Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/77573

Please be advised that this information was generated on 2017-12-06 and may be subject to change.

Prognostic biomarkers in ovarian carcinoma cyst fluid

Eva Kolwijck

Publication of this thesis was generously sponsored by:
Stichting Nationaal Fonds tegen Kanker - voor onderzoek naar reguliere en aanvullende therapieën te Amsterdam, Schering-Plough B.V., Medical Dynamics, Zebra Bioscience, DDL Diagnostic Laboratory.

Cover & Lay-out: Irene Pieper, FAdesign, Enschede
Printed by: Ipskamp, Nijmegen

ISBN: 978-90-9025434-0

@ 2010 E. Kolwijck

All rights reserved. No parts of this publication may be reproduced or transmitted in any form or by any means without prior written permission of the author.

Prognostic biomarkers in ovarian carcinoma cyst fluid

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen

Proefschrift

Ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann
volgens besluit van het college van decanen
in het openbaar te verdedigen op
woensdag 30 juni 2010
om 13.30 uur precies

door

Eva Kolwijck
geboren op 12 april 1980
te Nijmegen

Promotores

Prof. dr. L.F.A.G. Massuger

Prof. dr. R.A. Wevers

Prof. dr. C.G.J. Sweep

Manuscriptcommissie

Prof. dr. W.T.A. van der Graaf (voorzitter)

Prof. dr. A. Heerschap

Prof. dr. A.G.J. van der Zee (UMC Groningen)

Voor mijn opa en oma

Contents


1

General introduction and outline of this thesis

Introduction

Ovarian cancer represents a great clinical challenge. First, early detection of the disease remains problematic as there are no good screening tests and there is a lack of a clearly defined precursor lesion. A further concern is the response to current therapy. Despite improvement in cytoreductive surgery and the recent introduction of platinum- and taxol-based (intraperitoneal) chemotherapy, gains in survival rates over the last three decades are rather modest.[1] Although 65-80% of the patients respond to first-line chemotherapy, most patients will relapse with drug-resistant disease.[2] Consequently, the 5-year survival rate of patients with advanced stage disease is only 5-30%.[3] For this reason, it is necessary to obtain insight in mechanisms of cellular drug resistance of ovarian cancer. However, studying ovarian cancer pathology is complicated, as the disease does not appear as a single entity. Instead, ovarian cancer comprises a heterogeneous group of histological subtypes each with its own expression profiles of genes, proteins and metabolites.[4-7] These difficulties have limited the introduction of successful biomarkers that can predict clinical response and guide treatment.

Biology and pathology of ovarian cancer

There are more than 25 major types of ovarian neoplasms. This broad range of histological features displayed by ovarian tumors reflects the diverse anatomical structure of the ovary itself. Approximately 90% of the ovarian tumors are histologically classified as surface epithelial neoplasms. The malignant potential of these ovarian epithelial tumors is categorized on the basis of the extent of epithelial proliferation, nuclear atypia and stroma invasion as benign, borderline or malignant.[8]

During embryonic life, the celomic cavity is lined by a mesothelium, parts of which become specialized to form the serosal epithelium covering the gonadal ridge. The same mesothelial lining gives rise to the müllerian ducts, from which arise the fallopian tubes, uterus and endocervix. Epithelial ovarian cancer (EOC) exhibits a variety of müllerian-type differentiations. This histological subclassification of EOC is based entirely on tumor cell morphology and is categorized according to the World Health Organization (WHO) histological classification system.[4] The four most common subtypes of EOC, referred to as serous, mucinous, endometrioid and clear cell tumors, bear strong resemblance with the normal cells lining the fallopian tube, endocervix and endometrium, respectively (Figure 1).[9,10] This has recently been confirmed at the molecular level, as the genes that were expressed in different EOC subtypes also were concordantly expressed in the normal tissue that they resemble histologically.[11] Less frequently diagnosed are transitional (Brenner), squamous cell, mixed type and undifferentiated EOC.[8] In addition to the type of differentiation, EOC can be subclassified based on the degree of differentiation (tumor grade). During the last decades, several grading systems have been used in different institutions. The most commonly used have been proposed by the International Federation of Gynecology and Obstetrics (FIGO) [12] and WHO [13]. At present, a conceptual change

occurs as in the approach of EOC, the heterogeneity of the disease is taken into account. Accordingly, pathologists believe that a histospecific grading system is more appropriate than a universal grading system to reflect the diversity of the disease.[14,15] However, there is as yet no universally accepted grading system for EOC.

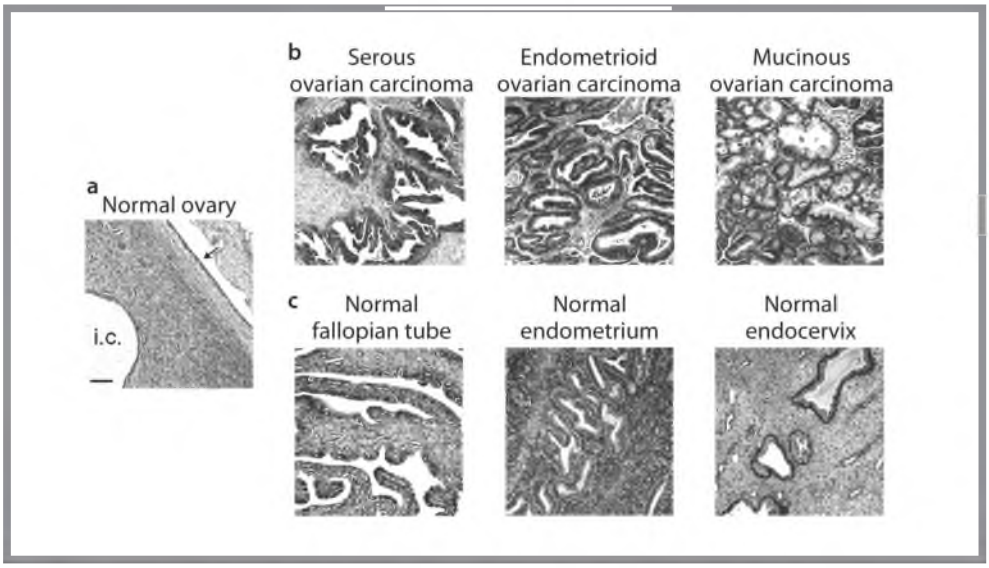

Figure 1. Morphological features of epithelial ovarian cancers. (a) Hematoxylin-eosin-stained section of normal human ovary surrounded by monolayered epithelium (arrow) and containing an inclusion cyst (i.c.). (b) Sections of ovarian carcinomas: serous, with papillary features; endometrioid, with glandular features; and mucinous, with cribriform features. (c) Sections of normal fallopian tube, endometrium en endocervix.(source: Naora et al. 2007 [10])

Ovarian neoplasms are characterized by their cystic appearance. Serous carcinomas may range from microscopic to about 20 cm in diameter. They are typically multilocular and cystic, with soft, friable papillae containing serous, turbid or bloody fluid.[8] Mucinous carcinomas are generally larger than serous carcinomas, with a diameter sometimes up to 50 cm. They are mostly cystic and multilocular, display mucoid surfaces and are filled with thick tenacious gelatinous material that is usually pale yellow but may be turbid or brown. Endometrioid carcinomas range from 12 to 20 cm, are frequently cystic and typically contain friable soft masses and bloody cyst fluid.[8]

A short history on ovarian cyst fluid

In the days before the first abdominal surgical extirpation was performed, massive cystic enlargement of the ovary must have been one of the most disabling diseases among women. Due to the lack of a proper treatment, the abdomen often increased to a size of a full-term pregnancy which could result

in severe respiratory and gastro-intestinal problems caused by mechanical pressure from the ovarian tumor. In addition, symptoms of hypoalbuminemia were strikingly common those days, attributed to the accumulation of protein in the large amount of ovarian cyst fluid.[16]

In the first half of the 19th century, only pioneering surgeons performed abdominal surgery.[17,18] Therefore, the common treatment that was offered to a patient with an ovarian tumor comprised palliation by "tapping" *per vaginam*.[18] Due to a quick refill of the cyst with fluid, in most of the cases these tapping procedures often had to be performed on a regular base. Of the numerous reports on repetitive tapping, a case of a women who underwent 253 tappings in 8 years, with a total production of more than 1,000 liters of cyst fluid, is perhaps one of the most remarkable.[19] As a result, pathologists became aware and subsequently fascinated by the large differences in aspect(s) of the cyst fluid between ovarian tumors. As availability of tissue specimens was mostly limited to autopsies, for many years cyst fluid had been the most important source for pathologic examination. This is evidenced by the extensive pathological description of the characteristics of the fluid in most of the reported cases.[16-18]

By performing chemical analyses, pathologists discovered that transparent cyst fluid consisted almost completely of water, with a few constituents. Red cyst fluid was found to contain large numbers of erythrocytes, whereas thick cyst fluid, nowadays referred to as mucinous fluid, was reported to contain a large amount of protein(s) and slimy compounds. Green colored cyst fluid was thought to be related to human bile and it was found to contain mostly anorganic compounds. The fatty aspect of some cyst fluid was attributed to the existence of crystals and/or cholesterol.[18,19] More importantly, until the days that abdominal surgery was introduced, ovarian cyst fluid was also analyzed from a prognostic point of view. In his work on ovarian dropsy published in 1862, Brown stated that "an increase of density in the dropsical fluid, whether that increase manifests itself by a mucilaginous consistence, a more plentiful production of flaky, or gruel, honey, or brainlike matter, betokens a more depraved or morbid condition of the cysts, and indeed of the general health, and consequently a condition less amenable to cure."[18]

The introduction of the abdominal ovariectomy during the late 19th century gradually led to a greater emphasis on pathological inspection of tissue specimens removed at operation.[20] Slowly it became clear that preoperative punction of ovarian cyst fluid was dangerous in case of ovarian cancer because it could cause iatrogenic spread of malignant cells in the abdomen.[21] Therefore, ovarian cyst fluid was no longer regarded to be of clinical value. After pathological inspection of the ovarian tumor, the pathologist of the 21th century generally discards the cyst fluid and uses the ovarian tissue for histopathological diagnosis. Thus, although ovarian cyst fluid had been a main focus of interest until the 20th century, it more or less has lost its clinical importance thereafter.

Predictive and prognostic biomarkers for ovarian cancer

Over the past several decades, considerable investment has been made in the screening and early detection of EOC. In the 1980s, cancer antigen 125 (CA 125) was introduced for diagnosis of EOC.[22] Later on, dozens of proteins or protein fragments have been suggested as new candidates for the detection of EOC. However, none of these proposed biomarkers had enough sensitivity and specificity to be of clinical value.[23,24] Currently, a shift in biomarker research takes place and the main focus is on tests that may predict response to therapy (predictive biomarkers) and/or patient survival (prognostic biomarkers). With the introduction of many preclinical therapeutic agents, appropriate markers can be used to determine which tumor(s) will respond to which treatment(s) in order to predict the likelihood of drug resistance and the efficacy of the therapeutics. A severe limitation for translational research however, is the extreme histological diversity of EOC. It has become clear that, due to their diverse cellular differentiation, the histological subtypes of EOC respond differently to current chemotherapeutics, [25-27] and each of them seems to have its own characteristic biomarker profile.[27-30] Therefore, during the search for predictive and prognostic biomarkers, the complex histological presentation of various subtypes has to be seriously taken into account. In an attempt to improve biomarker studies or find tumor-derived biomarkers, different sources of biospecimen have been used, all with their own advantages and disadvantages (Table 1).

Table 1. Advantages and disadvantages of different biological sources for EOC research

Material	Advantages	Disadvantages
Blood	Easy to obtain Multiple longitudinal assessments possible	Components are not always tumor-derived Tumor-derived components not always present
Tissue	Components of the tumor directly assessed	Surgery required Heterogeneity of cells Single biopsy not always representative
Cyst fluid	Tumor-derived components Serum-based assessments	Tumor spill when preoperatively obtained Surgery required Not present in all ovarian tumors

Biomarker studies using blood

Most studies that investigated potential predictive and prognostic biomarkers for patients with EOC have used blood as starting material.[24,31,32] The reason is clear as blood is directly available and most assessments in serum or plasma are inexpensive. Of all biomarkers that have been investigated during the years, serum CA 125 has most frequently been documented. It has been shown that the kinetics

of serum CA 125 levels during chemotherapy has prognostic significance, but the predictive role of the preoperative serum CA 125 concentration for response to chemotherapy still remains questionable.[24,33] In addition, not any of the other investigated preoperative serum biomarkers has proven its clinical value for prediction of prognosis of the disease.[24,31,32] The difficulty of developing an appropriate prognostic test in serum might be caused by the many subtypes of EOC resulting in distinct histologybased biomarkers patterns present in blood.[28] Also, the concentration of circulating compounds in blood may be influenced by physiological processes. In addition, the mechanisms and conditions responsible for the release of tumor-derived components into the circulation are poorly understood.

Tissue biomarker studies

Although investigated less frequently than serum, ovarian cancer tissue has also been studied to identify predictive and/or prognostic biomarkers.[24,34-42] Most of the research has been performed using immunohisto- and immunocytochemical techniques, by which the degree of staining can be compared between different EOC tissues. These methods are limited by interobserver variation in identifying stained cells and different subjective scoring systems. The introduction of techniques measuring biomarkers in tissue extracts (e.g. cytosol), or in DNA and RNA, has basically solved this problem as such assessments can be done quantitatively. Nevertheless, tissue biomarker research in general is severely limited by the extreme heterogeneity of EOC tissue, resulting in tumor areas with diverse cellular differentiation within the same tissue specimen.[43-45] As a consequence, the expression of tumor-derived compounds differs significantly within the ovarian cancer tissue specimen, which increases the risk of sampling bias.[37,46-53] Table 2 lists the studies that compared expression of biomarkers between different sections of the same tumor. These studies have demonstrated that ovarian intratumoral heterogeneity is present in almost half of the cases (range: 18-53%).[54-58] A single biopsy might thus not be representative for the whole ovarian tumor, whereas the analysis of multiple regions of the same ovarian tumor would be extremely time-consuming and impractical for clinical purposes.

Table 2. Studies that compared biomarker variation within the same EOC tissue using multiple tissue sections of the tumor

Author	No.*	Biomarker	Number of tissue sections	Detection Technique	Intratumoral heterogeneity
Quinn *et al.*[54]	10	ER and PR	2-3	IHC	44%
Zangwill *et al.*[55]	19	DNA ploidy	3-12	FCM	47%
Takahashi *et al.*[58]	41	DNA ploidy	3-10	FCM	49%
Zborovskaya *et al.*[56]	25	LOH	3-8	IHC	18-53%
Tuefferd *et al.*[57]	320	HER2	2	IHC/FISH	46%

**Number of ovarian carcinomas in the study; ER = estrogen receptor; PR = progesterone receptor; HER2 = Human Epidermal growth factor Receptor 2; LOH = Loss of heterozygosity; IHC = immunohis-tochemistry; FCM = flow cytometry; FISH = fluorescent in situ hybridization*

Cyst fluid biomarker studies

Although not as frequently investigated as blood and tissue, ovarian cancer cyst fluid has been studied as a source for biomarkers. It has been demonstrated that the concentration of analytes in ovarian cyst fluid often reflects the biological processes taking place within the ovarian epithelial cyst wall.[51-53,59-63] Using immunohisto- and immunocytochemical staining, a significant association between expression of several tumor-associated antigens in tissue sections and cyst fluid cells was found.[47,51-53,59-63] Additional evidence for the direct release of tissue-derived compounds into the ovarian cyst fluid is provided by the fact that absolute levels of tumor-associated antigens in ovarian cyst fluid were found to be significantly higher than in serum of the same patients.[64-73] Because of its adjacent relation to the tumor surface, ovarian cyst fluid might therefore offer an interesting source for biomarker studies.

Thus far, biomarker research using ovarian cyst fluid typically had an explorative design and has been focused on the differences in the concentration of various biomarker candidates between cyst fluid of benign and malignant ovarian tumors.[64] As preoperative aspiration of ovarian cyst fluid might cause iatrogenic metastasis of tumor cells, the analysis of biomarkers in ovarian cyst fluid may not be used for the preoperative diagnosis of EOC.[21,68] Aspiration of the ovarian cyst after removal of the tumor at surgery is permitted and the fluid can be subsequently used for the analysis of predictive and prognostic biomarkers contributing to the understanding of the biology of ovarian tumors. Surprisingly, despite the shift in focus of biomarker research as described earlier, to the best of our knowledge no studies have been published that focused on biomarkers in ovarian cyst fluid for prognosis and/or prediction of response to chemotherapy.

Traditional biomarkers

Tumor-associated antigens

Tumors are known to induce the release of many proteins which might be used as biomarkers for detection and progression of cancer. The first recognized test for a specific type of cancer was reported in 1965, when the carcinoembryonic antigen (CEA) level was found to be elevated in serum of patients with colon cancer.[74] By the end of the 1970s, potential tumor-associated serum tests had been developed for a variety of cancers.[75] In 1981, Bast *et al.* discovered that a large membrane bound glycoprotein, cancer antigen (CA) 125, was present on tumor cells of the majority of patients with EOC.[22] An immunoassay for the quantification of CA 125 in serum was developed using the monoclonal antibody OC 125.[76] After initial interest in this molecule for its potential use as a serologic biomarker for early EOC diagnosis, studies revealed that the sensitivity and specificity of this marker were insufficient for use in populationbased screening.[77] A plethora of studies have subsequently been performed

to investigate the role of serum CA 125 as a predictive and/or prognostic marker for EOC. The monitoring of serum CA 125 levels during early chemotherapy has now proven its relevance and is currently used in clinical practice.[78] However, the clinical value of preoperative serum CA 125 levels in prediction of disease outcome or response to chemotherapy for EOC patients remains controversial. Although the majority of studies could not demonstrate a prognostic significance, [38,79-84] some studies found that preoperative serum CA 125 correlated significantly with survival.[85,86]

CA 125 levels in ovarian cyst fluid of EOC patients have found to be 100 to 1,000 fold higher than in serum, [68] suggesting a direct release of CA 125 from the tumor tissue into the ovarian cyst fluid. No attempts have been made so far to investigate whether CA 125 in ovarian cyst fluid might serve as a prognostic biomarker for EOC.

Proteases

A major step in cancer growth and dissemination involves the degradation of the extracellular matrix (ECM). This process is mediated by multiple degradative actions of proteolytic enzymes or proteases.[87] Proteolysis is a physiological mechanism the cell employs in order to regulate the function of its proteins. Tumor cells have the capacity to directly alter the surrounding ECM by quantitatively influencing the production of proteolytic enzymes.[88,89] Different proteases use different strategies to hydrolyze proteins and on this basis they can be grouped into four major classes: the aspartyl and cysteine proteases (lysosomal cysteine proteases), which are mainly involved in intracellular proteolysis, and the metallo and serine proteases, which are responsible for extracellular proteolysis.[87,88]

Activation of the proteolytic cascade participating in ECM degradation, in general, is induced by the lysosomal cysteine proteases.[88] Lysosomal cysteine proteases or cathepsins, comprise a large family of enzymes divided into subgroups (B, H, L, S, C, K, O, F, V, X and W) on the basis of their catalytic mechanisms.[90] Under physiological circumstances, cathepsins are localized in lysosomes but during cancer development and progression, they are often translocated to the cell surface or even secreted into surrounding fluids.[91] Cathepsins are distributed ubiquitously in most cancer tissues and studies have shown a correlation between cancer development and differential expression and localization of cathepsins.[91-93] Due to their increased levels in extracellular fluid, cathepsins have been implicated as biomarkers for the prognosis of cancer.[91] For this purpose, cathepsin B and H have most frequently been investigated and serum levels have been associated with a shorter survival of patients with colorectal, lung, breast and head and neck cancer.[91] Similar results were found when cathepsins were explored in urine, [94,95] and cerebrospinal [96] and pleural fluid [97] of cancer patients. Until now, the prognostic value of cathepsins in ovarian cyst fluid for EOC has not been established.

Enzymes involved in the metabolism of drugs

Multiple enzymes are present in cells to protect molecules such as nucleic acids, proteins, and lipids against drugs and oxidative stress. The process of detoxification by these enzymes usually involves two distinct stages, commonly referred to as phase I and phase II reactions. Phase I reactions, exemplified by the P-450 isoenzyme family, provide the target molecule with hydroxyl- or amino-groups. Phase II reactions, mainly executed by the glutathione S-transferase (GST) family, involve the addition of hydrophilic moieties to the target molecule, creating a more watersoluble conjugate, which may be less toxic and more readily excretable.[98] Therefore, phase II reactions are generally cytoprotective. The human GST superfamily comprises cytosolic isoenzymes, which have been assigned to at least four generic classes: Alpha (A), Mu (M), Pi (P) and Theta (T).[99] The Pi class GSTs (GSTP1-1) are believed to interact with platinum-based compounds and are frequently found to be overexpressed in a variety of neoplastic tissues, including EOC.[99,100] It has been shown that high GSTP1-1 activity results in an increased metabolism of platinum-based compounds, which subsequently results in a diminished cytotoxic effect on tumor cells.[98,100,101] Studies have shown that overexpression of GSTP1-1 in ovarian cancer tissue corresponded with a reduced response to chemotherapy of EOC patients.[39,102-105] These results are promising for the potential implication of GSTP1-1 as a biomarker for chemoresistant EOC. Therefore, there is a need for studies that investigate the feasibility of GSTP1-1 as a biomarker in biological fluids of patients with EOC.

