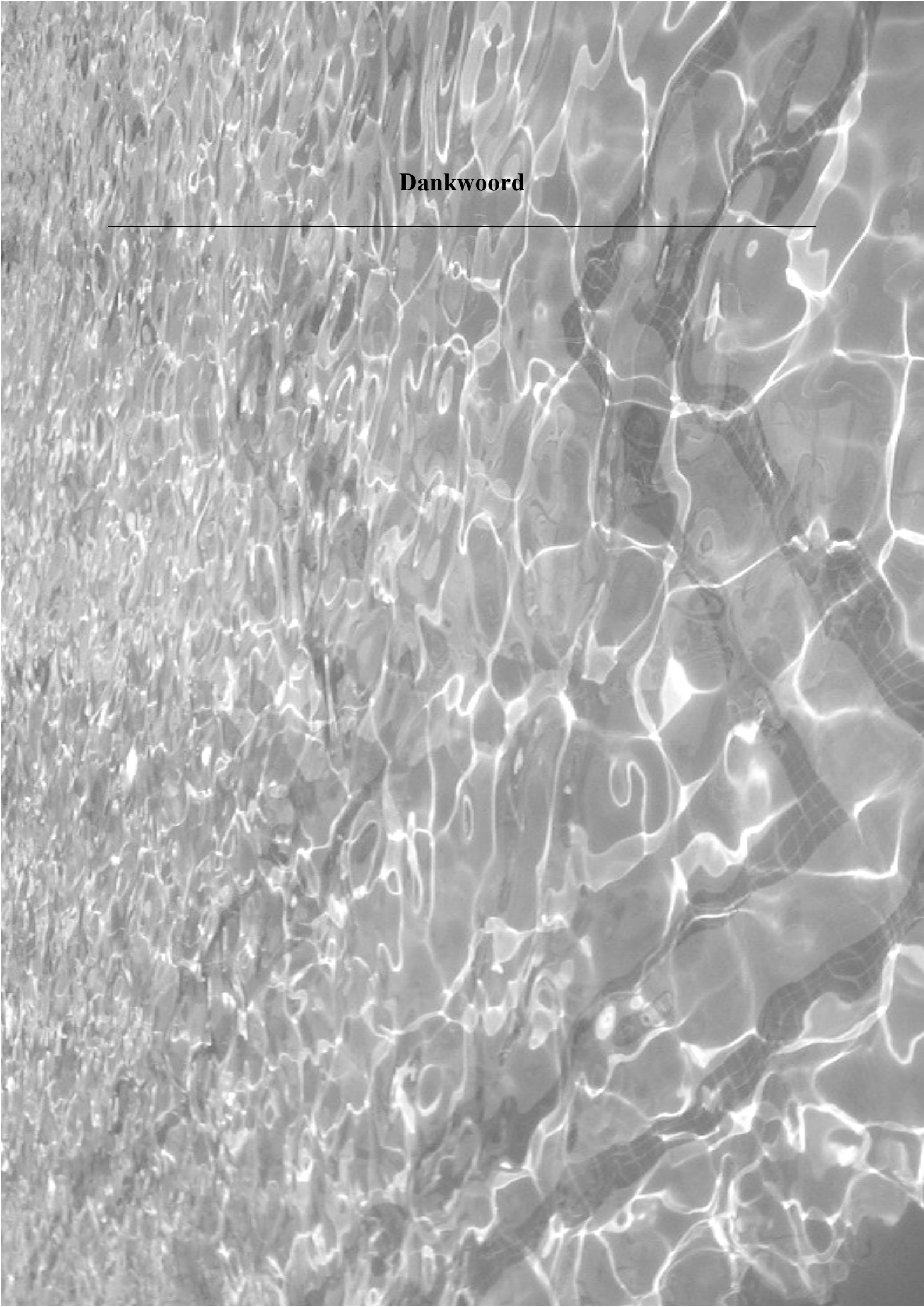
In **hoofdstuk 4** zijn mutaties in het BRAF gen bepaald in 30 SBTs en in de implants en wordt gekeken naar een correlatie tussen mutatie-status en klinisch gedrag van de tumor. Een V599E mutatie in BRAF werd gevonden in 41% van de SBTs en tumoren met een BRAF mutatie waren vaker bilateraal (36%), presenteerden zich vaak met een lager FIGO stadium en een groter tumorvolume en waren minder vaak aneuploid. De hypothese hierbij is dat BRAF mutatie positieve SBTs een subgroep vormen die zich klinisch meer benigne gedraagt.

In **hoofdstuk 5** beschrijven we array-CGH op DNA van een progressieve SBT in de tijd. Tumormateriaal afgenomen op verschillende tijdspunten gedurende tien jaar follow-up van een progressieve SBT werd bestudeerd op genomische onbalans. Er werd niet veel genetische instabiliteit gevonden in het tumor DNA. Dit wijst erop dat in SBTs andere, waarschijnlijk epigenetische factoren een rol spelen bij progressie en invasie. Genen gelegen op de locaties 1q41 en 12q24.33 (gains) en 1p13, 1p13.2, 1p22, 1p31, 1p31.1 en 1p36 spelen mogelijk een rol bij progressie van SBTs.