Identification of new prognostic biomarkers

Genomics and proteomics

Until recently, the discovery of individual cancer biomarkers advanced rather slowly. Each candidate biomarker must be identified among an enormous amount of proteins and metabolites. In addition, clinical validation by means of very costly large multicenter randomized prospective trials is subsequently mandatory.

The last decade, genomic technologies such as microarrays have made it possible to study several thousands of genes simultaneously to obtain a global gene expression profile of tissue samples. To date, a variety of studies have employed gene expression profiling to classify ovarian carcinomas into clinically relevant subtypes, while more recent papers entirely focused on the development of gene expression profiles that correlate with chemoresistance or survival of EOC patients.[44,106-112] However, genomic research is limited by the fact that DNA alterations or RNA expression profiles are not always representative for the expression of the protein they encode. About 60% of the human genome is expressed while only 40% is translated into protein.[113]

The progress in proteomic approaches to identify novel biomarkers has relied on large scale protein expression profiling analysis by mass spectrometry. After the first attempts were made to use spectra of overexpressed proteins for discrimination between malignant and benign ovarian tumors, [114-116] more recently, a few studies have focused on the overexpression of proteins in chemoresistant ovarian cancer cell lines.[117-122] Again, large scale proteomic research has been limited to tissue and blood of patients with EOC.

Metabolomics

Metabolomics involves the study of the metabolome, the total repertoire of small molecules present in tissues, isolated cells and body fluids. It is a rapidly expanding field of scientific research which has become, after genomics and proteomics, the latest "omic" science. Metabolites are the end product of gene and protein activity. They are small, low-molecular weight compounds that predominantly serve as substrates and products in various metabolic pathways.[123] The number of different metabolites in humans is unknown; estimates range from 3,000 to 20,000, compared with approximately 23,000 genes and 60,000 proteins.[124] Individual metabolites have already been used as disease markers for years, such as glucose in diabetes mellitus or creatinine clearance in renal failure. By the introduction of proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, it became possible to provide a full spectrum of proton-containing compounds in a given fluid sample. So far, a view studies have been published using ^{1}H-NMR spectroscopy to explore differences in metabolic profiles between fluid samples of patients with malignant and benign ovarian tumors.[7,125,126] Further research is necessary to identify whether or not metabolomics will serve as a complementary or even an alternative tool to traditional approaches to define biomarkers for prediction of disease outcome in EOC.

Outline of this thesis

Despite improvements in aggressive debulking surgery and the initial good response of patients to platinum- and paclitaxel-based chemotherapeutics, there has been little improvement in the survival rates of EOC patients over the last three decades. Unfortunately, there are no reliable biomarkers for predicting clinical response and guiding treatment regimens. Blood en tissue of EOC patients have been traditionally used as starting material for biomarker research. In contrast, compounds in ovarian cyst fluid have never been investigated for their prognostic and/or predictive value. Ovarian cyst fluid has several advantages over blood and tissue. The aim of this thesis is to investigate the prognostic and predictive value of a number of potential biomarkers in ovarian carcinoma cyst fluid. To achieve this objective, at first, the prognostic value of three classical (groups of) biomarkers is studied. Hereafter, novel biomarkers are revealed by metabolomic profiling and subsequently studied for their prognostic significance. In chapter 1, the selection of the studied biomarkers is described in detail. Although cyst fluid can be obtained easily from ovarian tumors after primary surgery, not all EOCs contain cyst fluid. Therefore, availability is the main problem when using cyst fluid biomarkers for clinical purposes. In chapter 2 the exact prevalence of cysts in EOC is studied as well as tumor characteristic that may be related to the presence of cysts.

CA 125 is the only biomarker for EOC that is used in daily clinical practice worldwide. Serum CA 125 is valuable for monitoring during chemotherapy. However, the role of pre-operative serum CA 125 as a prognostic marker remains questionable. In chapter 3 the prognostic value of CA 125 in ovarian cyst fluid is studied for patients with EOC.

Another group of potential prognostic biomarkers might be the proteases. With their capacity to degrade tumor basement membrane components and other structural proteins, they have been frequently associated with tumor progression and metastasis. For several types of cancer, extracellular presence of proteases and their endogenous inhibitors turned out to be of prognostic value. In chapter 4, cathepsin B, L and cystatin C are explored in the cyst fluid of ovarian tumors. Hereafter, in chapter 5, the prognostic value of cyst fluid cathepsin B, H, L, and X and their endogenous inhibitor cystatin C is studied for patients with EOC.

Detoxification enzymes are involved in the metabolism of drugs and by-products of oxidative stress. Overexpression of GSTP1-1, a member of the phase II detoxification enzymes, in cancer tissue has found to be related to a reduced response to platinum-based chemotherapy in cancer patients. In chapter 6, the predictive role of GSTP1-1 in ovarian cyst fluid as a biomarker for resistance to platinum-based chemotherapy is investigated.

By the introduction of ^{1}H-NMR spectroscopy, it became possible to provide a full spectrum of proton-containing compounds in a given fluid sample. In chapter 7, ^{1}H-NMR spectroscopy is used to reveal ovarian cancer biomarkers by comparing ovarian cyst fluid profiles between different histopathological

subgroups. N-acetylaspartic acid (NAA) and drug binding protein Alpha 1-acid glycoprotein (AGP) were subsequently identified and validated by quantitative techniques. NAA is the second most abundant free amino acid in the human brain but synthesis has never been found in human tissues outside the cerebrospinal system.[127] In chapter 8, the relation between cyst fluid NAA and different ovarian tumor subtypes is further investigated. The second biomarker, AGP, is an acute phase protein with drug binding properties. During pathological conditions, the synthesis of AGP increases due to an acute phase response.[128] A high serum concentration of AGP has found to be an indicator for poor survival in patients with cancer. However, little is known about the direct relation between treatment failure and binding of AGP to chemotherapeutics. In chapter 9, the prognostic and predictive value of AGP is determined for EOC patients treated with chemotherapy. In addition, the binding affinity is established between AGP and paclitaxel/cisplatin.

Finally, in chapter 10, the results of this thesis are discussed in general.

References

1. Colombo N, Van Gorp T, Parma G et al. Ovarian cancer. Crit Rev Oncol Hematol 2006;60:159-79.
2. du Bois A, Quinn M, Thigpen T et al. 2004 consensus statements on the management of ovarian cancer: final document of the 3rd International Gynecologic Cancer Intergroup Ovarian Cancer Consensus Conference GCIG OCCC 2004). Ann Oncol 2005;16 Suppl 8:viii7-viii12.
3. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96.
4. Soslow RA. Histologic subtypes of ovarian carcinoma: an overview. Int J Gynecol Pathol 2008;27:161-74.
5. Zhu Y, Wu R, Sangha N et al. Classifications of ovarian cancer tissues by proteomic patterns. Proteomics 2006;6:5846-56.
6. Konstantinopoulos PA, Spentzos D, Cannistra SA. Gene-expression profiling in epithelial ovarian cancer. Nat Clin Pract Oncol 2008;5:577-87.
7. Boss EA, Moolenaar SH, Massuger LF et al. High-resolution proton nuclear magnetic resonance spectroscopy of ovarian cyst fluid. NMR Biomed 2000;13:297-305.
8. Kurman RJ. Blaustein's pathology of the female genital tract, 5th ed. New York: Springer-Verlag; 2002.
9. Dubeau L. The cell of origin of ovarian epithelial tumours. Lancet Oncol 2008;9:1191-7.
10. Naora H. The heterogeneity of epithelial ovarian cancers: reconciling old and new paradigms. Expert Rev Mol Med 2007;9:1-12.
11. Marquez RT, Baggerly KA, Patterson AP et al. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. Clin Cancer Res 2005;11:6116-26.
12. Classification and staging of malignant tumours in the female pelvis. Acta Obstet Gynecol Scand 1971;50:1-7.
13. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
14. Malpica A. Grading of ovarian cancer: a histotype-specific approach. Int J Gynecol Pathol 2008;27:175-81.
15. Silverberg SG. Histopathologic grading of ovarian carcinoma: a review and proposal. Int J Gynecol Pathol 2000;19:7-15.
16. Treub H. Gynaecology by H. Treub, 2nd ed. Leiden: S.C. van Doesburgh; 1895.
17. Wells TS. Diseases of the ovaries, 1st ed. London: Spottis Woode and Co.; 1865.
18. Brown IB. Ovarian dropsy, 1st ed. London: Savill and Edwards; 1862.
19. Peelen P. Diseases of the female tract of Kiwisch's clinical lectures, 3th ed. Tiel: 1852.
20. Young RH. The history of British gynaecological pathology. Histopathology 2009;54:144-55.
21. Trimbos JB, Hacker NF. The case against aspirating ovarian cysts. Cancer 1993;72:828-31.
22. Bast RC Jr., Feeney M, Lazarus H et al. Reactivity of a monoclonal antibody with human ovarian carcinoma. J Clin Invest 1981;68:1331-7.
23. Terry KL, Sluss PM, Skates SJ et al. Blood and urine markers for ovarian cancer: a comprehensive review. Dis Markers 2004;20:53-70.
24. Gadducci A, Cosio S, Tana R et al. Serum and tissue biomarkers as predictive and prognostic variables in epithelial ovarian cancer. Crit Rev Oncol Hematol 2009;96:12-27.
25. Eisenhauer EA, Vermorken JB, van Glabbeke M. Predictors of response to subsequent chemotherapy in platinum pretreated ovarian cancer: a multivariate analysis of 704 patients. Ann Oncol 1997;8:963-8.
26. Omura GA, Brady MF, Homesley HD et al. Long-term follow-up and prognostic factor analysis in advanced ovarian carcinoma: the Gynecologic Oncology Group experience. J Clin Oncol 1991;9:1138-50.
27. Cloven NG, Kyshtoobayeva A, Burger RA et al. In vitro chemoresistance and biomarker profiles are unique for histologic subtypes of epithelial ovarian cancer. Gynecol Oncol 2004;92:160-6.
28. Nolen B, Marrangoni A, Velikokhatnaya L et al. A serum based analysis of ovarian epithelial tumorigenesis. Gynecol Oncol 2009;112:47-54.

29. Kobel M, Kalloger SE, Boyd N et al. Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS Med 2008;5:e232.
30. Crijns AP, Duiker EW, de Jong S et al. Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. Int J Gynecol Cancer 2006;16 Suppl 1:152-65.
31. Gadducci A, Cosio S, Carpi A et al. Serum tumor markers in the management of ovarian, endometrial and cervical cancer. Biomed Pharmacother 2004;58:24-38.
32. Canevari S, Garibaldi M, Reid JF et al. Molecular predictors of response and outcome in ovarian cancer. Crit Rev Oncol Hematol 2006;60:19-37.
33. Høgdall E. Cancer antigen 125 and prognosis. Curr Opin Obstet Gynecol 2008;20:4-8.
34. De Graeff P, Crijns AP, ten Hoor KA et al. The ErbB signalling pathway: protein expression and prognostic value in epithelial ovarian cancer. Br J Cancer 2008;99:341-9.
35. Cheng WF, Huang CY, Chang MC et al. High mesothelin correlates with chemoresistance and poor survival in epithelial ovarian carcinoma. Br J Cancer 2009;100:1144-53.
36. Kupryjanczyk J, Szymanska T, Madry R et al. Evaluation of clinical significance of TP53, BCL-2, BAX and MEK1 expression in 229 ovarian carcinomas treated with platinum-based regimen. Br J Cancer 2003;88:848-54.
37. Kommoss F, Pfisterer J, Thome M et al. Steroid receptors in ovarian carcinoma: immunohistochemical determination may lead to new aspects. Gynecol Oncol 1992;47:317-22.
38. Høgdall EV, Christensen L, Kjaer SK et al. CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study. Gynecol Oncol 2007;104:508-15.
39. Hamada S, Kamada M, Furumoto H et al. Expression of glutathione S-transferase-pi in human ovarian cancer as an indicator of resistance to chemotherapy. Gynecol Oncol 1994;52:313-9.
40. Silvestrini R, Daidone MG, Veneroni S et al. The clinical predictivity of biomarkers of stage III-IV epithelial ovarian cancer in a prospective randomized treatment protocol. Cancer 1998;82:159-67.
41. Scorilas A, Fotiou S, Tsiambas E et al. Determination of cathepsin B expression may offer additional prognostic information for ovarian cancer patients. Biol Chem 2002;383:1297-303.
42. Howells RE, Dhar KK, Hoban PR et al. Association between glutathione-S-transferase GSTP1 genotypes, GSTP1 over-expression, and outcome in epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:242-50.
43. Edwards PA. Heterogeneous expression of cell-surface antigens in normal epithelia and their tumours, revealed by monoclonal antibodies. Br J Cancer 1985;51:149-60.
44. Crijns AP, Fehrmann RS, de Jong S et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:e24.
45. Khalique L, Ayhan A, Weale ME et al. Genetic intra-tumour heterogeneity in epithelial ovarian cancer and its implications for molecular diagnosis of tumours. J Pathol 2007;211:286-95.
46. Rubin SC, Finstad CL, Federici MG et al. Prevalence and significance of HER-2/neu expression in early epithelial ovarian cancer. Cancer 1994;73:1456-9.
47. Harlozinska A, Bar J, Montenarh M. Analysis of the immunoreactivity of three anti-p53 antibodies and estimation of the relations between p53 status and MDM2 protein expression in ovarian carcinomas. Anticancer Res 2000;20:1049-56.
48. Welch WR, Niloff JM, Anderson D et al. Antigenic heterogeneity in human ovarian cancer. Gynecol Oncol 1990;38:12-6.
49. Woloszynska-Read A, Mhawech-Fauceglia P, Yu J et al. Intertumor and intratumor NY-ESO-1 expression heterogeneity is associated with promoter-specific and global DNA methylation status in ovarian cancer. Clin Cancer Res 2008;14:3283-90.

50. Lee FY, Vessey A, Rofstad E et al. Heterogeneity of glutathione content in human ovarian cancer. Cancer Res 1989;49:5244-8.
51. Bar JK, Harlozinska A, Sobanska E et al. Relation between ovarian carcinoma-associated antigens in tumor tissue and detached cyst fluid cells of patients with ovarian neoplasms. Tumori 1994;80:50-5.
52. Harlozinska A, Bar JK. Relationship between p53 and c-erbB-2 overexpression in tissue sections and cyst fluid cells of patients with ovarian cancer. Tumour Biol 1994;15:223-9.
53. Harlozinska A, Bar JK, Jothy S et al. Carcinoembryonic antigen isotypes in tissue sections and loose cyst fluid cells of ovarian neoplasms. Tumour Biol 1993;14:1-8.
54. Quinn MA, Rome RM, Cauchi M et al. Steroid receptors and ovarian tumors: variation within primary tumors and between primary tumors and metastases. Gynecol Oncol 1988;31:424-9.
55. Zangwill BC, Balsara G, Dunton C et al. Ovarian carcinoma heterogeneity as demonstrated by DNA ploidy. Cancer 1993;71:2261-7.
56. Zborovskaya I, Gasparian A, Karseladze A et al. Somatic genetic alterations (LOH) in benign, borderline and invasive ovarian tumours: intratumoral molecular heterogeneity. Int J Cancer 1999;82:822-6.
57. Tuefferd M, Couturier J, Penault-Llorca F et al. HER2 status in ovarian carcinomas: a multicenter GINECO study of 320 patients. PLoS ONE 2007;2:e1138.
58. Takahashi Y, Takenaka A, Ishiguro T et al. Intratumoral DNA heterogeneity correlated with lymph node involvement and surgical staging in epithelial ovarian cancer by flow cytometry. Cancer 1994;73:3011-4.
59. Harlozinska A, Bar JK, Sedlaczek P et al. Expression of p53 protein and Ki-67 reactivity in ovarian neoplasms. Correlation with histopathology. Am J Clin Pathol 1996;105:334-40.
60. Harlozinska A, Bar JK, Gerber J. nm23 expression in tissue sections and tumor effusion cells of ovarian neoplasms. Int J Cancer 1996;69:415-9.
61. Harlozinska A, Bar JK, Sobanska E et al. Epidermal growth factor receptor and c-erbB-2 oncoproteins in tissue and tumor effusion cells of histopathologically different ovarian neoplasms. Tumour Biol 1998;19:364-73.
62. Montenarh M, Harlozinska A, Bar JK et al. p53 autoantibodies in the sera, cyst and ascitic fluids of patients with ovarian cancer. Int J Oncol 1998;13:605-10.
63. Bar JK, Harlozinska A, Popiela A et al. Expression and mutation of p53 in tumor effusion cells of patients with ovarian carcinoma: response to cisplatin-based chemotherapy. Tumour Biol 2001;22:83-91.
64. Boss EA. Clinical value of analytes in cyst fluid of ovarian tumors. Thesis, 2003: Radboud University Nijmegen Medical Center.
65. Thomas CM, Boss EA, Boonstra H et al. Gonadotropins and female sex steroid hormones in cyst fluid and serum from patients with ovarian tumors. Eur J Gynaecol Oncol 2008;29:468-72.
66. Sedlaczek P, Frydecka I, Gabrys M et al. Comparative analysis of CA125, tissue polypeptide specific antigen, and soluble interleukin-2 receptor alpha levels in sera, cyst, and ascitic fluids from patients with ovarian carcinoma. Cancer 2002;95:1886-93.
67. Ivarsson K, Runesson E, Sundfeldt K et al. The chemotactic cytokine interleukin-8--a cyst fluid marker for malignant epithelial ovarian cancer? Gynecol Oncol 1998;71:420-3.
68. Fleuren GJ, Nap M, Aalders JG et al. Explanation of the limited correlation between tumor CA 125 content and serum CA 125 antigen levels in patients with ovarian tumors. Cancer 1987;60:2437-42.
69. Harlozinska A, Sedlaczek P, Kulpa J et al. Vascular endothelial growth factor (VEGF) concentration in sera and tumor effusions from patients with ovarian carcinoma. Anticancer Res 2004;24:1149-57.
70. Arts HJ, de Jong S, Hollema H et al. Fas and Fas ligand in cyst fluids, serum and tumors of patients with benign and (borderline) malignant ovarian tumors. Int J Oncol 2005;26:379-84.
71. Chudecka-Glaz A, Rzepka-Gorska I. Activin A levels in serum and cyst fluid in epithelial tumors of the ovary. Int J Gynaecol Obstet 2005;89:160-2.

72. Koivunen E, Itkonen O, Halila H et al. Cyst fluid of ovarian cancer patients contains high concentrations of trypsinogen-2. Cancer Res 1990;50:2375-8.
73. Halila H, Huhtala ML, Haglund C et al. Tumour-associated trypsin inhibitor (TATI) in human ovarian cyst fluid. Comparison with CA 125 and CEA. Br J Cancer 1987;56:153-6.
74. Gold P, Freedman SO. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J Exp Med 1965;121:439-62.
75. Jacobs EL, Haskell CM. Clinical use of tumor markers in oncology. Curr Probl Cancer 1991;15:299-360.
76. Bast RC, Jr., Klug TL, St John E et al. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. N Engl J Med 1983;309:883-7.
77. Bast RC, Jr., Xu FJ, Yu YH et al. CA 125: the past and the future. Int J Biol Markers 1998;13:179-87.
78. Gadducci A, Cosio S, Tana R et al. Serum and tissue biomarkers as predictive and prognostic variables in epithelial ovarian cancer. Crit Rev Oncol Hematol 2009;69:12-27.
79. Tingulstad S, Skjeldestad FE, Halvorsen TB et al. Survival and prognostic factors in patients with ovarian cancer. Obstet Gynecol 2003;101:885-91.
80. Geisler JP, Miller GA, Lee TH et al. Relationship of preoperative serum CA-125 to survival in epithelial ovarian carcinoma. J Reprod Med 1996;41:140-2.
81. Nagele F, Petru E, Medl M et al. Preoperative CA 125: an independent prognostic factor in patients with stage I epithelial ovarian cancer. Obstet Gynecol 1995;86:259-64.
82. Rossi AC, DiVG, Cormio G et al. A retrospective study of preoperative CA 125 levels in 82 patients with ovarian cancer. Arch Gynecol Obstet 2004;269:263-5.
83. Gadducci A, Zola P, Landoni F et al. Serum half-life of CA 125 during early chemotherapy as an independent prognostic variable for patients with advanced epithelial ovarian cancer: results of a multicentric Italian study. Gynecol Oncol 1995;58:42-7.
84. Cruickshank DJ, Fullerton WT, Klopper A. The clinical significance of pre-operative serum CA 125 in ovarian cancer. Br J Obstet Gynaecol 1987;94:692-5.
85. Makar AP, Kristensen GB, Kaern J et al. Prognostic value of pre- and postoperative serum CA 125 levels in ovarian cancer: new aspects and multivariate analysis. Obstet Gynecol 1992;79:1002-10.
86. Cooper BC, Sood AK, Davis CS et al. Preoperative CA 125 levels: an independent prognostic factor for epithelial ovarian cancer. Obstet Gynecol 2002;100:59-64.
87. Chapman HA, Riese RJ, Shi GP. Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 1997;59:63-88.
88. Skrzydlewska E, Sulkowska M, Koda M et al. Proteolytic-antiproteolytic balance and its regulation in carcinogenesis. World J Gastroenterol 2005;11:1251-66.
89. Aznavoorian S, Murphy AN, Stetler-Stevenson WG et al. Molecular aspects of tumor cell invasion and metastasis. Cancer 1993;71:1368-83.
90. Turk V, Turk B, Guncar G et al. Lysosomal cathepsins: structure, role in antigen processing and presentation, and cancer. Adv Enzyme Regul 2002;42:285-303.
91. Kos J, Werle B, Lah T et al. Cysteine proteinases and their inhibitors in extracellular fluids: markers for diagnosis and prognosis in cancer. Int J Biol Markers 2000;15:84-9.
92. Duffy MJ. Proteases as prognostic markers in cancer. Clin Cancer Res 1996;2:613-8.
93. Kos J, Lah TT. Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer (review). Oncol Rep 1998;5:1349-61.
94. Staack A, Koenig F, Daniltchenko D et al. Cathepsins B, H, and L activities in urine of patients with transitional cell carcinoma of the bladder. Urology 2002;59:308-12.
95. Hirano T, Manabe T, Takeuchi S. Serum cathepsin B levels and urinary excretion of cathepsin B in the cancer patients with remote metastasis. Cancer Lett 1993;70:41-4.

96. Nagai A, Terashima M, Harada T et al. Cathepsin B and H activities and cystatin C concentrations in cerebrospinal fluid from patients with leptomeningeal metastasis. Clin Chim Acta 2003;329:53-60.
97. Bunatova K, Obermajer N, Kotyza J et al. Levels of cathepsins S and H in pleural fluids of inflammatory and neoplastic origin. Int J Biol Markers 2009;24:47-51.
98. Tew KD. Glutathione-associated enzymes in anticancer drug resistance. Cancer Res 1994;54:4313-20.
99. Beckett GJ, Hayes JD. Glutathione S-transferases: biomedical applications. Adv Clin Chem 1993;30:281-380.
100. Raunio H, Husgafvel-Pursiainen K, Anttila S et al. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility-a review. Gene 1995;159:113-21.
101. McIlwain CC, Townsend DM, Tew KD. Glutathione S-transferase polymorphisms: cancer incidence and therapy. Oncogene 2006;25:1639-48.
102. Green JA, Robertson LJ, Clark AH. Glutathione S-transferase expression in benign and malignant ovarian tumours. Br J Cancer 1993;68:235-9.
103. Cheng X, Kigawa J, Minagawa Y et al. Glutathione S-transferase-pi expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. Cancer 1997;79:521-7.
104. Hirazono K, Shinozuka T, Kuroshima Y et al. Immunohistochemical expression of glutathione S-transferase pi (GST-pi) and chemotherapy response in malignant ovarian tumors. J Obstet Gynaecol 1995;21:305-12.
105. Satoh T, Nishida M, Tsunoda H et al. Expression of glutathione S-transferase pi (GST-pi) in human malignant ovarian tumors. Eur J Obstet Gynecol Reprod Biol 2001;96:202-8.
106. Spentzos D, Levine DA, Ramoni MF et al. Gene expression signature with independent prognostic significance in epithelial ovarian cancer. J Clin Oncol 2004;22:4700-10.
107. Berchuck A, Iversen ES, Lancaster JM et al. Patterns of gene expression that characterize long-term survival in advanced stage serous ovarian cancers. Clin Cancer Res 2005;11:3686-96.
108. Lancaster JM, Dressman HK, Whitaker RS et al. Gene expression patterns that characterize advanced stage serous ovarian cancers. J Soc Gynecol Investig 2004;11:51-9.
109. Hibbs K, Skubitz KM, Pambuccian SE et al. Differential gene expression in ovarian carcinoma: identification of potential biomarkers. Am J Pathol 2004;165:397-414.
110. Collins Y, Tan DF, Pejovic T et al. Identification of differentially expressed genes in clinically distinct groups of serous ovarian carcinomas using cDNA microarray. Int J Mol Med 2004;14:43-53.
111. Helleman J, Jansen MP, Span PN et al. Molecular profiling of platinum resistant ovarian cancer. Int J Cancer 2006;118:1963-71.
112. Helleman J, Jansen MP, Burger C et al. Integrated genomics of chemotherapy resistant ovarian cancer: A role for extracellular matrix, TGFbeta and regulating microRNAs. Int J Biochem Cell Biol 2010;42:25-30.
113. Carninci P, Kasukawa T, Katayama S et al. The transcriptional landscape of the mammalian genome. Science 2005;309:1559-63.
114. Alaiya AA, Franzen B, Fujioka K et al. Phenotypic analysis of ovarian carcinoma: polypeptide expression in benign, borderline and malignant tumors. Int J Cancer 1997;73:678-83.
115. Cadron I, Van Gorp T, Timmerman D et al. Application of proteomics in ovarian cancer: Which sample should be used? Gynecol Oncol 2009;115:497-503.
116. Alaiya AA, Franzen B, Hagman A et al. Classification of human ovarian tumors using multivariate data analysis of polypeptide expression patterns. Int J Cancer 2000;86:731-6.
117. Yan XD, Pan LY, Yuan Y et al. Identification of platinum-resistance associated proteins through proteomic analysis of human ovarian cancer cells and their platinum-resistant sublines. J Proteome Res 2007;6:772-80.
118. Song J, Shih I, Salani R et al. Annexin XI is associated with cisplatin resistance and related to tumor recurrence in ovarian cancer patients. Clin Cancer Res 2007;13:6842-9.
119. Stewart JJ, White JT, Yan X et al. Proteins associated with Cisplatin resistance in ovarian cancer cells identified by quantitative proteomic technology and integrated with mRNA expression levels. Mol Cell Proteomics 2006;5:433-43.

120. Le Moguen K, Lincet H, Deslandes E et al. Comparative proteomic analysis of cisplatin sensitive IGROV1 ovarian carcinoma cell line and its resistant counterpart IGROV1-R10. Proteomics 2006;6:5183-92.
121. Aggarwal S, He T, Fitzhugh W et al. Immune modulator CD70 as a potential cisplatin resistance predictive marker in ovarian cancer. Gynecol Oncol 2009;115:430-7.
122. Helleman J, van der Vlies V, Jansen MP et al. Serum proteomic patterns for ovarian cancer monitoring. Int J Gynecol Cancer 2008;18:985-95.
123. Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. Annu Rev Pharmacol Toxicol 2008;48:653-83.
124. Schmidt CW. Metabolomics: what's happening downstream of DNA. Environ Health Perspect 2004;112:A410-A415.
125. Massuger LF, van Vierzen PB, Engelke U et al. 1H-magnetic resonance spectroscopy: a new technique to discriminate benign from malignant ovarian tumors. Cancer 1998;82:1726-30.
126. Odunsi K, Wollman RM, Ambrosone CB et al. Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. Int J Cancer 2005;113:782-8.
127. Baslow MH. N-acetylaspartate in the vertebrate brain: metabolism and function. Neurochem Res 2003;28:941-53.
128. Israili ZH, Dayton PG. Human alpha-1-glycoprotein and its interactions with drugs. Drug Metab Rev 2001;33:161-235.


2

Prevalence of cysts in epithelial ovarian cancer

Kolwijck E, Lybøl C, Bulten J, Vollebergh JHA, Wevers RA, Massuger LFAG

European Journal of Obstetrics & Gynecology and Reproductive Biology,2010; in press

Abstract

Ovarian carcinomas mostly appear as large cystic masses. However, the exact prevalence of cystic epithelial ovarian cancer (EOC) has never been documented as well as the tumor factors that are related to the presence of cysts. Proving prevalence of cysts in EOC is of essential for research focused on predictive and prognostic biomarkers in ovarian cyst fluid. From 233 patients with primary EOC who underwent surgery, pathological data were collected from pathology reports. Univariate and multivariate logistic regression were used to analyze the relationship between the presence of cysts and other tumor characteristics. Cysts in EOC were present in 83.7% of the patients and were mostly (61%) multilocular. The most common histological subtypes (serous, mucinous, endometrioid, clear cell) contained cysts in more than 85% of the cases. In univariate regression analysis, early FIGO stage, low tumor grade and a large tumor size were significantly associated with the presence of cysts (OR (95% CI) = 5.312 (1.81-15.57), 6.906 (2.31-20.66) and 1.169 (1.08-1.27), respectively). In multivariate regression analysis, apart from tumor size, only tumor grade was independently associated with the presence of cysts (adjusted OR (95% CI) = 4.234 (1.36-13.22)). The large majority of all EOCs contained cysts. Histological subtype, FIGO stage, tumor necrosis, and age were not associated with the presence of cystic EOC. This means that cystic EOCs represent a subgroup of larger and more well-differentiated tumors. The evident relationship between the presence of cysts and differentiation grade is interesting from a clinical point of view as grading is especially important for the prognosis and treatment of patients with stage I EOC.

Introduction

Epithelial ovarian cancer (EOC) accounts for almost 90% of all malignant ovarian tumors and is the most lethal gynecological malignancy worldwide. Its high mortality rate is mainly caused by the inability to detect the disease in an early stage, due to the absence of specific clinical symptoms and availability of proper screening techniques.[1]

These days, there is a gaining interest for biomarkers that are able to predict survival and response to treatment of patients with EOC at time of diagnosis, and gynecologic oncologists have emphasized the need for these biomarkers to select patients for individually based treatment.[2] During the last decade, many researchers have attempted to identify clinically relevant biomarkers in EOC tissue. However, results of such studies are difficult to compare due to sampling errors, inappropriate pathological evaluation of ovarian tumors, the use of different histological techniques, and most importantly, the intratumoral tissue heterogeneity.[3] To avoid these difficulties, researchers have become increasingly interested in the analysis of the biochemical composition of ovarian cyst fluid to find relevant biomarkers.

Ovarian tumors, benign as well as malignant, often present as cystic masses which contain variable amounts of fluid.[4] These cysts generally are encapsulated within the tumor tissue. Cyst fluid therefore contains compounds released by the tumor tissue thus reflecting the biological processes of the surrounding tumor cells.[5] Also, in contrast to ovarian cancer tissue, compounds in cyst fluid are homogeneously distributed and analyses are quantitative and often uncomplicated.[6] Since preoperative punction of cyst fluid might cause tumor spill, ovarian cyst fluid has to be obtained after surgical removal of the tumor and cyst fluid biomarkers can only be clinically used as predictors of survival and/or response to treatment. For example, glutathione S-transferase pi (GSTP1-1) levels in post-operatively obtained ovarian cyst fluid have recently been shown to be of independent prognostic value for patients with EOC.[7] However, the exact prevalence of cystic epithelial ovarian cancer (EOC) has never been documented as well as the tumor factors that are related to the presence of cysts. Proving prevalence of cysts in EOC is a serious prerequisite for research focused on various predictive and prognostic biomarkers in ovarian cyst fluid.

In the present study, prevalence of cysts was investigated in a large series of EOCs and pathological tumor characteristics were related to the presence of cysts.

Materials and Methods

Patients and pathological reports

The present study was performed with pathological data of 233 patients diagnosed with primary EOC who had surgery between January 1985 and August 2008 at the Radboud University Nijmegen Medical Centre (RUNMC), and Bernhoven Hospital (BH), locations Oss and Veghel, the Netherlands. In the national pathological database, the pathological anatomical national automatized archive (PALGA), a data search was performed with the terms: "ovary" and "malignancy" which resulted in 475 pathology reports of patients who had surgery at RUNMC or BH. From these reports, 58 were excluded because it concerned biopsies or revisions, 114 were excluded because patients had a benign or atypical proliferative (borderline) ovarian tumor, [8-10] and 29 were excluded because of preoperative chemotherapy. In addition, 37 pathological reports were not conclusive on stage and 1 was not conclusive on aspect of the tumor (solid or cystic). Of the remaining reports, 3 tumors were of non-epithelial origin and were excluded as well. Complete pathological reports of 233 patients with primary EOC could be included.

Histopathological characteristics

For the purpose of this study, pathological reports were retrospectively obtained and reviewed for macroscopic and microscopic features.

Macroscopic features that were scored were: bilaterality or unilaterality of the ovarian tumor, largest diameter of the tumor (cm), the presence of cysts and the number of cysts. According to the Dutch guidelines for Pathologists, after macroscopic incision of the tumor, EOC has to be defined as unilocular, multilocular, or purely solid. Whenever a tumor was mentioned to macroscopically contain both cystic and solid parts, for this study purposes the tumor was scored as cystic.

Microscopic features that were achieved were: presence of necrosis, histological subtype, and tumor grade. Histological tumor type was scored according to the World Health Organization histological classification of ovarian tumors.[9] Adenocarcinomas "not otherwise specified" (NOS) were epithelial tumors of which the pathological report was not conclusive on histology. Tumor grade was scored as reported by the initial pathologists. For grading, the pathologists formerly relied on cytological atypia and architecture. In present years, grading has often been performed according to Silverberg.[11] In patients with bilateral ovarian cancer, pathological features of both ovaries were scored, and eventually, only features of the most unfavorable tumor, based on surface involvement and tumor grade, were included for analysis. Stage of disease was determined according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO).[12] Staging was based on the data from complete histopathological reports.

Statistical analyses

Statistical analysis was performed to investigate which factors were related to the presence of cysts in EOC. Association between clinicopathologic features and the presence of a cystic tumor were tested using the Chi-square test. Differences of medians of continuous variables between the groups were analyzed using a non-parametric test (Mann-Whitney). Univariate and multivariate logarithmic regression analyses were performed to determine which clinicopathological factors were significantly related to the presence of cysts in EOC. Because the small number of patients with FIGO stage II (n = 20, 8.6%) and IV (n = 18, 7.7%) and tumor grade 1 (n = 30, 12.9%) (Table 1), for the purpose of univariate and multivariate analyses FIGO stage and tumor grade were grouped. FIGO stage was grouped into early stage (I-IIa) and advanced stage (IIb-IV). Early stage FIGO stage (I-IIa) comprised 75 patients with stage I and 4 patients with stage IIa. Advanced FIGO stage (IIb-IV) comprised 16 patients with stage IIb and IIc, 120 patients with stage III and 18 patients with stage IV. Tumor grade was grouped into grade 1 and 2 versus grade 3. The group with grade 1 and 2 tumors comprised 89 patients and the group with grade 3 comprised 106 patients. Tumor grade was scored as 1 and 2 versus grade 3 (instead of the more common classification of grade 1 versus grade 2 and 3) because of statistical reasons as none of the 30 tumors with grade 1 was solid. For histological subtypes, reports of patients with transitional tumors and undifferentiated tumors were excluded from uni- and multivariate regression analyses due to low sample sizes (Table 1). Adenocarcinomas NOS were excluded from uni- and multivariate regression analyses because it was unclear which histological subtype was represented. Variables found to be significantly related to the presence of a cystic tumor by univariate logistic regression were entered into a multivariate logistic regression model with forward selection procedures to identify the variables that were independently associated with the presence of a cystic tumor. Reports with missing data were excluded from the multivariate analysis. In all tests a p-value <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS software version 16.0 (Chicago, Illinois, USA).

Results

Patient and tumor characteristics

The study population included 233 patients with primary EOC of whom complete pathological reports were reviewed. Median age at diagnosis was 58 years (range: 17-88 years). The median diameter of the cystic tumors was 12 cm (range: 4-44 cm) whereas solid tumors had a median diameter of 6.8 cm (range: 2.5-18 cm) (p <0.001, Mann-Whitney test). Of the 4 most common histopathological subtypes, serous tumors had a median diameter of 9 cm (range: 2-30 cm), and mucinous, endometrioid and clear cell tumors had a median diameter of 20 cm (range: 4.5-44), 13 cm (range: 6.5-22 cm) and 12 cm (range: 6-23 cm), respectively. Table 1 represents the remaining clinicopathological characteristics of the study population.

Table 1 Clinicopathological characteristics of 233 patients with primary EOC

Clinicopathological characteristics		total *n* (%)	cystic tumors *n* (%)	solid tumors *n* (%)
FIGO stage	I	75 (32.2)	72 (36.9)	3 (7.9)
	II	20 (8.6)	18 (9.2)	2 (5.3)
	III	120 (51.5)	89 (45.7)	31(81.5)
	IV	18 (7.7)	16 (8.2)	2 (5.3)
Tumor grade	1	30 (12.9)	30 (15.4)	0
	2	59 (25.3)	55 (28.2)	4 (10.5)
	3	106 (45.5)	80 (41.0)	26 (68.4)
	unknown	38 (16.3)	30 (15.4)	8 (21.1)
Histological subtype	serous	97 (41.6)	83 (42.6)	14 (36.8)
	mucinous	28 (12.0)	26 (13.3)	2 (5.3)
	endometrioid	36 (15.5)	31 (15.9)	5 (13.1)
	clear cell	14 (6.0)	12 (6.2)	2 (5.3)
	transitional cell	1 (0.4)	1 (0.5)	0
	mixed	19 (8.2)	15 (7.7)	4 (10.5)
	undifferentiated	5 (2.1)	3 (1.5)	2 (5.3)
	NOS	33 (14.2)	24 (12.3)	9 (23.7)
Laterality	bilateral	114 (48.9)	91 (46.7)	23 (60.5)
	unilateral	119 (51.1)	104 (53.3)	15 (39.5)
Presence of necrosis	yes	120 (51.5)	101 (51.8)	19 (50.0)
	no	113 (48.5)	94 (48.2)	19 (50.0)
Total		233 (100)	195 (83.7)	38 (16.3)

Prevalence of cystic EOC

For all clinicopathological variables scored, prevalences of cystic and solid tumors are listed in Table 1. The majority of the patients (n = 195; 83.7%) presented with EOC that contained one or more cysts. Prevalence of these cysts was more than 88% in patients with FIGO stage I, II and IV (96%, 90%, and 89%, respectively). Only EOC stage III had a lower prevalence of cysts (74%). All well-differentiated tumors in this study appeared to be cystic, which was also true for almost all moderately differentiated tumors (93.2%). In contrast, 87.7% of the solid tumors were poorly differentiated. Of the three most common histological subtypes (serous, mucinous and endometrioid), more than 85% of the tumors appeared to contain one or more cysts (86%, 93%, and 86%, respectively). Also, clear cell tumors were found to be cystic in more than 85% of the cases. Only mixed type and undifferentiated adenocarcinomas showed lower percentages of cysts (78.9% and 60% respectively). The majority of cystic EOC was multilocular (n = 119; 61%), 45 (23.1%) were unilocular and in 31 (15.9%) only the presence of a cystic component was reported without being conclusive of the number of cysts.

Logistic regression analysis

Variables significantly associated with the presence of cysts in EOC were: largest tumor diameter, tumor grade and FIGO stage (all $p < 0.001$, Table 2). Age, histological subtype, laterality and necrosis were not associated with the prevalence of cystic EOC ($p = 0.354$, $p = 0.754$, $p = 0.118$, and $p = 0.840$, respectively, Table 2). No differences were found in presence of cysts when tumors were compared between the prior grading guidelines ($n = 149$) and the current system according to Silverberg ($n = 84$) ($p = 0.912$; solid: 16.1% versus 16.7%; cystic: 83.9% versus 83.3%, respectively). Variables significantly associated with the presence of cysts in EOC (largest tumor diameter, tumor grade and FIGO stage) were entered into logistic regression analysis. After univariate logistic regression, tumor size (OR = 1.169, 95% CI = 1.08-1.27, $p < 0.001$), tumor grade 1 or 2 (OR = 6.906; 95% CI = 2.31-20.66, $p = 0.001$), and FIGO stage I-IIa (OR = 5.312, 95% CI = 1.81-15.57, $p = 0.002$) were significantly associated with the presence of cysts (Table 2). The analysis was concluded by performing a multivariate logistic regression on the factors that showed a significant relation with cystic EOC prevalence.

According to this analysis, size of the tumor and tumor grade were independently associated with the presence of cysts in EOC (multivariate regression, Table 2). FIGO stage was not independently associated with the presence of cysts in EOC by multivariate analysis. Tumor grade 1 and 2 appeared to be most strongly related to the presence of cystic EOC (adjusted OR = 4.234, $p = 0.013$), followed by tumor size (adjusted OR = 1.192, $p = 0.002$).

Discussion

It has been generally accepted that most EOCs contain one or more cysts. However, the exact prevalence of cystic EOC has never been documented. Also, it has not been clarified which clinicopathological factors are related to the presence of cysts in EOC. These epidemiologic data are important to determine in which way ovarian cyst fluid is clinically available as a source of prognostic and predictive biomarkers.

The present study has demonstrated that 83.7% of the ovarian adenocarcinomas macroscopically contained a cystic component, which was mostly multilocular (61%). Moreover, the four most common histological EOC subtypes (serous, mucinous, endometrioid and clear cell), were cystic in more than 85% of the cases. Tumor grade and tumor size were independently related to the presence of cystic EOC. This means that cystic EOCs represent a subgroup of larger and more well-differentiated tumors. Histological subtype, FIGO stage, tumor necrosis, and age were not independently associated with the presence of cystic EOC.