In **hoofdstuk 6** worden genetische verschillen tussen SBTs en hooggradige sereuze ovariumcarcinomen (OVCAs) bestudeerd met behulp van “multiplex ligation dependent probe amplification” (MLPA). Hierbij worden in SBTs veel minder genomische afwijkingen gevonden dan in OVCAs. Uit een dataset van 18 SBTs en 20 OVCAs werden 6 mathematische modellen gedistilleerd bestaande uit genen die voorspellen of DNA materiaal afkomstig is van een SBT of een OVCA. Deze modellen werden vervolgens getest op een tweede set van 23 SBTs en 27 OVCAs. Vier van de zes modellen voorspelden het tumortype correct en kunnen mogelijk gebruikt worden voor klinische toepassing. Wij concluderen dat deze resultaten de theorie dat SBTs geen voorstadium van hooggradige sereuze ovarium carcinomen zijn onderstrepen en dat SBTs een aparte entiteit zijn binnen het ovariële tumorspectrum.

In **hoofdstuk 7** wordt een casus met “loss of heterozygosity” van het BRCA2 gen besproken in een patiënte met een SBT. Deze patiente was een bekende BRCA2 mutatiedraagster en ontwikkelde een SBT. Om te onderzoeken of deze tumor kon worden toegeschreven aan de BRCA2 mutatiestatus en dus kan worden toegevoegd aan het BRCA2-gerelateerde tumorspectrum, werd tumor DNA onderzocht om te zien of er verlies van het wildtype BRCA2 was. In plaats daarvan vonden we verlies van het gemuteerde BRCA2 allel. Tevens werd in de literatuur gezocht naar een correlatie tussen SBTs en BRCA1/2 mutaties hetgeen niet kon worden gevonden. Er wordt geconcludeerd dat SBTs geen onderdeel van het BRCA1/2 gerelateerde tumorspectrum zijn.

In **hoofdstuk 8** worden de resultaten van dit proefschrift bediscussieerd. Er wordt geconcludeerd dat SBTs waarschijnlijk geen voorlopers zijn van hooggradige ovarium carcinomen maar dat ze wel in zeldzame gevallen kunnen doorontwikkelen tot micropapillaire sereuze carcinomen en tot laaggradige ovariumcarcinomen. Verder zijn morfometrie en DNA cytologie geen goede voorspellers voor het klinisch gedrag van deze tumoren, BRAF mutaties worden frequent gezien in bilaterale SBTs en de bijbehorende implants en zouden wel eens een prognostisch gunstige subgroep kunnen vormen, SBTs kunnen zich presenteren met extra-abdominale implants en zijn geen onderdeel van het BRCA1/2 gerelateerde tumorspectrum. Een richting voor verder onderzoek wordt aangegeven, bijvoorbeeld verder onderzoek naar mutaties in het tumor-DNA van SBTs en laag FIGO-stadium hooggradige ovariumcarcinomen met behulp van CGH of MLPA.



Dankwoord

Dankwoord

Aan het einde van dit boekje het dankwoord, vaak het eerste dat wordt bekeken door de lezer. Het is duidelijk dat veel mensen hebben bijgedragen aan het schrijven van dit proefschrift. Graag betuig ik mijn dank aan de volgende personen:

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Mijn paranimfen: **Corine Penning** en **Marlies Heiligenberg**. Corine, in 1992 begonnen we samen aan de studie Biomedische Wetenschappen in Leiden. Tijdens het WOM practicum (Wegen, Oplossen en Meten) werd de basis gelegd voor een vriendschap. Negen jaar geleden was ik jouw paranimf, nu is het dan eindelijk tijd om de rollen om te draaien. Ik ben blij dat je erbij bent! Marlies, mijn kleine nichtje. Inmiddels ook arts geworden en hard op weg te promoveren. Ik vind het geweldig om dit met jou te delen.

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Curriculum vitae

Marjolijn Barbara Verbruggen werd op 22 januari 1974 geboren als oudste van 3 kinderen van Ingeborg Verbruggen-Heiligenberg en Henk Verbruggen. In 1992 behaalde zij haar diploma aan het Stedelijk gymnasium te Haarlem (gymnasium β). Na uitloting voor de studie Geneeskunde startte zij datzelfde jaar met de studie Biomedische wetenschappen aan de Universiteit Leiden. In december 1996 werd het doctoraaldiploma behaald. Inmiddels was zij na vier maal uitloten via een beroep op de hardheidsclausule toegelaten tot de studie Geneeskunde aan de Universiteit Leiden. In juni 1999 behaalde zij haar doctoraaldiploma geneeskunde en op 20 april 2001 de artsenbul. Hierna werkte zij een half jaar als Medical adviser bij Merck, Sharp & Dohme BV. Aansluitend werkte zij tot december 2002 als ANIOS verloskunde & gynaecologie in het Kennemer Gasthuis te Haarlem. Van december 2002 tot oktober 2004 deed zij wetenschappelijk onderzoek op de afdeling gynaecologische oncologie van het VU medisch centrum te Amsterdam onder begeleiding van prof. dr. René Verheijen en prof. dr. Paul van Diest. In oktober 2004 startte zij met de specialisatie tot gynaecoloog in het St. Lucas/Andreas ziekenhuis (opleider: prof. dr. Fedde Scheele) en het VU medisch centrum (opleiders: prof. dr. Herman van Geijn en prof. dr. Hans Brölmann) te Amsterdam. Zij is sinds mei 2005 getrouwd met Ronald Zweemer. Samen hebben zij een dochter; Floor (1 juli 2008) en zorgen zij part-time voor Lieke en Tim.

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