We found that tumor grade and tumor size were independently associated with the presence of cysts in EOC. The significantly larger tumor size found in cystic EOC can almost certainly be explained by

Table 2. Prevalence of ovarian cystic EOC for clinicopathological characteristics using univariate and multivariate logistic regression analysis

	total *n*	cystic *n* (%)	EOC median (range)	*p*-value	OR	(95% CI)	*p*-value	Adj. OR	(95% CI)	*p*-value
Age (years)	233		57 (17-88)	0.354^	0.984	(0.96-1.01)	0.275			
FIGO stage	233			<0.001*						
I-IIa	79	75 (38.5)			5.312	(1.81-15.57)	0.002			
IIb-IV	154	120 (61.5)			1	(ref)				
Grade	195			<0.001*						
1+2	89	30 (51.5)			6.906	(2.31-20.66)	0.001	4.234	(1.36-13.22)	0.013
3	106	80 (48.5)			1	(ref)		1	(ref)	
Histology	194			0.754*						
serous	97	83 (49.7)			1	(ref)				
mucinous	28	26 (15.6)			2.193	(0.47-10.29)	0.319			
endometrioid	36	31 (18.6)			1.046	(0.35-3.15)	0.937			
clear cell	14	12 (7.2)			1.012	(0.20-5.02)	0.988			
mixed	19	15 (9.0)			0.633	(0.18-2.19)	0.469			
Laterality	233			0.118*						
unilateral	119	104 (53.3)			1.752	(0.86-3.56)	0.121			
bilateral	114	91 (46.7)			1	(ref)				
Tumor necrosis	233			0.840*						
yes	120	101 (51.8)			1.074	(0.54-2.15)	0.840			
no	113	94 (48.2)			1	(ref)				
Tumor size (cm)	220		12 (2-44)	<0.001^	1.169	(1.08-1.27)	<0.001	1.192	(1.07-1.33)	0.002

*Sample sizes change because of missing values in the pathological reports. n = number; CI = Confidence Interval; P = p value; OR = Odds Ratio; ^ Mann Whitney; * Chi-square; ref = reference*

the large volume of cyst fluid that is usually present in these tumors.[4] Interestingly, histological subtype was not an independent variable in multivariate analysis for the presence of cysts even though mucinous tumors are known to have the largest diameter of all histological subtypes due to sometimes enormous amounts of cyst fluid.[4] Although FIGO stage was significantly associated with cystic EOC in univariate analysis, no independent relationship was found in multivariate analysis. Therefore, we conclude that cystic EOCs are larger and less poorly differentiated than solid EOCs. As not any of the solid tumors in our study was well-differentiated, statistical analysis had to be performed with grade 1 and 2 versus grade 3 instead of the common classification of grade 1 versus grade 2 and 3 (low-grade versus high-grade). This means that if low-grade (grade 1) and high-grade (2 and 3) tumors would have been compared, a stronger relationship between grade and presence of cysts in EOC had most likely been found. From a clinical point of view grading is especially important for the prognosis and treatment of patients with stage I EOC. It has been shown that patients with stage Ia-Ib and grade 1 tumors face a good prognosis and do not need chemotherapeutic treatment after surgery.[13] Kang *et al.* recently demonstrated that patients with stage Ia-Ib grade 3 tumors, patients with Ic tumors and patients with clear cell tumors, were of high risk compared to the other patients with stage I EOC.[14] In addition, in another study, the 5-year survival of patients with stage Ia and grade 1 or 2 was found to be more than 90%.[15] These data emphasize the clinical significance of the unification of grade 1 and 2 versus grade 3. The evident relationship between the presence of cysts and differentiation grade in the present study is also interesting in terms of the popular theory that claims that EOC arises by two different pathways of carcinogenesis.[16] The type I EOCs, which are classified as low-grade tumors, are thought to arise from benign or borderline ovarian tumors and develop slowly. These benign and borderline tumors are well known for their cystic nature.[4] In contrast, the type II EOCs, which are classified as high-grade tumors, are thought to arise de novo and progress far more rapidly.[16]

Epidemiologic data from our study population regarding age, FIGO stage, tumor grade and histopathological subtypes are consistent with the distribution found in previous literature.[4,17,18] However, compared to most other epidemiological overviews, the present study contained somewhat less serous type carcinomas. This is probably due to the fact that we included a subtype of adenocarcinoma NOS (not otherwise specified) in our study. This group of unspecified carcinomas probably consisted for a large part of serous carcinomas, because the remaining histological subtypes correspond well to previous found rates.[4,17,19] Moreover, our findings regarding histological subtypes are comparable to the series of Vernooij *et al.* [18] who also added a group of adenocarcinoma NOS to their classification of histopathological subtypes which resulted in 38% serous EOCs.

The present study might have been biased by the retrospective analysis of the pathology reports, because not always all pathological features were mentioned (differentiation grade and histology) and inter-observer variation might have occurred. In addition, two different grading systems were used during the time-interval of this study. The system used in the past was based on cytological atypia and architectural pattern. Recently, however, the grading system of Silverberg [11] has been introduced. Using this system, besides atypia and architectural pattern, mitotic activity has to be scored as well.

However, no differences in presence of cysts in EOC were found when results were compared between the prior grading system and the new grading system according to Silverberg.

To the best of our knowledge, we report the first structured study in which the prevalence of cysts in EOC was investigated. Besides the fact that these data have never been documented and might be of interest for pathological and epidemiological statistics, the present study also determined the factors related to the presence of cystic EOC.

Acknowledgements

The authors thank Dr. Jan Hendriks for his statistical help in this study.

References

1. Colombo N, Van Gorp T, Parma G et al.. Ovarian cancer. Crit Rev Oncol Hematol 2006;60:159-79.
2. Tingulstad S, Skjeldestad FE, Hagen B. The effect of centralization of primary surgery on survival in ovarian cancer patients. Obstet Gynecol 2003;102:499-505.
3. Howells RE, Dhar KK, Hoban PR et al. Association between glutathione-S-transferase GSTP1 genotypes, GSTP1 over-expression, and outcome in epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:242-50.
4. Kurman RJ. Blaustein's pathology of the female genital tract, 5th ed. New York: Springer-Verlag;2002.
5. Bar JK, Harlozinska A, Sobanska E et al. Relation between ovarian carcinoma-associated antigens in tumor tissue and detached cyst fluid cells of patients with ovarian neoplasms. Tumori 1994;80:50-5.
6. Boss EA, Massuger LF, Thomas CM et al. Vascular endothelial growth factor in ovarian cyst fluid. Cancer 2001;91:371-7.
7. Kolwijck E, Zusterzeel PL, Roelofs HM et al. GSTP1-1 in ovarian cyst fluid and disease outcome of patients with ovarian cancer. Cancer Epidemiol Biomarkers Prev 2009; 18:2176-81.
8. Seidman JD, Soslow RA, Vang R et al. Borderline ovarian tumors: diverse contemporary viewpoints on terminology and diagnostic criteria with illustrative images. Hum Pathol 2004;35:918-33.
9. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
10. Changes in definitions of clinical staging for carcinoma of the cervix and ovary: International Federation of Gynecology and Obstetrics. Am J Obstet Gynecol 1987;156:263-4.
11. Silverberg SG. Histopathologic grading of ovarian carcinoma: a review and proposal. Int J Gynecol Pathol 2000;19:7-15. Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
13. Eisenhauer EA, Gore M, Neijt JP. Ovarian cancer: should we be managing patients with good and bad prognostic factors in the same manner? Ann Oncol 1999;10 Suppl 1:9-15.
14. Kang WD, Choi HS, Kim SM. Value of serum CA125 levels in patients with high-risk, early stage epithelial ovarian cancer. Gynecol Oncol 2010;116:57-60.
15. Young RC, Walton LA, Ellenberg SS et al. Adjuvant therapy in stage I and stage II epithelial ovarian cancer. Results of two prospective randomized trials. N Engl J Med 1990;322:1021-7.
16. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
17. Naora H. The heterogeneity of epithelial ovarian cancers: reconciling old and new paradigms. Expert Rev Mol Med 2007;9:1-12.
18. Vernooij F, Heintz AP, Coebergh JW et al. Specialized and high-volume care leads to better outcomes of ovarian cancer treatment in The Netherlands. Gynecol Oncol 2009;112:455-61.
19. Heintz AP, Odicino F, Maisonneuve P et al. Carcinoma of the ovary. J Epidemiol Biostat 2001;6:107-38.

3

Prognostic value of CA 125 in ovarian cyst fluid of patients with epithelial ovarian cancer

Kolwijck E, Span PN, Thomas CMG, Bulten J, Sweep FCGJ, Massuger LFAG

Oncology Reports 2009;19:1335-8.

Abstract

Most ovarian tumors contain ovarian cyst fluid (oCF) which can be easily obtained during surgery. This is the first study that explored if CA 125 in oCF could be of prognostic value for patients with epithelial ovarian cancer (EOC). Of 54 patients with primary EOC, oCF and preoperative serum were collected and clinicopathological data were retrospectively obtained. CA 125 was measured with the commercially available CA 125 assay. CA 125 in oCF (n = 54, median: 55,500 U/ml, range: 590-10,200,000 U/ml) was always higher than in the corresponding serum (n = 51, median: 179 U/ml, range: 13-11,000 U/ml) (p <0.001) and values were moderately correlated (R = 0.337, p = 0.016). CA 125 in oCF was associated with histology (p <0.001) and tumor grade (p = 0.038). High levels of oCF CA 125 (> median) were significantly associated with a poor disease free survival (DFS) (log-rank p = 0.002, and p = 0.005 univariate Cox-regression). Other factors associated with a poor DFS in univariate analysis were advanced FIGO stage, suboptimal debulking (both p <0.001), high tumor grade (p = 0.025), serous histology (p = 0.003) and high serum (>median) CA 125 (p = 0.009). In multivariate analysis, only FIGO stage was of independent predictive value. These findings indicate that, although high levels of oCF CA 125 were significantly associated with a poor survival of EOC patients, CA 125 in oCF was not of independent predictive value and might therefore not be useful as a prognostic biomarker for EOC.

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancies in developed countries. Due to the absence of specific signs and symptoms in an early stage of disease, the majority of patients are diagnosed when the tumor has already metastasized to the upper abdomen with a subsequent 5-year overall survival of less than 20%.[1,2]

Prognostic biomarkers are gaining attention as they are able to predict survival and response to treatment of patients with EOC at time of diagnosis, and many gynecologic oncologists have emphasized the need for these markers to select patients for individually-based treatment.[3,4] Cancer Antigen (CA) 125 is the best known biomarker for EOC and the only biomarker that is used in daily practice worldwide. It was discovered by Bast *et al.* [5] in 1981 and has been used ever since. Clinically, post-treatment serum CA 125 has proven its value and is used for the follow-up of EOC patients.[4,6,7] However, the role of preoperative serum CA 125 as a prognostic marker for survival remains questionable. The majority of studies could not demonstrate a prognostic significance, [8-12] while other studies found that preoperative serum CA 125 correlated significantly with survival in univariate analysis, [3,13-16] although the prognostic effect was lost in multivariate analysis in most of these studies.[3,13,15]

EOC typically presents as a large cystic mass which contains variable amounts of fluid.[17] Ovarian cyst fluid (oCF) is encapsulated in the ovarian tumor and therefore, it represents a micro-environment which contains compounds directly released by the tumor tissue. Since preoperative punction of oCF might cause tumor spill, it is advised to obtain oCF only during or after surgery. Therefore, the clinical value of biomarkers in oCF is limited to prediction of survival and/or response to treatment of patients with EOC. A number of studies have compared oCF CA 125 levels between patients with benign and malignant ovarian tumors.[18-21] However, to our knowledge, no studies have been published investigating the prognostic value of oCF CA 125 for patients with EOC.

We performed the first study that explored the prognostic value of oCF CA 125 for the prediction of survival of patients with EOC. We also investigated the relation between oCF CA 125 levels and important clinicopathological parameters of patients with EOC.

Material and Methods

Patients

In the period between January 2000 and January 2008, oCF was collected from patients who underwent primary surgery for an ovarian tumor at the Radboud University Nijmegen Medical Centre (RUNMC) and stored at our cyst fluid biobank. During or after primary debulking or diagnostic surgery, oCF was collected when a cystic ovarian tumor was diagnosed as malignant by frozen section examination. Cyst fluid collection was performed during primary debulking or diagnostic surgery, never during interval debulking surgery. From 68 patients, oCF was obtained. Four patients were excluded because final paraffin examination revealed that the origin of the primary tumor was not ovarian derived. Another 10 patients were excluded because the ovarian malignancy was non-epithelial. Included in this study were patients with histologically proven primary EOC (n = 54 patients). Informed consent was obtained from all participants.

Sample collection

Cyst fluid samples of the ovarian tumors were collected by aseptic fine-needle aspiration at the Department of Pathology, immediately after surgical removal of the tumor. Preoperative serum samples of patients with EOC (n = 51) were taken within one week prior to surgery. From 3 patients, preoperative serum could not be obtained. After cooled transport to our laboratory, the oCF and blood samples were centrifuged at 3000 x g for 10 min and the supernatant was aliquoted and stored at -35 °C until use.

CA 125 measurements

CA 125 measurements in oCF and serum were performed with the commercially available CA 125 assay (AxSYM, Abbott Laboratories, Chicago, Illinois, USA) with a minimum detectable concentration of 2 U/ml. This assay is based on Microparticle Enzyme Immunoassay technology and uses the murine monoclonal antibody OC 125 as catching antibody. Determination of CA 125 was carried out without prior knowledge of the patients' clinical outcome.

Clinicopathological characteristics

Complete pathological reports of all EOC patients were reviewed for correct histopathological diagnosis (primary ovarian carcinoma, histological tumor subtype and tumor grade) by one pathologist (JB), specialized in gynecological oncology. Surgery and FIGO staging was always performed by a gynecologist specialized in oncology from the RUMCN. From the medical records, the following clinicopathological

characteristics were retrospectively retrieved: age at diagnosis, International Federation of Gynecology and Obstetrics (FIGO) stage, residual tumor after surgery, tumor recurrence, and date of death. For some patients, information about one or more clinicopathological parameters could not be retrieved (Table 1). Staging was performed according to the criteria of the FIGO.[22] Patients were divided into two groups: patients with early stage (FIGO stage Ia-IIa) and patients with advanced stage (FIGO stage IIb-IV) EOC.[23] Histopathological tumor type and grade were classified according to the World Health Organization criteria.[24] Existence of residual tumor after surgery was divided into two groups by the criteria for optimal cytoreduction [25]; patients who had no residual disease or a residual lesion less than 1 cm, and patients with a suboptimal debulking of one or more centimeters residual tumor. Recurrence of disease was defined as a measurable lesion during follow-up evaluated by computed tomography, magnetic resonance imaging and/or ultrasonography.

Statistical analyses

Statistical analyses were carried out using SPSS 16.0.2 software (SPSS Benelux BV, Gorinchem, the Netherlands). Correlations between oCF CA 125 and serum CA 125 values were analyzed by Spearman's rank correlation testing. Normality of value distributions was assessed using Kolmogorov-Smirnov testing, and obtained by normal-log (Ln) transformation of both serum and oCF CA125 levels. Differences in levels of CA125 in serum or oCF samples from EOC patients categorized by clinicopathological characteristics, as grouping variables, were assessed with parametric Student's t-tests or ANOVA with post-hoc Tukeys HSD tests where appropriate. The median of CA125 values (for both serum and oCF CA 125) was used as the cut off value for dichotomizing oCF and serum CA 125. Disease-free survival (DFS) time was used as follow-up endpoint and was defined as the time interval from the date of surgery to the date of recurrence or death, and censored at last follow-up. Survival curves were generated using the method of Kaplan and Meyer. Equality of survival distributions was tested using log-rank testing. The hazard ratio's (HR) with the corresponding 95% confidence interval (CI) are presented. Multivariate Cox regression analysis was performed to find the clinicopathological parameters that independently contribute to a decreased time to recurrence. *P*-values of < 0.05 were regarded as statistically significant.

Results

Study population

The patient's age at diagnosis ranged from 32 to 89 years, with a median age of 56 years. Numbers of patients in various clinicopathological subgroups are listed in Table 1.

Table 1. Association of CA125 (U/ml) in oCF and serum and clinicopathologically defined patient categories

Category	*n* (%)	CA125 in oCF (U/ml) median (IQR)	*P**	CA125 in serum (U/ml) median (IQR)	*P**
Age			0.905		0.858
≤ 56 years	27 (50)	68,000 (111,300)		138 (729)	
> 56 years	27 (50)	55,000 (116,100)		195 (596)	
Histology			<0.001	387 (1,187)	0.014
serous	24 (44)	79,500 (152,250)	a	59 (111)	a
mucinous	14 (26)	5,250 (10,200)	b	387 (647)	b
endometrioid	8 (15)	109,000 (185,000)	a	176 (1,218)	
other	8 (15)	78,000 (198,325)	a		
FIGO stage			0.076	66 (94)	<0.001
Ia-IIa	19 (35)	20,500 (87,150)		387 (1,374)	
IIb-IV	34 (63)	71,000 (137,300)			
unknown	1 (2)				
Tumor grade			0.038		0.007
1	14 (26)	5,950 (21,275)	a	55 (117)	a
2	16 (30)	21,500 (95,675)		321 (738)	
3	20 (37)	89,000 (123,250)	b	225 (1,492)	b
unknown	4 (7)	322,500 (845,500)		300 (650)	
Residual disease			0.188		0.109
< 1 cm	43 (80)	24,000 (114,200)		153 (492)	
≥ 1 cm	11 (20)	85,000 (164,000)		198 (1,186)	

**p-value of student's t-test or ANOVA of Ln normalized values; a and b denote categories that differ significantly in post-hoc Tukey's HSD analysis; IQR = interquartile range*

Serous carcinomas were found in 24 patients (44%), 14 patients had mucinous carcinomas (26%), and 8 patients had endometrioid type carcinomas (15%). Of the remaining 8 patients in the category "other", 2 patients had clear cell carcinomas (4%), 1 patient had an undifferentiated adenocarcinoma (2%) and 5 patients had ovarian adenocarcinomas not otherwise specified (NOS) (9%). Of the 19 patients with early FIGO stage, 11 had Ia, 1 had Ib and 7 patients had FIGO Ic. Of the 34 patients with advanced FIGO stage, 2 had IIb, 2 had IIc, 3 had IIIa, 8 had IIIb, 13 had IIIc, and 6 patients had FIGO stage IV. For 1 patient, FIGO stage could not be obtained. Of the total number of 54 patients, 31 (58%) were treated with 6 courses of adjuvant platinum-based chemotherapy after primary surgery, 5 (9%) patients received

neo-adjuvant chemotherapy before interval surgery, and 18 (33%) patients did not receive chemotherapy at all. Median follow-up time was 42 months (range: 10-120 months). None of the patients was lost to follow-up. DFS ranged from 1 to 120 months, with a median of 16 months. Within the follow-up period, 25 patients (46%) showed recurrent disease and 17 patients died (32%).

CA 125 levels in oCF and preoperative serum

Values for CA125 in oCF ($n = 54$) of patients with EOC ranged from 590 up to 10,200,000 (median 55,500) U/ml. Preoperative serum CA125 values could be obtained from 51 patients, and ranged from 13 to 11,000 (median 179) U/ml. CA125 in oCF was always higher than in serum (p <0.001, Student's t-test, Figure 1A). Serum and oCF CA 125 within single patients were moderately correlated (R = 0.337, p = 0.016, Spearman's correlation test, Figure 1B).

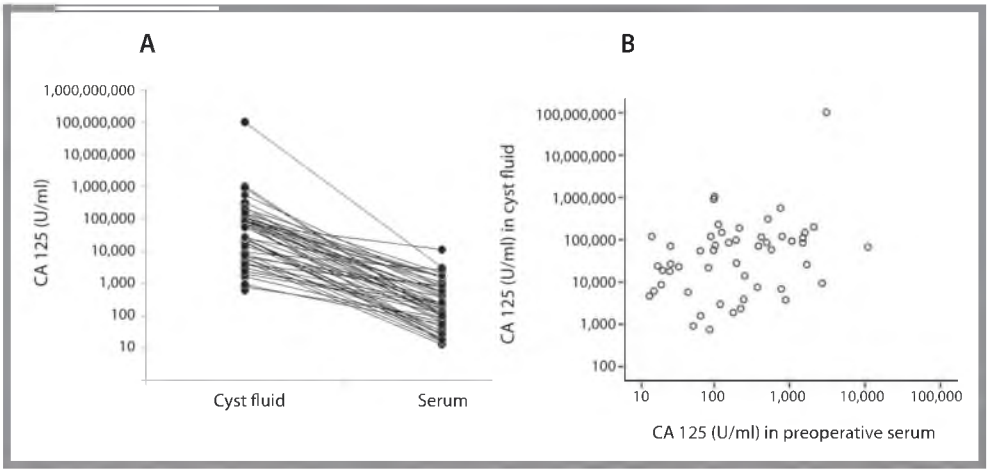

Figure 1. (A) Serum and OCF levels of CA 125 in patients with EOC. CA 125 in oCF is always higher than in serum (p <0.001, student's t-test). (B) CA 125 levels between oCF and serum only moderately correlate (p= 0.016, R = 0.337, Spearman's correlation test).

Differences between histopathological patient categories

Table 1 lists the association of CA 125 in oCF and CA 125 in preoperative serum with different clinicopathological patient characteristics. CA125 in oCF was significantly lower in patients with mucinous tumors compared to patients with other histological subtypes (p <0.001), and was significantly lower in patients with well differentiated tumors compared to patients with poorly differentiated tumors (p = 0.038). Serum CA125 was also lower in patients with mucinous tumors (p = 0.014) compared to patients with serous tumors and correlated with tumor grade (p = 0.007) as well. In addition, serum CA 125 was significantly higher in patients with advanced FIGO stage (p <0.001) compared to patients with early FIGO stage.

Table 2. Univariate Cox regression analysis of clinicopathological parameters and oCF CA 125 as prognostic factors for DFS in patients with EOC

Variable	DFS HR (95% CI)	*p*-value
Age		0.493
≤ 56 years	(Reference)	
> 56 years	1.30 (0.61-2.79)	
Histology		<0.001
serous	10.96 (2.28-52.67)	0.003
mucinous	(Reference)	
endometrioid	1.30 (0.12-14.72)	0.834
other	11.17 (2.25-55.51)	0.003
FIGO stage		<0.001
Ia-IIa	(Reference)	
IIb-IV	31.12 (4.11-235)	
Tumor grade		0.025
1	(Reference)	
2+3	60.61 (1.68-2191)	
Residual disease		<0.001
< 1 cm	(Reference)	
≥ 1 cm	5.82 (2.44-13.93)	
CA125 serum		0.009
< median	(Reference)	
> median	3.11 (1.29-7.51)	
CA125 oCF		0.005
< median	(Reference)	
> median	3.40 (1.45-7.97)	

Survival analysis

Figure 2 shows the Kaplan-Meier DFS curves of the patients with oCF CA 125 values above the median of 55,500 U/ml and below 55,500 U/ml (Figure 2). High levels of oCF CA125 were significantly associated with a poor DFS ($p = 0.002$, log-rank test). Table 2 shows the hazard ratio with 95% CI, using the univariate proportional hazard model. For the variables "FIGO stage", "Tumor grade", and "Serum CA 125", one, four and three cases were missing, respectively. Advanced FIGO stage, serous histology, high tumor grade, a suboptimal debulking, high preoperative serum CA 125 and high oCF CA 125 levels were significant predictors of a poor DFS. However, using the multivariate proportional hazard model with selection procedure, FIGO stage was the only independent predictor that had impact on DFS (HR = 87.1; 95% CI: 1.11-6845; $p = 0.045$). As a result, the other factors did not contribute additionally to FIGO stage to predict the time to recurrence.

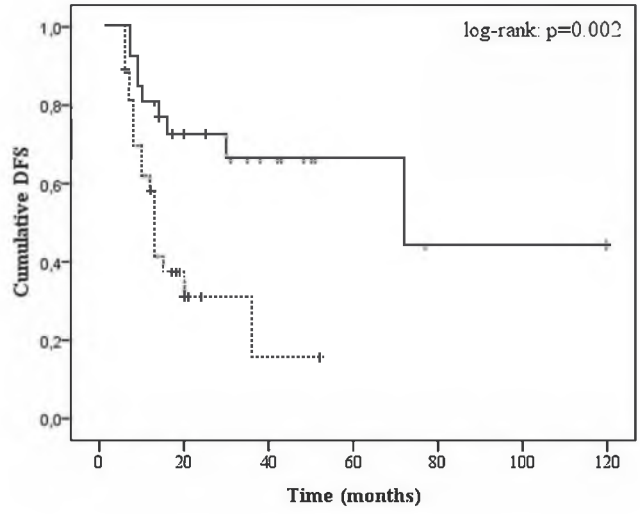

Figure 2. Kaplan-Meyer estimates of DFS of patients with EOC (n = 54). Low oCF CA 125 values (n = 27; < median) and high oCF CA 125 values (n = 27; > median) are represented by the solid and broken line, respectively. Vertical bars indicate patients with censored data.

Discussion

This is the first study that explored if CA 125 in oCF could be of prognostic value for patients with EOC. Ovarian cyst fluid is in close contact with the ovarian tumor epithelium and can be easily obtained by fine-needle aspiration after removal of the ovarian tumor at primary surgery. CA 125 measurement in oCF is possible with the standard CA 125 assay and therefore, seems to be applicable in clinical practice. In the present study high levels of oCF CA 125 were significantly associated with a poor prognosis of EOC patients. In addition, CA 125 in oCF did not show any relationship with FIGO stage and/or debulking status, which have shown to be the most important prognostic factors for EOC.[3] These findings did suggest an independent prognostic role for oCF CA 125. However, multivariate survival analysis showed that FIGO stage was the only independent predictor of the time to recurrence. Therefore, we concluded that CA 125 in oCF was not of additional value as a predictive marker for patients with EOC.

Prognostic markers are of major importance for the clinical management of patients with EOC. Therefore, identification of the group of patients with a poor disease outcome should occur as early

as possible. Currently, serum CA 125 is used during chemotherapeutic treatment to monitor response. It has also proven its value to predict recurrence and disease progression after primary therapy.[4,6,7] However, the role of preoperative serum CA 125 as a prognostic tumor marker remains unclear as most studies could not show a significantly independent predictive effect.[3,4,9,11-14,26] We found that preoperative serum CA 125 was a significant predictor of DFS in univariate analysis. However, it correlated strongly with FIGO stage, which also has been demonstrated in previous studies.[3,9,14,27] Therefore, similar to oCF CA 125, preoperative serum CA 125 in the present study was not of additional prognostic value either.

Only two reports have evaluated the prognostic value of CA 125 expression in ovarian tissue. De la Cuesta *et al.* [28] found that patients with a high expression of the analyte had a significantly poorer prognosis compared to patients with no expression. More recently, Høgdall *et al.* [8] reported for a large series of ovarian tissue samples that increased tissue expression of CA 125, as determined by immunohistochemistry, was related to poor prognosis, but only in the group of patients with advanced stage EOC. In the present study, we also found that patients with high levels of CA 125 in oCF had a significantly poorer prognosis and also that these oCF CA 125 levels were not related to FIGO stage. Therefore, we believe that oCF CA 125 might give a representative reflection of the tumor tissue biology.

The present study demonstrated that oCF levels varied between different histopathological subtypes. More specifically, mucinous tumors contained significantly lower amounts of oCF CA 125 than all other subtypes. It has been shown previously that the proportion of CA 125- producing cells differs between EOCs due to its well-known variety of histopathological subtypes, [8,20,29] of which mucinous tumors produce less CA 125.[8] In their series of 584 ovarian cancer tissue samples, Høgdall *et al.* found CA 125 expression in 12%, 40%, 65% and 85% of mucinous, clear cell, endometrioid and serous samples, respectively.[8]

The mechanism of CA 125 release from epithelial cells of the ovary into oCF or serum has been poorly understood. Jacobs *et al.* [30] reported that elevation of preoperative serum CA 125 is not always related to the production of the CA 125 analyte in ovarian tissue. In FIGO stage I EOC, when the tumor is still confined to the ovaries, the phenomenon of high tissue CA 125 levels in association with normal serum CA 125 levels has been demonstrated.[30] A similar observation was found in patients with benign ovarian tumors, where high CA 125 levels in oCF were accompanied by low serum CA 125 levels.[20] This might suggest that CA 125 is released into the serum only in case of extensive infiltrative growth and wide spread malignancy, probably because of loss of permeability of the basement membrane and the substantial contribution of metastatic lesions to the CA 125 production.[20] Therefore, preoperative serum CA 125 seems to be a biomarker for disease progression, which might explain its high correlation with FIGO stage of disease. On the other hand, CA 125 in oCF seems to result from a more continuous production brought about by the malignant ovarian epithelium. In the present study, oCF CA 125 was only correlated with histology and grade, which are pathological characteristics of the tumor tissue itself

This study was performed to explore the predictive possibilities of analysis of CA 125 in cyst fluid of patients with EOC. We realize that our retrospective study comprised a relatively small and heterogeneous cohort of patients with EOC. Although we found that high levels of CA 125 were significantly associated with a poor DFS in univariate analysis, a prospective study with more uniform samples and increased power is required to replicate our findings. On base of the results of the present explorative study, we suppose that analyzing CA 125 in oCF has no additional value in prediction of recurrence for patients with EOC.

References

1. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96.
2. Colombo N, Van Gorp T, Parma G et al. Ovarian cancer. Crit Rev Oncol Hematol 2006;60:159-79.
3. Tingulstad S, Skjeldestad FE, Halvorsen TB et al. Survival and prognostic factors in patients with ovarian cancer. Obstet Gynecol 2003;101:885-91.
4. Høgdall E. Cancer antigen 125 and prognosis. Curr Opin Obstet Gynecol 2008;20:4-8.
5. Bast RC, Jr., Feeney M, Lazarus H et al. Reactivity of a monoclonal antibody with human ovarian carcinoma. J Clin Invest 1981;68:1331-7.
6. Yedema CA, Kenemans P, Voorhorst F et al. CA 125 half-life in ovarian cancer: a multivariate survival analysis. Br J Cancer 1993;67:1361-7.
7. Duffy MJ, Bonfrer JM, Kulpa J et al. CA125 in ovarian cancer: European Group on Tumor Markers guidelines for clinical use. Int J Gynecol Cancer 2005;15:679-91.
8. Høgdall EV, Christensen L, Kjaer SK et al. CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study. Gynecol Oncol 2007;104:508-15.
9. Rossi AC, Di Vagno G, Cormio G et al. A retrospective study of preoperative CA 125 levels in 82 patients with ovarian cancer. Arch Gynecol Obstet 2004;269:263-5.
10. Gadducci A, Zola P, Landoni F et al. Serum half-life of CA 125 during early chemotherapy as an independent prognostic variable for patients with advanced epithelial ovarian cancer: results of a multicentric Italian study. Gynecol Oncol 1995;58:42-7.
11. Cruickshank DJ, Fullerton WT, Klopper A. The clinical significance of pre-operative serum CA 125 in ovarian cancer. Br J Obstet Gynaecol 1987;94:692-5.
12. Sevelda P, Schemper M, Spona J. CA 125 as an independent prognostic factor for survival in patients with epithelial ovarian cancer. Am J Obstet Gynecol 1989;161:1213-6.
13. Geisler JP, Miller GA, Lee TH et al. Relationship of preoperative serum CA-125 to survival in epithelial ovarian carcinoma. J Reprod Med 1996;41:140-2.
14. Makar AP, Kristensen GB, Kaern J et al. Prognostic value of pre- and postoperative serum CA 125 levels in ovarian cancer: new aspects and multivariate analysis. Obstet Gynecol 1992;79:1002-10.
15. Nagele F, Petru E, Medl M et al. Preoperative CA 125: an independent prognostic factor in patients with stage I epithelial ovarian cancer. Obstet Gynecol 1995;86:259-64.
16. Cooper BC, Sood AK, Davis CS et al. Preoperative CA 125 levels: an independent prognostic factor for epithelial ovarian cancer. Obstet Gynecol 2002;100:59-64.
17. Kurman RJ. Blaustein's pathology of the female genital tract, 5th ed. New York: Springer-Verlag;2002.
18. Menczer J, Ben-Baruch G, Moran O et al. Cyst fluid CA 125 levels in ovarian epithelial neoplasms. Obstet Gynecol 1993;81:25-8.
19. Sedlaczek P, Frydecka I, Gabrys M et al. Comparative analysis of CA125, tissue polypeptide specific antigen, and soluble interleukin-2 receptor alpha levels in sera, cyst, and ascitic fluids from patients with ovarian carcinoma. Cancer 2002;95:1886-93.
20. Fleuren GJ, Nap M, Aalders JG et al. Explanation of the limited correlation between tumor CA 125 content and serum CA 125 antigen levels in patients with ovarian tumors. Cancer 1987;60:2437-42.
21. Candido Dos Reis FJ, Moreira de AJ, Bighetti S. CA 125 and vascular endothelial growth factor in the differential diagnosis of epithelial ovarian tumors. Gynecol Obstet Invest 2002;54:132-6.
22. Pecorelli S, Benedet JL, Creasman WT et al. FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
23. Vergote I, Trimbos BJ. Treatment of patients with early epithelial ovarian cancer. Curr Opin Oncol 2003;15:452-5.

24. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
25. Chi DS, Eisenhauer EL, Lang J et al. What is the optimal goal of primary cytoreductive surgery for bulky stage IIIC epithelial ovarian carcinoma (EOC)? Gynecol Oncol 2006;103:559-64.
26. Gadducci A, Cosio S, Tana R et al. Serum and tissue biomarkers as predictive and prognostic variables in epithelial ovarian cancer. Crit Rev Oncol Hematol 2009;69:12-27.
27. Clark TG, Stewart ME, Altman DG et al. A prognostic model for ovarian cancer. Br J Cancer 2001;85:944-52.
28. De la Cuesta R, Maestro ML, Solana J et al. Tissue quantification of CA 125 in epithelial ovarian cancer. Int J Biol Markers 1999;14:106-14.
29. Kabawat SE, Bast RC, Welch WR et al. Immunopathologic characterization of a monoclonal antibody that recognizes common surface antigens of human ovarian tumors of serous, endometrioid, and clear cell types. Am J Clin Pathol 1983;79:98-104.
30. Jacobs I, Bast RC Jr. The CA 125 tumour-associated antigen: a review of the literature. Hum Reprod 1989;4:1-12.

4

Cathepsins B, L and cystatin C in cyst fluid of ovarian tumors

Kolwijck E, Massuger LFAG, Thomas CMG, Span PN, Krasovec M, Kos J, Sweep FCGJ

Journal of Cancer Research and Clinical Oncology, 2010;136(5):771-8

Abstract

In cancer, an extracellular and membrane bound localization of cathepsins contributes to the invasion of tumor cells at the basement membrane. This is the first study that explored levels of cathepsins B (CatB), L (CatL) and their inhibitor cystatin C (CysC) in the ovarian cyst fluid (oCF) of ovarian tumors (n = 110). Ovarian cyst fluid contained considerable amounts of CatB, CatL and CysC. Remarkable differences in CatB and CatL and cystatin C oCF levels were found between different histopathological tumor subtypes. Levels of CatB and CysC were significantly higher in oCF of malignant serous tumors compared to those found in benign serous tumors (p = 0.010 and p = 0.001, respectively), whereas levels of CatL were significantly higher in oCF of malignant mucinous tumors compared to those found in benign mucinous tumors (p = 0.035). CatB and CysC showed a strong correlation in the group of patients with malignant serous tumors (p <0.001; R = 0.921) suggesting that the increase in CatB might be balanced by a corresponding increase in CysC. Further studies are warranted to investigate cathepsins as possible prognostic biomarkers for the aggressiveness of ovarian cancer.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy worldwide. Because of the absence of early symptoms, approximately 70% of the patients is diagnosed with International Federation of Gynecologists and Obstetricians (FIGO) stage III or IV, when tumor cells already have metastasized to the upper abdomen.[1] Compared to other types of cancer, insight in the carcinogenesis and progression of ovarian cancer is limited by the lack of a well-defined precursor lesion and by the lack of data from early stage cancers.[2]

In neoplastic transformation, the epithelial surface first becomes multicell layered. Tumor cells then degrade the basement membrane and invade the stroma or become detached from the primary tumors and metastasize.[3-6] It is now widely believed that the degradation of the basement membrane is catalyzed by proteolytic enzymes.[7-9] Among these enzymes are the lysosomal cysteine proteinases or cathepsins, which have an important physiological function in regulation of intracellular protein metabolism. The human family of cathepsins has 11 members (cathepsin B, C, F, H, K, L, O, S, V, W and X), which share a conserved active site that is formed by cysteine, histidine and asparagine residues. In cancer tissue, an increased expression of cathepsins has been described and an extracellular and membrane bound localization of particularly cathepsin B (CatB) and cathepsin L (CatL) has been demonstrated, which might contribute to the invasion of tumor cells at the basement membranes. [8,10,11]

Numerous reports have now been published investigating cancer tissue expression of cathepsins. [9,12,13] Although quantitation of cathepsins in extracellular fluid has several advantages compared to measurement in tissue, reports investigating extracellular cathepsin levels are rather limited.[10] Higher levels of CatB and CatL were reported in sera of patients with several types of cancer, [14-20] including ovarian cancer, [21,22] compared to serum levels in healthy subjects or patients with benign lesions. The possibility of measuring cathepsins in blood increases its clinical value as a biomarker but reported levels in the serum are significantly lower than levels in tumor cells of cancer patients.[10] Lah *et al*. [23] have shown that CatB was present in ascites of patients with ovarian carcinoma. Moreover, they showed that extracellularly released CatB was inhibited most strongly by cystatin C (CysC), which is an extracellular cysteine proteinase inhibitor.[23] Recent studies on tissue of breast and ovarian cancer, and melanoma showed that an imbalance of CatB/CysC was correlated with a more aggressive tumor behavior.[15,24,25]

Most ovarian tumors exhibit, sometimes multiple, cysts which can be large. The ovarian cyst fluid (oCF) is in close contact with the tumor tissue. To our knowledge, no studies have been performed to investigate the levels of cathepsins or their endogenous inhibitors in this oCF. In the present study, we explored CatB, CatL as well as CysC levels in oCF of patients with benign, borderline and malignant ovarian tumors.

Material and methods

Patients and cyst fluid collection

From our biobank containing oCF samples of patients with ovarian tumors who underwent primary surgery at the Radboud University Nijmegen Medical Center in the period between 1988 and 1998, all 110 oCF samples were retrieved for analysis. Samples in this biobank, were collected by aseptic fine needle aspiration at the Department of Pathology immediately after surgical removal of the tumor. After cooled transport to the laboratory, the oCF samples were centrifuged at 3000 x *g* for 10 minutes and the supernatant was stored at -35 °C in small portions until use. Histopathological diagnosis was performed by a pathologist specialized in gynecology and revealed 74 benign, 26 malignant and 10 borderline epithelial ovarian tumors. Informed consent was obtained from all participants.

ELISA procedures

For the quantitative analysis of CatB and CatL and CysC in oCF, commercially available specific ELISAs (sandwich ELISAs, Krka d.d., Novo mesto, Slovenia) were used, as developed by Kos *et al.*[15] The components were purified and characterized, and the tests were optimized and performed as described.[26] To define the optimal dilution, oCF samples were serially diluted to the levels encompassing the range of the assays. The linearity of the response was evaluated by comparing the measured values with the calibration curves. The recovery was tested by the addition of different amounts of the antigen to the samples with known antigen concentration and varied from 88 to 108%, comparing expected versus observed concentrations. The intra-assay coefficient of variance varied from 7.6 to 12.7 %. CF samples were diluted 1:4 for CatB and CatL and 1:100 for CysC prior to being applied to the wells of microtiter plate. A microplate reader (SLT Rainbow, Salzburg, Austria) was used to measure absorbance. Determination of the levels of cathepsins and CysC was carried out without prior knowledge of the histological or clinical outcome.

Parameters for aggressiveness of the tumor

From the pathological and medical reports of the patients with EOC, the following parameters were retrieved: histopathologic subtype and histopathologic grade, age at diagnosis and FIGO stage of disease. Histopathologic subtype and tumor grade were classified according to the World Health Organization criteria.[27] Staging was performed using the FIGO criteria.[28] In case of uncertainty about the primary location of the tumor, extensive immunohistochemical staining was performed. Only patients with confirmed primary EOC were included.

Statistical analyses

Values are shown as median with range (ng/ml). Normality of distributions was analyzed by Kolmogorov-Smirnov testing. Data regarding CatB, CatL and CysC could be normalized by Ln transformation. Differences between two groups were tested by Student's t-test, and for more than 2 groups by ANOVA and subsequent post hoc Tukey's HSD tests. Correlations were assessed by Pearson correlation tests of the Ln transformed values. A priori significance was set at a two-sided $p < 0.05$. All statistical analyses were performed using SPSS version 16.0.01 (SPSS Inc. Chicago IL, USA).

Results

Patients

The mean age at diagnosis was 40 years (SD ± 14) for the patients with benign ovarian tumors ($n = 74$), 42 years (SD ± 14) for the patients with borderline ovarian tumors ($n = 10$) and 57 years (SD ± 11) for the patients with EOC ($n = 26$). The mean age of patients with ovarian cancer was significantly higher than the mean age of patients with benign ($p < 0.001$, Student's t-test) and borderline tumors ($p = 0.009$, Student's t-test). No differences in mean age were found between patients with benign and borderline tumors ($p = 0.898$, Student's t-test).

Histopathological subtype

Table 1 shows the median (range) concentrations of CatB, CatL and CysC in ovarian oCF of patients with malignant, borderline and benign ovarian tumors, grouped by histological subtype. Within the benign group, oCF levels of CatL were significantly different between serous cystadenomas and mucinous cystadenomas ($p = 0.003$) and between endometriosis cysts and mucinous cystadenomas ($p < 0.001$). Although generally not significantly different from the other benign histological subtypes, the median concentrations of CatB and CatL in cystic endometriosis were remarkably high, and comparable to those found in oCF of malignant tumors. CysC concentrations were in the same range for all benign subclasses. Within the group of borderline and malignant tumors, oCF levels of CatB, CatL and CysC did not differ significantly between histopathological subtypes.

Table 1. Median levels (range) of cathepsin B, L and cystatin C (ng/ml) in cyst fluid of ovarian tumors (n = 110) grouped by histopathological subtype

Histology	*n* (%)	Cathepsin B	Cathepsin L	Cystatin C
Benign				
serous cystadenoma	29 (39%)	12 (1-269)	31 (9-121)[A]	572 (131-1,781)
mucinous cystadenoma	21 (28%)	16 (1-227)	11 (1-95)[A,B]	578 (39-9,984)
dermoid cyst	5 (7%)	11 (1-108)	10 (2-97)	618 (117-876)
cystic endometriosis	16 (22%)	71 (1-177)	69 (8-233)[B]	338 (92-1,931)
corpus luteum cyst	3 (4%)	18 (6-98)	15 (15-133)	644 (520-1,805)
Borderline				
serous borderline tumor	2 (20%)	14 (8-21)	28 (18-38)	1,449 (1125-1,772)
mucinous borderline tumor	6 (60%)	31 (2-118)	75 (8-166)	697 (474-3,897)
mixed type borderline tumor*	1 (10%)	227	92	340
unknown	1 (10%)			
Malignant				
serous carcinoma	10 (38%)	65 (3-269)	61 (8-163)	1,443 (448-5,972)
mucinous carcinoma	8 (31%)	33 (5-269)	31 (13-187)	959 (399-9,984)
endometrioid carcinoma	6 (23%)	85 (1-269)	71 (14-181)	1,200 (64-2,907)
mixed type carcinoma*	1 (4%)	95	67	1,232
undifferentiated carcinoma*	1 (4%)	110	75	109

** single concentration is listed. Statistical significance according to ANOVA ($p = 0.001$) with Tukey's HSD Post-hoc test of values normalized after Ln transformation; [A] $p = 0.003$ for mucinous cystadenomas compared to serous cystadenomas; [B] $p < 0.001$ for mucinous cystadenomas compared to cystic endometriosis*

Table 2 and Figure 1 show median (range) oCF levels of CatB, CatL and CysC in benign (cystadenoma), borderline and malignant (cystadenocarcinoma) ovarian tumors according to the histopathological subtype. In serous tumors ($n = 41$), oCF levels of CatB were significantly higher for patients with malignant tumors compared to patients with benign tumors ($p = 0.010$), whereas CatL levels did not differ significantly within this histological subgroup. In contrast, in mucinous tumors ($n = 35$), oCF levels of CatL were significantly higher for patients with malignant tumors compared to patients with benign tumors ($p = 0.035$), whereas CatB levels did not differ within this histological subgroup. Ovarian cyst fluid levels of CysC only differed between patients with malignant and benign tumors in the subgroup of serous tumors ($p = 0.001$).

Parameters of aggressiveness of the tumor

Table 3 shows clinicopathological parameters of tumor aggressiveness and median (range) concentrations of CatB, CatL and CysC of the 26 patients with EOC. Since no differences in oCF levels of CatB, Cat L and CysC were found between serous, mucinous and endometrioid cystadenocarcinomas (Table 1), data of patients with EOC were merged. When patients were grouped according to FIGO stage of disease, tumor grading and presence of ascites, no significant differences in oCF levels of CatB, CatL and/or CysC were found.

Correlation between cathepsins and cystatin C

Figure 2 shows the scatter plots of the correlation between cathepsins and CysC for patients with serous (Figure 2A and 2B; $n = 41$) and mucinous (Figure 2C; $n = 35$) tumors. For serous tumors, oCF levels of CysC and CatB were significantly correlated ($p < 0.001$, $R = 0.652$, Figure 2A). The correlation between oCF levels of CysC and CatB was highest for the malignant group ($p < 0.001$, $R = 0.921$). For mucinous tumors, oCF levels of CysC and CatB did not show any relationship ($p = 0.923$, Figure not shown). Correlations between oCF levels of CysC and CatL were significant for both serous and mucinous tumors ($p = 0.001$, $R = 0.491$, Figure 2B and $p = 0.003$, $R = 0.481$, Figure 2C, respectively). For mucinous tumors, the correlation between oCF levels of CysC and CatL was highest for the benign group ($p < 0.001$, $R = 0.665$)

Discussion

In this study, we examined oCF levels of CatB, CatL, and their extracellular inhibitor CysC. In benign tumors, remarkable differences in oCF levels of CatB and CatL were found between different histopathological subtypes. For serous tumors, levels of CatB and CysC were significantly higher in the oCF of patients with EOC compared to oCF levels of patients with benign ovarian tumors. For mucinous tumors, levels of CatL were significantly higher in the oCF of patients with EOC compared to oCF levels of patients with benign ovarian tumors. Furthermore, correlations between cyst fluid levels of CatB and CysC and between CatL and CysC were found. Finally, we investigated the relationship between levels of CatB, CatL and CysC and parameters of tumor aggressiveness of EOC but could not find significant differences between the subgroups of patients.

We are the first to describe that oCF contains considerable amounts of CatB, CatL and CysC. In ascitic fluid of patients with ovarian cancer, Lah *et al.* [23] found CatB and a large pool of free CysC.

We showed that oCF levels of these enzymes were higher in ovarian cancer patients compared to patients with benign ovarian tumors. Serum studies in patients with other types of cancer have demonstrated similar results, as levels of CatB, CatL and CysC were found to be higher in serum of patients with cancer compared to serum levels of patients with benign tumors or healthy controls. [14-19,29-34] In serum of patients with ovarian cancer, Warwas *et al.* [22] found higher levels of CatB compared to levels in patients with benign ovarian tumors. On the other hand, Nishikawa *et al.* [24] could not observe a significant difference in serum CatB level between patients with benign ovarian tumors and EOC although they did find a significantly higher level of CysC in the serum of patients with ovarian cancer. Only one study has been published investigating CatL in the serum of patients with ovarian tumors.[21] The authors found that serum levels of CatL were elevated in 8/10 patients with ovarian cancer compared to 2/10 patients with benign ovarian tumors.

Table 2. Median levels (range) of CatB, CatL and CysC (ng/ml) in oCF ($n = 67$) of benign, borderline and malignant tumors grouped by serous and mucinous subtype

Subtype	*n* (%)	Cathepsin B	*p*-value*	Cathepsin L	*p*-value*	Cathepsin C	*p*-value*
Serous	41				0.131		0.001
serous cystadenoma	29 (71%)	12 (1-269)[A]	0.031	31 (9-121)		572 (131-1,780)[B]	
serous borderline tumor	2 (5%)	14 (8-21)		28 (18-38)		1,449 (1,125-1,772)	
serous cystadenocarcinoma	10 (24%)	65 (3-269)[A]		61 (8-163)		1,443 (448-5,972)[B]	
Mucinous	35				0.017		0.136
mucinous cystadenoma	21 (60%)	16 (1-227)		11 (1-95)[C]		578 (39-9,984)	
mucinous borderline tumor	6 (17%)	32 (2-118)	0.315	75 (8-166)		697 (474-3,897)	
mucinous cystadenocarcinoma	8 (23%)	33 (5-269)		31 (13-187)[C]		959 (399-9,984)	

*Statistical significance according to ANOVA * and Student's t-test[A,B,C] of values normalized after Ln transformation; [A] $p = 0.010$ for serous cystadenoma compared to serous carcinoma; [B] $p = 0.001$ for serous cystadenoma compared to serous carcinoma; [C] $p = 0.035$ for mucinous cystadenoma compared to mucinous carcinoma*

Table 3. Median levels (range) of cathepsin B, L and cystatin C (ng/ml) in malignant ovarian tumor cyst fluid ($n = 26$) according to clinicopathological parameters for aggressiveness of the tumor

Subtype	*n* (%)	Cathepsin B	*p*-value*	Cathepsin L	*p*-value*	Cathepsin C	*p*-value*
FIGO stage			0.613 [A]		0.984 [A]		0.406 [A]
Ia-IIa	13 (50 %)	48 (28-93)		40 (29-78)		1,040 (594-2,355)	
≥IIb	12 (46%)	72 (16-109)		63 (18-125)		1,233 (1,055-1,500)	
unknown	1 (4%)						
Tumor grade			0.554 [B]		0.595 [B]		0.535 [B]
1	6 (23%)	38 (10-269)		31 (14-187)		959 (465-9,984)	
2	5 (19%)	71 (38-269)		62 (38-163)		1,076 (399-1,502)	
3	13 (50%)	64 (3-11)		64 (8-181)		1,324 (448-2,908)	
unknown	2 (8%)						
Ascites			0.632 [A]		0.379 [A]		0.569 [A]
yes	11 (42%)	76 (5-269)		64 (13-181)		1,330 (1,048-2,908)	
no	12 (46%)	55 (10-269)		48 (14-187)		970 (399-9,984)	
unknown	3 (12%)						

Statistical significance according to [A] Student's t test or [B] ANOVA of values normalized after Ln transformation

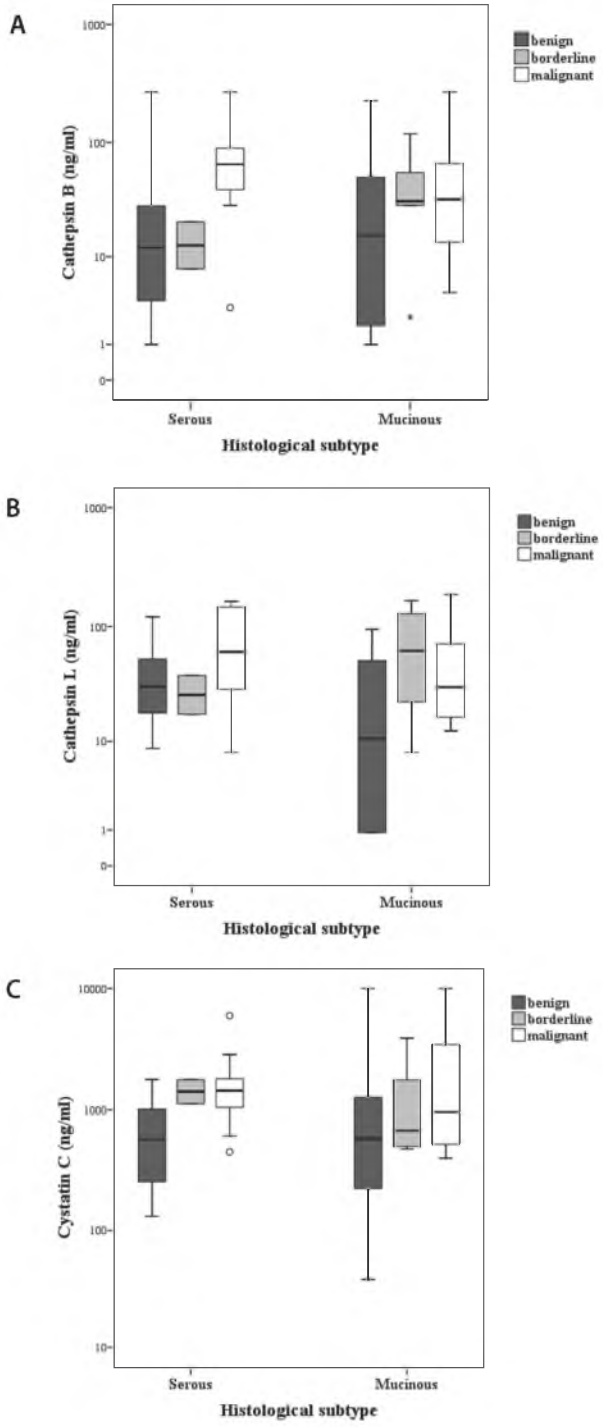

Figure 1. Boxplots of (A) CatB, (B) CatL, and (C) CysC (ng/ml) concentration (logarithmic scale) in ovarian cyst fluid for patients with malignant, borderline and benign ovarian tumors, clustered by histological subtype (serous and mucinous tumors).

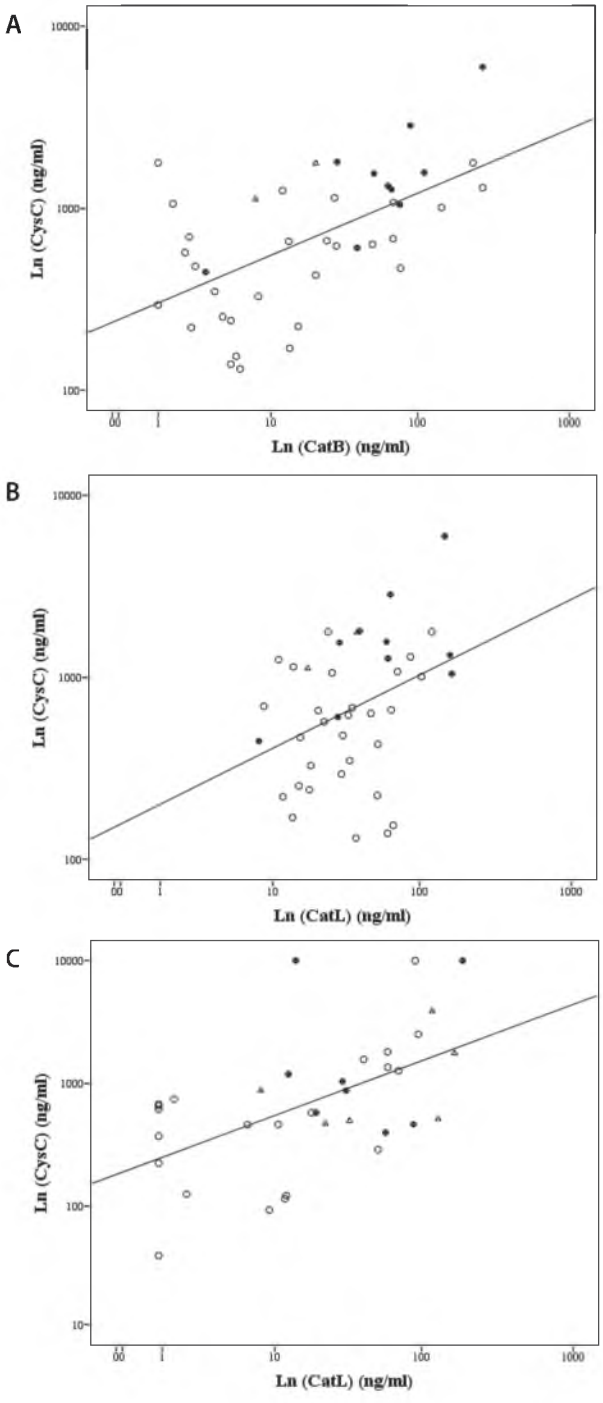

Figure 2. Scatter plots of Ln-transformed values of oCF (A) CysC with CatB and (B) CysC with CatL for patients with serous tumors ($n = 41$) and (C) CysC with CatL for patients with mucinous tumors ($n = 35$). Statistical significance ($p < 0.05$) and correlation coefficient (R) according to Pearson's correlation test. * = malignant; Δ = borderline; ○ = benign

The mean age of patients with ovarian cancer is known to be significantly higher than the mean age of patients with benign ovarian tumors.[35] This was also found in our study group. This might confound our results, as there is definite evidence that renal function decreases with age, [36] which results in a reduced glomerular filtration rate in older people. CysC is known to be almost completely reabsorbed and catabolized in the proximal renal tubular cells.[37] This might explain the higher serum levels of CysC that were reported earlier in patients with EOC compared to serum levels of CysC in patients with benign ovarian tumors. However, we found no correlation in oCF between age and CysC level within the subgroups of patients. In addition, it is unclear whether CysC levels in oCF, in contrast to serum, are influenced by renal function. Finally, the group of patients with borderline tumors is of similar age as the patients with benign tumors, yet exhibited CatB and CatL and CysC levels similar to those of patients with EOC. The level of CysC might be changed also due to pathological renal failures, changing the glomerular filtration rate in cancer patients.[37] However, patients, included in this study, did not exhibit any impaired kidney function. Therefore, we believe that the results of CysC in this study have not been biased by age-related or pathological changes of kidney function.

Remarkably, CatB and CysC oCF levels were significantly higher in malignant serous tumors compared to benign serous tumors, whereas oCF levels of CatL were significantly higher in malignant mucinous tumors compared to benign mucinous tumors. This finding contributes to the theory that malignant serous and mucinous ovarian tumors are different diseases, with subtype-specific biomarker profiles. [38-40] In addition, a significant correlation between CatB and CysC was found for patients with serous tumors. Moreover, the correlation of CatB and CysC was extremely strong in the group of patients with malignant serous tumors ($R = 0.921$), which might indicate that an increase of oCF CatB in malignant tumors is balanced by a corresponding increase in oCF CysC level. This finding could be interesting as it was proposed in previous studies that a relative imbalance between CatB and CysC in patients with cancer might contribute to the invasion and metastasis of tumor cells.[10,24,41]

Although not significant, median oCF levels of CatB and CatL were up to seven times higher in cystic endometriosis compared to the other histopathological subtypes and levels were comparable to oCF levels of ovarian cancer patients with unfavorable tumor characteristics. Noteworthy is that CysC did not differ between cystic endometriosis and other benign subtypes, which might suggest a different balance between CatB and CysC in endometriosis cysts compared to the other histological subtypes. This might be explained by the continuous breakdown of components of blood and stromal tissue in endometriosis without a malignant invasion or metastasis. However, it is now well established that endometriosis does have neoplastic potential and it has been associated with the development of endometrioid and clear-cell ovarian carcinoma.[5,42]

Since ovarian cancer tissue is highly heterogeneous, multiple biopsies are necessary for a careful examination.[43,44] This means that quantitation of cathepsins and cystatins in biological fluids of ovarian cancer patients has several clinical advantages above measurements in ovarian cancer tissue. We found that levels of cathepsins and CysC in oCF were much higher than levels in serum of cancer

patients as have been reported in literature. More specifically, up to 6 times more CatB was found in oCF than in the serum of patients with colorectal carcinoma [16] and melanoma [15], up to 10 times more CatL was found in oCF than in serum of patients with EOC, [21] and up to 8 times more CysC was found in oCF than in the serum of patients with EOC [24]. This might indicate that CatB, CatL as well as CysC are released from tumor cells into the oCF to a greater extent than the release of these compounds into the serum of cancer patients. In this way, oCF values may give a better reflection of local changes of the ovarian tumor than levels of cathepsins and its inhibitors in serum, which also might be affected by the systemic response on malignant disease and circadian variations.[45,46] From a clinical perspective, preoperative aspiration of oCF might cause iatrogenic metastasis of tumor cells, and oCF can only be used as a source of biomarkers for predicting prognosis and response to therapy. We therefore investigated the relationship between clinicopathological parameters that correspond with tumor aggressiveness and the levels of CatB, CatL and CysC in patients with EOC. However, when patients were grouped according to FIGO stage of disease, tumor grading and presence of ascites, no significant differences in oCF levels of CatB, CatL and/or CysC were found. This could be caused by the relatively small size of oCF samples from malignant tumors in the present study.

Ovarian cyst fluid levels of CatB, and its endogenous inhibitor CysC were significantly higher in serous EOC compared to benign serous ovarian tumors, whereas oCF levels of Cat L were significantly higher in mucinous EOC compared to benign mucinous ovarian tumors. The correlation between CatB and CysC was extremely strong for patients with serous EOC, which might indicate that an increase in CatB of these patients is balanced by a corresponding increase in CysC level. We feel that further studies with a larger series of samples from malignant tumors are needed to explore the possible prognostic value of cathepsins and cystatin in oCF.

References

1. Colombo N, Van Gorp T, Parma G et al. Ovarian cancer. Crit Rev Oncol Hematol 2006;60:159-79.
2. Landen Jr. CN, Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. J Clin Oncol 2008;26:995-1005.
3. Capo-Chichi CD, Smith ER, Yang DH et al. Dynamic alterations of the extracellular environment of ovarian surface epithelial cells in premalignant transformation, tumorigenicity, and metastasis. Cancer 2002;95:1802-15.
4. Scully RE. Pathology of ovarian cancer precursors. J Cell Biochem Suppl 1995;23:208-18.
5. Feeley KM, Wells M. Precursor lesions of ovarian epithelial malignancy. Histopathology 2001;38:87-95.
6. Aznavoorian S, Murphy AN, Stetler-Stevenson WG et al. Molecular aspects of tumor cell invasion and metastasis. Cancer 1993;71:1368-83.
7. Liotta LA, Tryggvason K, Garbisa S et al. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980;284:67-8.
8. Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta 2000;1477:98-111.
9. Duffy MJ. Proteases as prognostic markers in cancer. Clin Cancer Res 1996;2:613-8.
10. Kos J, Werle B, Lah T et al. Cysteine proteinases and their inhibitors in extracellular fluids: markers for diagnosis and prognosis in cancer. Int J Biol Markers 2000;15:84-9.
11. Premzl A, Turk V, Kos J. Intracellular proteolytic activity of cathepsin B is associated with capillary-like tube formation by endothelial cells in vitro. J Cell Biochem 2006;97:1230-40.
12. Schwartz MK. Tissue cathepsins as tumor markers. Clin Chim Acta 1995;237:67-78.
13. Sloane BF, Moin K, Krepela E et al. Cathepsin B and its endogenous inhibitors: the role in tumor malignancy. Cancer Metastasis Rev 1990;9:333-52.
14. Gabrijelcic D, Svetic B, Spaic D et al. Cathepsins B, H and L in human breast carcinoma. Eur J Clin Chem Clin Biochem 1992;30:69-74.
15. Kos J, Stabuc B, Schweiger A et al. Cathepsins B, H, and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. Clin Cancer Res 1997;3:1815-22.
16. Kos J, Nielsen HJ, Krasovec M et al. Prognostic values of cathepsin B and carcinoembryonic antigen in sera of patients with colorectal cancer. Clin Cancer Res 1998;4:1511-6.
17. Leto G, Tumminello FM, Pizzolanti G et al. Lysosomal cathepsins B and L and Stefin A blood levels in patients with hepatocellular carcinoma and/or liver cirrhosis: potential clinical implications. Oncology 1997;54:79-83.
18. Strojan P, Budihna M, Smid L et al. Cathepsin B and L and stefin A and B levels as serum tumor markers in squamous cell carcinoma of the head and neck. Neoplasma 2001;48:66-71.
19. Zore I, Krasovec M, Cimerman N et al. Cathepsin B/cystatin C complex levels in sera from patients with lung and colorectal cancer. Biol Chem 2001;382:805-10.
20. Bhuvarahamurthy V, Govindasamy S. Extracellular matrix components and proteolytic enzymes in uterine cervical carcinoma. Mol Cell Biochem 1995;144:35-43.
21. Nishida Y, Kohno K, Kawamata T et al. Increased cathepsin L levels in serum in some patients with ovarian cancer: comparison with CA125 and CA72-4. Gynecol Oncol 1995;56:357-61.
22. Warwas M, Haczynska H, Gerber J et al. Cathepsin B-like activity as a serum tumour marker in ovarian carcinoma. Eur J Clin Chem Clin Biochem 1997;35:301-4.
23. Lah TT, Kokalj-Kunovar M, Kastelic L et al. Cystatins and stefins in ascites fluid from ovarian carcinoma. Cancer Lett 1992;61:243-53.
24. Nishikawa H, Ozaki Y, Nakanishi T et al. The role of cathepsin B and cystatin C in the mechanisms of invasion by ovarian cancer. Gynecol Oncol 2004;92:881-6.
25. Yano M, Hirai K, Naito Z et al. Expression of cathepsin B and cystatin C in human breast cancer. Surg Today 2001;31:385-9.

26. Kos J, Smid A, Krasovec M et al. Lysosomal proteases cathepsins D, B, H, L and their inhibitors stefins A and B in head and neck cancer. Biol Chem Hoppe Seyler 1995;376:401-5.
27. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
28. Pecorelli S, Benedet JL, Creasman WT et al. FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
29. Leto G, Tumminello FM, Pizzolanti G et al. Lysosomal aspartic and cysteine proteinases serum levels in patients with pancreatic cancer or pancreatitis. Pancreas 1997;14:22-7.
30. Yano M, Hirai K, Naito Z et al. Expression of cathepsin B and cystatin C in human breast cancer. Surg Today 2001;31:385-9.
31. Makarewicz R, Drewa G, Szymanski W et al. Cathepsin B in predicting the extent of the cervix carcinoma. Neoplasma 1995;42:21-4.
32. Miyake H, Hara I, Eto H. Serum level of cathepsin B and its density in men with prostate cancer as novel markers of disease progression. Anticancer Res 2004;24:2573-7.
33. Strojan P, Svetic B, Smid L et al. Serum cystatin C in patients with head and neck carcinoma. Clin Chim Acta 2004;344:155-61.
34. Mulaomerovic A, Halilbasic A, Cickusic E et al. Cystatin C as a potential marker for relapse in patients with non-Hodgkin B-cell lymphoma. Cancer Lett 2007;248:192-7.
35. Merino MJ, Jaffe G. Age contrast in ovarian pathology. Cancer 1993;71:537-44.
36. Lindeman RD, Goldman R. Anatomic and physiologic age changes in the kidney. Exp Gerontol 1986;21:379-406.
37. Stabuc B, Vrhovec L, Stabuc-Silih M et al. Improved prediction of decreased creatinine clearance by serum cystatin C: use in cancer patients before and during chemotherapy. Clin Chem 2000;46:193-7.
38. Heinzelmann-Schwarz VA, Gardiner-Garden M, Henshall SM et al. A distinct molecular profile associated with mucinous epithelial ovarian cancer. Br J Cancer 2006;94:904-13.
39. Kobel M, Kalloger SE, Boyd N et al. Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS Med 2008;5:e232.
40. Cloven NG, Kyshtoobayeva A, Burger RA et al. In vitro chemoresistance and biomarker profiles are unique for histologic subtypes of epithelial ovarian cancer. Gynecol Oncol 2004;92:160-6.
41. Lah TT, Strojnik T, Levicar N et al. Clinical and experimental studies of cysteine cathepsins and their inhibitors in human brain tumors. Int J Biol Markers 2000;15:90-3.
42. Wells M. Recent advances in endometriosis with emphasis on pathogenesis, molecular pathology, and neoplastic transformation. Int J Gynecol Pathol 2004;23:316-20.
43. Høgdall EV, Christensen L, Kjaer SK et al. CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study. Gynecol Oncol 2007;104:508-15.
44. Chauhan SC, Vinayek N, Maher DM et al. Combined staining of TAG-72, MUC1, and CA125 improves labeling sensitivity in ovarian cancer: antigens for multi-targeted antibody-guided therapy. J Histochem Cytochem 2007;55:867-75.
45. Cimerman N, Brguljan PM, Krasovec M et al. Twenty-four hour variations of cystatin C and total cysteine proteinase inhibitory activity in sera from healthy subjects. Clin Chim Acta 2000;291:89-95.
46. Cimerman N, Brguljan PM, Krasovec M et al. Circadian characteristics of cathepsins B, H, L, and stefins A and B, potential markers for disease, in normal sera. Clin Chim Acta 1999;282:211-8.


5

The balance between extracellular cathepsins and cystatin C is of importance for ovarian cancer

Kolwijck E, Kos J, Obermajer N, Span PN , Thomas CMG, Massuger LFAG, Sweep FCGJ

European Journal of Clinical Investigation,2010; in press

This work was supported by grant P4-0127 (J.K.) from the Slovenian Research Agency.

Abstract

A major step in cancer formation involves the degradation of the extracellular matrix, mediated by multiple degradative actions of (lysosomal) proteases. Extracellular release of lysosomal proteases (cathepsins) and their inhibitors has been associated with the development and progression of several types of cancer. We investigated whether cathepsins in ovarian cyst fluid (oCF) were associated with disease outcome in patients with epithelial ovarian cancer (EOC). The levels of cathepsin B (CatB), H (CatH), L (CatL), and X (CatX) and their most abundant extracellular inhibitor cystatin C (CysC) were determined in oCF of 50 EOC patients by quantitative ELISAs. The cathepsin levels and ratio's between cathepsins and CysC were related to clinicopathological parameters (Mann-Whitney and Kruskal-Wallis tests) and survival (Cox Regression analysis). Median (25^{th}-75^{th} percentile) levels of cathepsin B, H, L, X and CysC in oCF were 97 (42-203), 18 (12-32), 61 (37-108), 20 (13-47), and 657 (501-805) ng/ml, respectively. The ratio of CysC/CatB was significantly lower for patients with metastatic compared to localized EOC ($p = 0.025$). Ratio's of CysC/CatH and CysC/CatX differed significantly between histological subtypes ($p = 0.012$ and $p = 0.035$, respectively) and were significantly higher for high-grade tumors compared to low-grade tumors ($p = 0.031$ and $p = 0.039$, respectively). Neither cathepsins nor their ratio's were significant predictors of survival for EOC patients. Ratio's between CysC and cathepsins in oCF differed significantly between important clinicopathological subgroups. We believe that a complex cascade of proteolytic events, in which cathepsins play different roles, might be responsible for progression and metastasis in EOC.

Introduction

Tumor recurrence is the limiting factor and the main cause of death in epithelial ovarian cancer (EOC).[1] Due to its heterogeneity and lack of well-defined precursor lesions, development and progression of EOC are still poorly understood.[2] Insight in the carcinogenesis of EOC is of major importance for identification of biomarkers that predict early recurrence of disease for tailor-made treatment.

Tumor progression and metastasis are associated with proteolytic mechanisms to degrade the extracellular matrix (ECM) and other structural proteins.[3] Lysosomal cysteine proteases or cathepsins, comprise a large family of enzymes divided into subgroups (B, H, L, S, C, K, O, F, V, X and W) on the basis of their catalytic mechanisms.[4] Physiologically, cathepsins are localized in lysosomes but during cancer development and progression, they are often translocated to the cell surface or even secreted into biological fluids.[5] In extracellular fluids, cystatin C (CysC) is the most abundant inhibitor to minimize uncontrolled tissue damage from escaping cathepsins.[6] Cathepsins most likely collectively influence ECM degradation, tumor invasion and angiogenesis through proteolytic cascades, in which individual proteinases have distinct roles.[7] For several types of cancer, proteases and their endogenous inhibitors have shown to be of prognostic value characterizing aggressiveness of the tumor and disease outcome.[3,5] However, for EOC, cathepsins have less often been investigated and research has been limited to establish the prognostic value of the expression of cathepsin B, D or L.[8-12]

Recently, we explored levels of cathepsin B, L and their inhibitor CysC in cyst fluid of epithelial ovarian tumors and found significantly higher levels for patients with EOC compared to patients with benign lesions.[13] Ovarian tumors typically present as large cystic masses, consisting of variable amounts of fluid which has proven to contain many compounds that reflect the biological processes taking place within the ovarian epithelial tumor wall.[14] Clinically, oCF cannot be used for differentiation between benign and malignant tumors as pre-operative punction of the ovarian tumor might cause metastatic tumor spread. Therefore, oCF can only be obtained during or after surgery and compounds in the fluid might serve as biomarkers for the prediction of disease outcome of patients with EOC.

The aim of the present study was to investigate whether the concentration of cathepsins B (CatB), H (CatH), L (CatL) X (CatX) and CysC was associated with important clinicopathological parameters and survival of EOC patients. Based on the recently proposed hypothesis that an imbalance between cysteine proteases and their cysteine protease inhibitor might lead to cancer invasion and progression,[5,15,16] we also investigated if the ratio between cathepsins and CysC was related to clinicopathological parameters and survival.

Material and Methods

Patients and oCF samples

In the period between January 2000 and January 2008, oCF was collected from patients who underwent surgery for an ovarian tumor at the Radboud University Nijmegen Medical Centre (RUNMC) and stored in our cyst fluid biobank. Ovarian CF was collected through aseptic fine needle aspiration during or after primary debulking or diagnostic surgery, never during interval debulking surgery. All patients were treated with radical cytoreductive surgery. From 68 patients, oCF could be obtained. Ten patients were excluded from this study because final paraffin examination revealed that the origin of the primary tumor was not ovarian derived. Another 8 patients were excluded because the ovarian malignancy was non-epithelial. Included were patients with histologically proven primary EOC (n = 50). Informed consent was obtained from all participants. After cooled transport to our laboratory, the oCF samples were centrifuged at 3000 x g for 10 minutes and the supernatant was aliquoted and stored at -35°C until use.

Enzyme-linked immunoassays (ELISAs)

Human cathepsins B, L, H, and X, and CysC were analyzed using ELISAs developed by the group of J. Kos, University of Ljubljana. The components were purified and characterized, and the tests were optimized as described earlier.[17] To define the optimal dilution, the samples were serially diluted to the levels encompassing the range of the assays. For cathepsins, samples in a 1:4 dilution were added to the wells of microtiter plate. For CysC, samples in a 1:100 dilution were used in the assay.

Cathepsin B. Sheep and rabbit polyclonal antibodies (IgG), purified from antisera by immunoaffinity chromatography on immobilized human CatB, were used as capture and detection antibodies, respectively, as described earlier.[18] The detection limit of the assay was 0.9 ng/ml.

Cathepsin H. Sheep polyclonal antibody was used as capture antibody and murine 2E3 monoclonal antibody for detection, as described earlier.[19] They both recognize free antigen and enzyme-inhibitor complexes. The detection limit was 2 ng/ml.

Cathepsin L. Sheep anti-CatL IgG purified on immobilized antigen was used as capture antibody and as detection horseradish peroxidase-conjugated antibody.[20] The detection limit was 1.7 ng/nl.

Cathepsin X. In CatX ELISA 2F12 (recognizing mature CatX) and 3B10 (recognizing mature- and pro-form of CatX) monoclonal antibodies were used for capture and detection, respectively.[20] The detection limit was 2 ng/ml.

Cystatin C. Rabbit affinity-purified polyclonal antibody was used for capture and murine 1A2 M-antibody, conjugated with horseradish peroxidase, was used for detection.[18] The detection limit of the assay was 0.6 ng/ml.

Polyclonal antibodies used for cathepsin B, H, L and CysC ELISA recognize precursor molecule and enzyme/inhibitor complexes, as well as the mature form of the enzyme. The linearity of the response in all ELISAs was evaluated by comparing the measured values with the calibration curves. The recoveries were tested by the addition of different amounts of the antigens to the serum with known antigen concentrations and varied from 85-104% for all assays, comparing expected versus observed concentrations. The intra-assay coefficient of variance (CV) varied from 5.6 to 11.6% the inter-assay CV varied between 8.5% and 14.5% for the assays. A microplate reader (SLT Rainbow, Salzburg, Austria) was used to measure absorbance. Determination of the levels of cathepsins and CysC was carried out without prior knowledge of the histological or clinical outcome.

Clinicopathological characteristics

Complete cyto- and histopathological reports of all EOC patients were reviewed for correct histopathological diagnosis (primary ovarian carcinoma, histological tumor subtype and grade and presence of malignant cells in ascites) by one pathologist, specialized in gynecological pathology. Histopathological tumor type and grade were classified according to the World Health Organization criteria.[21] Surgery and International Federation of gynecologist and Obstetrics (FIGO) staging [22] was always performed by a gynecologist specialized in oncology. From the medical records of the patients, the following clinicopathological characteristics were retrospectively retrieved: age at diagnosis, FIGO stage, residual tumor after surgery, time of tumor recurrence, and date of death. For some patients, information about one or more clinicopathological parameters could not be retrieved (Table 1). Recurrence of disease was defined as a measurable lesion during follow-up evaluated by computed tomography, magnetic resonance imaging and/or ultrasonography.

Statistical analyses

Statistical analyses were carried out using SPSS 16.0.2 software (SPSS Benelux BV, Gorinchem, the Netherlands). Values are shown as median with 25^{th}-75^{th} percentile (ng/ml). Normality of distributions was confirmed by Kolmogorov-Smirnov testing for all variables except CatX and CysC, which could be normalized by Ln transformation. Differences between two groups were tested by Student's t-test, and for more than 2 groups by ANOVA and subsequent posthoc Tukey's HSD tests. Ratio's between CysC and all cathepsins were tested non-parametrically using Mann-Whitney and Kruskal-Wallis tests. For statistical analysis, variables regarding clinicopathological characteristics were grouped in the following manner: FIGO stage: I versus II, III and IV, as the first category refers to tumors that are localized in

Table 1. Patient and tumor characteristics

Characteristics	*n* (%)	Median (range)
Age (years)	50 (100)	57 (32-89)
Histology		
serous	23 (46)	
mucinous	14 (28)	
endometrioid	6 (12)	
clear cell	2 (4)	
undifferentiated	1 (2)	
NOS	4 (8)	
FIGO stage		
Ia	10 (20)	
Ib	1 (2)	
Ic	9 (18)	
IIa	0 (0)	
IIb	2 (4)	
IIc	2 (4)	
IIIa	2 (4)	
IIIb	7 (14)	
IIIc	12 (24)	
IV	5 (10)	
Differentiation grade		
good (1)	13 (26)	
moderate (2)	15 (30)	
poor (3)	18 (36)	
unknown	4 (8)	
Residual tumor after surgery		
< 1 cm	41 (82)	
≥ 1 cm	8 (16)	
unknown	1 (2)	

the ovaries and the second category refers to tumors that are metastasized; [22] histology: serous versus mucinous versus endometrioid, as the other subtypes consisted of less than 2 cases; differentiation grade: low-grade (well-differentiated) tumors versus high-grade (moderately and poorly differentiated) tumors; [23] age: median values; residual disease after surgery: lesions < 1cm versus lesions ≥ 1 cm. For survival analyses, the median of the cathepsins and CysC concentration and their ratio was used as the cut off value for dichotomizing oCF levels. Disease-free survival (DFS) time was used as follow-up endpoint and was defined as the time interval from the date of surgery to the date of recurrence or death, and censored at last follow-up. Equality of survival distributions was tested using univariate Cox regression analysis.

Results

Patient and tumor characteristics

Patient and tumor characteristics at time of surgery are listed in Table 1. All patients, except those with FIGO stage I, grade 1or 2 disease were given six cycles of platinum-based chemotherapy. Median follow-up time was 39 months (range: 9-160 months). DFS ranged from 1 to 117 months, with a median of 15 months. Within the follow-up period, 23 patients (46%) showed recurrent disease and 15 patients died (30%).

Cathepsins B, H, L, X and CysC levels in oCF

Median (25^{th}-75^{th} percentile) concentration of cathepsin B, H, L, X and CysC was: 97 (42-203), 18 (12-32), 61 (37-108), 20 (13-47), and 657 (501-805) ng/ml, respectively. The median (25^{th}-75^{th} percentile) cathepsin and CysC concentration for different clinicopathological subgroups is listed in Table 2. CatB was significantly higher in patients older than compared to patients younger than 57 years of age ($p = 0.049$). The CysC concentration differed significantly between histological subtypes ($p = 0.037$). Although not significantly different, CatH concentration was slightly higher in mucinous ($p = 0.055$) and low-grade tumors ($p = 0.059$).

Ratio values between cystatin C and cathepsin B, H, L and X in oCF

Median (25^{th}-75^{th} percentile) ratio's between CysC and cathepsin B, H, L, and X were 7 (3-13), 28 (17-56), 10 (7-21) , and 30 (13-54), respectively. Differences in ratio's between CysC and cathepsins for the clinicopathological subgroups are listed in Table 3. The ratio of CysC/CatB was significantly lower for patients with FIGO stage ≥ II compared to FIGO stage I ($p = 0.025$, Figure 1). The ratio of both CysC/CatH and CysC/CatX differed significantly between histological subtypes ($p = 0.012$, Figure 2 and $p = 0.035$, Figure 3, respectively) and was significantly higher for high-grade tumors compared to low-grade tumors ($p = 0.031$ and $p = 0.039$, respectively).

Cathepsins and CysC and survival of EOC patients

Median levels were used to dichotomize cathepsins, CysC and their ratio's to study their prognostic significance. Table 4 shows the hazard ratio (HR) with 95% confidence interval (CI), using the univariate proportional hazard model. Histological subtype, FIGO stage, differentiation grade, residual tumor after surgery and preoperative CA 125 level were found to be significant predictors of DFS in univariate analysis. None of the individual cathepsins, CysC or their ratio's were significant predictors of DFS.

Table 2. Relationship between median (25th-75th percentile) cathepsin B, H, L, X and cystatin C (ng/ml) in oCF and clinicopathological characteristics

Characteristics		Cathepsin B (ng/mL)	*p*-value*	Cathepsin H (ng/mL)	*p*-value*	Cathepsin L (ng/mL)	*p*-value*	Cathepsin X (ng/mL)	*p*-value**	Cystatin C (ng/mL)	*p*-value**
Age			0.049		0.931		0.950		0.403		0.673
	< median	64 (32-159)		19 (11-32)		67 (16-110)		21 (13-40)		640 (501-805)	
	≥ median	126 (60-251)		18 (12-29)		54 (40-104)		19 (11-49)		663 (491-805)	
Histology			0.791		0.055		0.485		0.329		0.037
	serous	86 (48-234)		18 (12-24)		66 (45-116)		19 (6-46)		640 (561-804)	
	mucinous	55 (23-196)		30 (12-62)		85 (1-108)		20 (16-45)		531 (296-805)	
	endometrioid	80 (15-162)		16 (1-36)		46 (1-89)		20 (10-58)		811 (594-1079)	
FIGO stage			0.087		0.654		0.502		0.913		0.415
	I	53 (21-164)		15 (9-51)		61 (1-109)		19 (14-39)		728 (360-822)	
	≥ II	115 (61-246)		18 (15-28)		61 (40-108)		20 (11-50)		645 (516-762)	
Differentiation grade			0.545		0.059		0.678		0.266		0.071
	low-grade (1)	45 (22-203)		25 (11-59)		67 (1-115)		21 (17-43)		501 (287-805)	
	high-grade (2+3)	99 (45-212)		18 (12-26)		57 (36-93)		19 (10-49)		665 (541-806)	
Residual disease after surgery			0.802		0.373		0.554		0.484		0.591
	< 1cm	94 (38-214)		18 (11-30)		64 (40-111)		20 (12-48)		650 (491-805)	
	≥ 1 cm	98 (54-236)		21 (18-46)		62 (27-80)		20 (17-42)		652 (540-798)	

*Statistical significance according to ANOVA and Student's t-test of values for * Cathepsin B, H and L and normalized after Ln transformation for ** Cathepsin X and Cystatin C.*

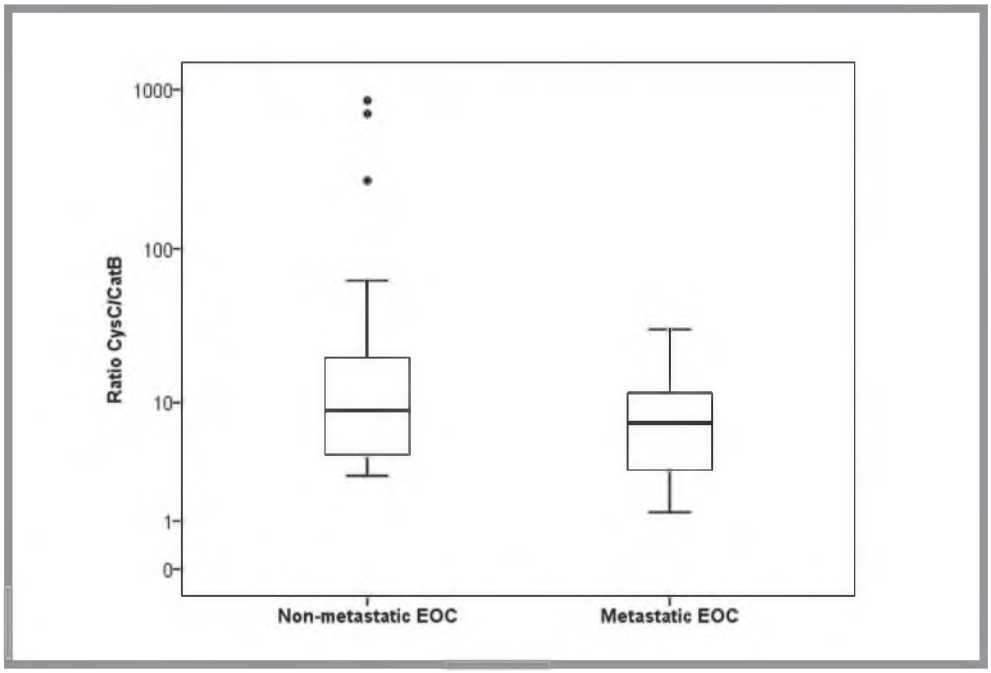

Figure 1. The difference in ratio values of oCF CysC and CatB between patients with ($n = 30$) and without ($n = 20$) metastatic EOC ($p = 0.025$).

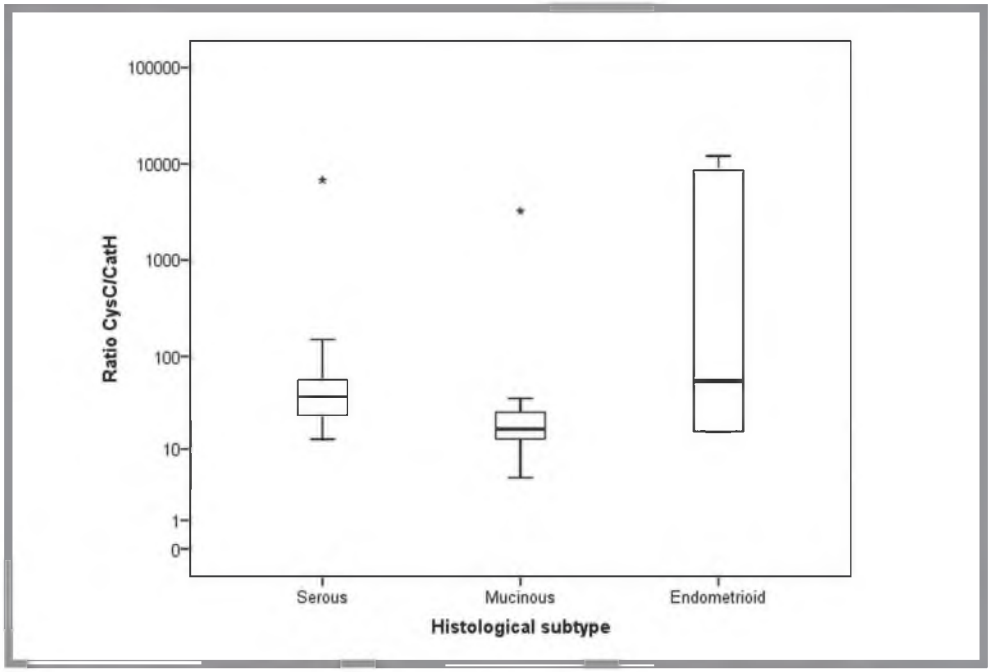

Figure 2. The difference in ratio values of oCF CysC and CatH between patients with serous ($n = 23$), mucinous ($n = 14$) and endometrioid ($n = 6$) type EOC ($p = 0.012$).

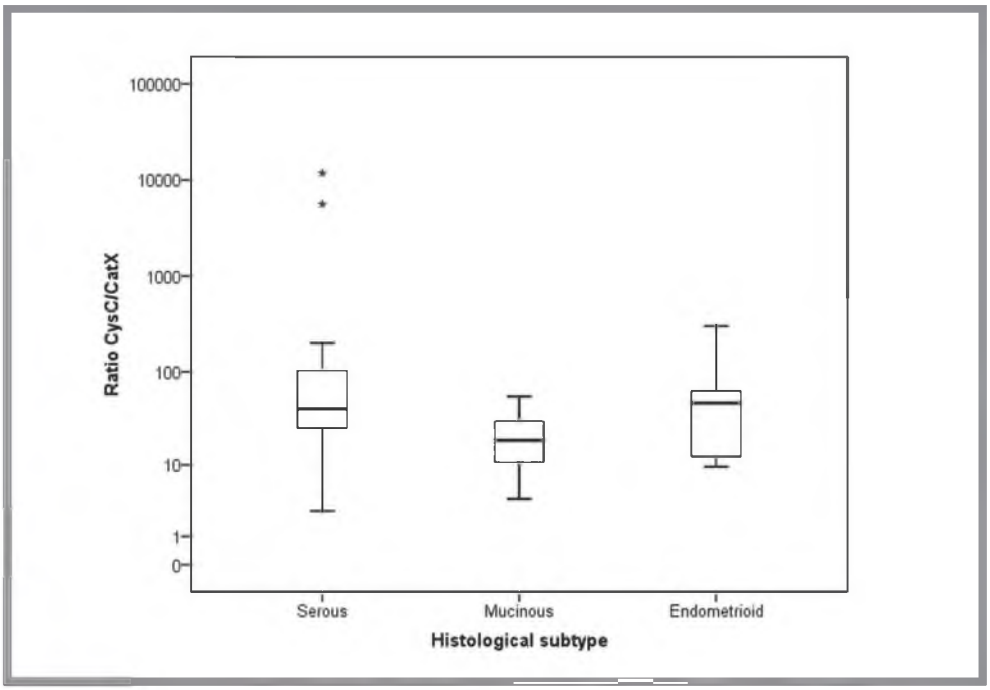

Figure 3. The difference in ratio values of oCF CysC and CatX between patients with serous ($n = 23$), mucinous ($n = 14$) and endometrioid ($n = 6$) type EOC ($p = 0.035$).

Discussion

Recently, several studies reported that extracellular release of cathepsins and their inhibitors is predictive for prognosis of cancer patients.[5] In serum of cancer patients, a high concentration of CatB, CatH and CysC was correlated with a shorter survival [5]. Similar results were found when cathepsins were explored in urine, [24,25] and cerebrospinal [26] and pleural [27] fluid. Compared to tissue, biological fluid samples are easier to obtain, homogeneous and compounds can be measured quantitatively.

Compared to reported serum values in cancer patients, [8,28-30] we found a high concentration of cathepsin B, H, L and X in oCF of EOC patients, suggesting an extracellular release from the ovarian tumor into the oCF. Individual levels of cathepsins in oCF showed no significant correlation with any of the clinicopathological parameters. However, ratio's between cathepsins B, H, X and CysC were significantly associated with important parameters of tumor progression and metastasis although they were not predictive for recurrence of disease. Scorilas *et al.* did find that CatB expressing EOC tissue was significantly associated with early recurrence and shorter survival.[10] However, these authors scored intracellular immunohistochemical expression of CatB as either positive or negative, whereas we measured extracellular cathepsins quantitatively by ELISA. To our knowledge, no other studies have been reported investigating the prognostic value of cathepsins or CysC for patients with EOC.

As cystatins have shown to be the most abundant and tight binding extracellularly inhibitors of all members of the cathepsin superfamily, [6] we suggest that an imbalance between the proteolytic cathepsins and their inhibitor might be essential for EOC progression and metastasis. This theory is supported by studies in which the ratio of CatB and CysC differed significantly between cancer patients and healthy controls.[16,18,26,31,32] We found that the ratio of CysC and CatB was significantly lower in oCF of advanced stage EOC patients compared to patients with localized disease. Since oCF CatB was found to be progressively increased with FIGO stage, high CatB concentration in metastasized EOC might be accompanied by less increased concentration of CysC. In agreement with our results, a high CatB concentration has been found in tissue and serum of advanced stage EOC patients as well.[10,33] In addition, for other types of tumors, a lower ratio between CysC and CatB has been found in serum and cerebrospinal fluid of patients with metastasized cancer compared to patients with early stage disease. [16,26] This lower ratio might be explained by a controlled decrease in cystatin concentration which results in an increase in activity of cathepsins.[7] It also has been suggested that a diminished binding affinity between CatB and CysC might be caused by changes in glycosylation, the presence of cathepsin activators or the binding of cathepsins with glycosaminoglycans [16]. We believe the CysC/CatB ratio might be of interest in order to obtain more insight into the complex carcinogenesis and progression of EOC. EOC does not appear as a single disease entity but comprises a heterogeneous group of tumors.[2] A further study with a larger series of samples is needed to investigate the association between CysC/CatB and the metastatic potential of EOC in more detail.

Ratio's between CysC and CatH and between CysC and CatX were significantly lower in mucinous EOC and low-grade tumors compared to other subtypes and high-grade tumors, respectively. This finding might be explained by the fact that, 92.3% of our low-grade tumors were of mucinous subtype (data not shown), whereas almost all high-grade tumors were of serous and endometrioid subtype. Previous studies have reported large variations in expression of individual cathepsins between different tissues and even between different types of cells within the same tissue.[34] Selective expression of individual cathepsins in different types of tumors suggest that they may participate in specialized cellular functions.[35] EOC is known for its many different histological subtypes.[36] Of these subtypes, mucinous carcinomas are slowly developing tumors, whereas serous and endometrioid carcinomas are mostly high-grade, rapidly growing tumors.[37] In agreement with our results, CatH differed significantly between histological subtypes in lung cancer as well.[38]

CatX was discovered only recently and is thought to be involved in phagocytosis and regulation of the immune response rather than in degradation of the ECM.[20] Only few reports have been published that investigated CatX in cancer patients. While CatX was found to be highly expressed in tissue sections from prostatic intraepithelial neoplasms and prostate carcinomas, [39] Kos *et al.* did not find a difference in expression of the enzyme between lung carcinomas and normal long tissue [20]. Cathepsin X was found to be increased also in gastric carcinomas, especially after infection by *Helicobacter pylori*.[40] The present study showed detectable quantities of CatX in oCF of EOC patients, which were 10 fold higher than the CatX concentration reported in sera of cancer patients, which suggests a direct release of CatX from the tumor tissue into the oCF.[41]

Table 3. Relationship between median (25th-75th percentile) cathepsin B, H, L, X and cystatin C (ng/ml) in oCF and clinicopathological characteristics

Characteristics		CysC/CatB	*p*-value*	CysC/CatH	*p*-value*	CysC/CatL	*p*-value*	CysC/CatX	*p*-value*
Age			0.070		0.691		0.635		0.517
	< median	10 (4-17)		26 (17-61)		11 (7-63)		30 (13-54)	
	≥ median	6 (3-11)		36 (18-55)		10 (7-21)		32 (13-56)	
Histology			0.717		0.012		0.216		0.035
	serous	9 (5-16)		38 (24-58)		10 (7-17)		40 (24-138)	
	mucinous	6 (3-13)		17 (13-27)		7 (5-150)		19 (10-30)	
	endometrioid	10 (4-261)		70 (16-943)		15 (9-993)		47 (12-122)	
FIGO stage			0.025		0.890		0.579		0.867
	I	9 (4-26)		28 (14-90)		12 (7-238)		25 (12-50)	
	≥ II	7 (3-12)		30 (21-52)		10 (7-20)		32 (13-56)	
Differentiation grade			0.724		0.031		0.414		0.039
	low-grade (1)	7 (3-13)		18 (12-33)		8 (5-194)		20 (10-30)	
	high-grade (2+3)	8 (3-15)		36 (21-62)		10 (8-21)		37 (13-70)	
Residual disease after surgery			0.871		0.482		0.465		0.808
	< 1 cm	8 (3-13)		27 (17-61)		10 (7-200)		30 (13-54)	
	≥ 1 cm	6 (3-16)		29 (18-36)		11 (9-25)		82 (17-40)	

**Statistical significance according to Mann-Whitney and Kruskal-Wallis test*

Table 4. Univariate analysis of Cathepsin B, H, L, X and cystatin C and other potential factors for DFS of 50 patients with EOC

	Cut-off value (median)	HR	(95% CI)	*p*-value
Age	57 years	1.50	(0.65-3.42)	0.345
Histology				
serous		5.36	(1.20-23.89)	0.028
mucinous		1.00	(reference)	
endometrioid		1.31	(0.12-14.43)	0.827
FIGO				
stage I		1.00	(reference)	
stage ≥ II		85.0	(2.44-2961)	0.014
Differentiation grade				
low (1)		1.00	(reference)	
high (2+3)		59.9	(1.13-3162)	0.043
Residual tumor after surgery				
< 1 cm		1.00	(reference)	
≥ 1 cm		5.63	(2.15-14.76)	<0.001
Preoperative CA 125 (serum)	126 U/ml	2.66	(1.04-6.85)	0.042
Cathepsin B (oCF)	97 ng/ml	1.29	(0.56-2.95)	0.551
Cathepsin H (oCF)	18 ng/ml	1.17	(0.51-2.67)	0.707
Cathepsin L (oCF)	61 ng/ml	1.03	(0.45-2.33)	0.952
Cathepsin X (oCF)	20 ng/ml	1.16	(0.51-2.64)	0.722
Cystatin C (oCF)	657 ng/ml	1.38	(0.59-3.21)	0.448
CysC/CatB (oCF)	7	1.13	(0.49-2.60)	0.771
CysC/CatH (oCF)	28	1.19	(0.52-2.73)	0.680
CysC/CatL (oCF)	10	1.14	(0.50-2.59)	0.759
CysC/CatX (oCF)	30	1.95	(0.82-4.66)	0.132

**Parameters with a p-value ≤ 0.05 were selected for multivariate analysis.*

Although cathepsins and CysC in oCF of EOC patients were not predictive for recurrence of EOC, we have shown that the interaction between CysC and cathepsins in oCF seems to be important. We believe that a complex cascade of proteolytic events, in which cathepsins play different roles, might be responsible for progression and metastasis in EOC. However, this study was explorative and to analyze the complex mechanism of proteolysis for EOC, further studies with larger series of patients have to be performed to confirm our results.

References

1. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96.
2. Naora H. The heterogeneity of epithelial ovarian cancers: reconciling old and new paradigms. Expert Rev Mol Med 2007;9:1-12.
3. Duffy MJ. Proteases as prognostic markers in cancer. Clin Cancer Res 1996;2:613-8.
4. Turk V, Turk B, Guncar G et al. Lysosomal cathepsins: structure, role in antigen processing and presentation, and cancer. Adv Enzyme Regul 2002;42:285-303.
5. Kos J, Werle B, Lah T et al. Cysteine proteinases and their inhibitors in extracellular fluids: markers for diagnosis and prognosis in cancer. Int J Biol Markers 2000;15:84-9.
6. Brix K, Dunkhorst A, Mayer K et al. Cysteine cathepsins: cellular roadmap to different functions. Biochimie 2008;90:194-207.
7. Turk V, Turk B, Turk D. Lysosomal cysteine proteases: facts and opportunities. EMBO J 2001;20:4629-33.
8. Nishida Y, Kohno K, Kawamata T et al. Increased cathepsin L levels in serum in some patients with ovarian cancer: comparison with CA125 and CA72-4. Gynecol Oncol 1995;56:357-61.
9. Protopapas A, Diakomanolis E, Bamias A et al. The prognostic significance of the immunohistochemical expression of p53, bcl-2, c-erb B-2 and cathepsin-D in ovarian cancer patients receiving platinum with cyclophosphamide or paclitaxel chemotherapy. Eur J Gynaecol Oncol 2004;25:225-9.
10. Scorilas A, Fotiou S, Tsiambas E et al. Determination of cathepsin B expression may offer additional prognostic information for ovarian cancer patients. Biol Chem 2002;383:1297-303.
11. Athanassiadou P, Sakellariou V, Petrakakou E et al. Cathepsin D immunoreactivity in ovarian cancer: correlation with prognostic factors. Pathol Oncol Res 1998;4:103-7.
12. Scambia G, Panici PB, Ferrandina G et al. Clinical significance of cathepsin D in primary ovarian cancer. Eur J Cancer 1994;30A:935-40.
13. Kolwijck E, Massuger LF, Thomas CM et al. Cathepsins B, L and cystatin C in cyst fluid of ovarian tumors. J Cancer Res Clin Oncol 2010;136:771-8.
14. Bar JK, Harlozinska A, Sobanska E et al. Relation between ovarian carcinoma-associated antigens in tumor tissue and detached cyst fluid cells of patients with ovarian neoplasms. Tumori 1994;80:50-5.
15. Nishikawa H, Ozaki Y, Nakanishi T et al. The role of cathepsin B and cystatin C in the mechanisms of invasion by ovarian cancer. Gynecol Oncol 2004;92:881-6.
16. Zore I, Krasovec M, Cimerman N et al. Cathepsin B/cystatin C complex levels in sera from patients with lung and colorectal cancer. Biol Chem 2001;382:805-10.
17. Kos J, Smid A, Krasovec M et al. Lysosomal proteases cathepsins D, B, H, L and their inhibitors stefins A and B in head and neck cancer. Biol Chem Hoppe Seyler 1995;376:401-5.
18. Kos J, Stabuc B, Schweiger A et al. Cathepsins B, H, and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. Clin Cancer Res 1997;3:1815-22.
19. Schweiger A, Stabuc B, Popovic T et al. Enzyme-linked immunosorbent assay for the detection of total cathepsin H in human tissue cytosols and sera. J Immunol Methods 1997;201:165-72.
20. Kos J, Sekirnik A, Premzl A et al. Carboxypeptidases cathepsins X and B display distinct protein profile in human cells and tissues. Exp Cell Res 2005;306:103-13.
21. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
22. Pecorelli S, Benedet JL, Creasman WT et al. FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
23. Silverberg SG. Histopathologic grading of ovarian carcinoma: a review and proposal. Int J Gynecol Pathol 2000;19:7-15.

24. Staack A, Koenig F, Daniltchenko D et al. Cathepsins B, H, and L activities in urine of patients with transitional cell carcinoma of the bladder. Urology 2002; 59:308-12.
25. Hirano T, Manabe T, Takeuchi S. Serum cathepsin B levels and urinary excretion of cathepsin B in the cancer patients with remote metastasis. Cancer Lett 1993;70:41-4.
26. Nagai A, Terashima M, Harada T et al. Cathepsin B and H activities and cystatin C concentrations in cerebrospinal fluid from patients with leptomeningeal metastasis. Clin Chim Acta 2003;329:53-60.
27. Bunatova K, Obermajer N, Kotyza J et al. Levels of cathepsins S and H in pleural fluids of inflammatory and neoplastic origin. Int J Biol Markers 2009;24:47-51.
28. Herszenyi L, Istvan G, Cardin R et al. Serum cathepsin B and plasma urokinase-type plasminogen activator levels in gastrointestinal tract cancers. Eur J Cancer Prev 2008;17:438-45.
29. Leto G, Tumminello FM, Pizzolanti G et al. Lysosomal aspartic and cysteine proteinases serum levels in patients with pancreatic cancer or pancreatitis. Pancreas 1997;14:22-7.
30. Siewinski M, Saleh Y, Gryboc M et al. Determination of cysteine peptidases-like activity and their inhibitors in the serum of patients with ovarian cancer treated by conventional chemotherapy and vitamin E. J Exp Ther Oncol 2004;4:189-93.
31. Hirai K, Yokoyama M, Asano G et al. Expression of cathepsin B and cystatin C in human colorectal cancer. Hum Pathol 1999;30:680-6.
32. Yano M, Hirai K, Naito Z et al. Expression of cathepsin B and cystatin C in human breast cancer. Surg Today 2001;31:385-9.
33. Warwas M, Haczynska H, Gerber J et al. Cathepsin B-like activity as a serum tumour marker in ovarian carcinoma. Eur J Clin Chem Clin Biochem 1997;35:301-4.
34. Qian F, Chan SJ, Gong QM et al. The expression of cathepsin B and other lysosomal proteinases in normal tissues and in tumors. Biomed Biochim Acta 1991;50:531-40.
35. Chapman HA, Riese RJ, Shi GP. Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 1997;59:63-88.
36. Soslow RA. Histologic subtypes of ovarian carcinoma: an overview. Int J Gynecol Pathol 2008;27:161-74.
37. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
38. Schweiger A, Staib A, Werle B et al. Cysteine proteinase cathepsin H in tumours and sera of lung cancer patients: relation to prognosis and cigarette smoking. Br J Cancer 2000;82:782-8.
39. Nagler DK, Kruger S, Kellner A et al. Up-regulation of cathepsin X in prostate cancer and prostatic intraepithelial neoplasia. Prostate 2004;60:109-19.
40. Krueger S, Kalinski T, Hundertmark T et al. Up-regulation of cathepsin X in Helicobacter pylori gastritis and gastric cancer. J Pathol 2005;207:32-42.
41. Decock J, Obermajer N, Vozelj S et al. Cathepsin B, cathepsin H, cathepsin X and cystatin C in sera of patients with early-stage and inflammatory breast cancer. Int J Biol Markers 2008;23:161-8.

6

GSTP1-1 in ovarian cyst fluid and disease outcome of patients with ovarian cancer

Kolwijck E, Zusterzeel PLM, Roelofs HMJ, Hendriks JC, Peters WHM, Massuger LFAG

Cancer Epidemiology Biomarkers and Prevention 2009; 18(8): 2176-2181

This study was supported by grants from the Wim Schellekens Foundation, the Netherlands

Abstract

Detoxification enzymes, especially glutathione S-transferase Pi (GSTP1-1), have been implicated in resistance to platinum-based chemotherapy. We studied GSTP1-1 levels in ovarian cyst fluid (oCF), obtained during surgery before chemotherapy, of patients with epithelial ovarian cancer (EOC) and clinical outcomes were correlated. GSTP1-1 was determined by ELISA in oCF of 56 patients with EOC and 109 noncancer controls (21 borderline and 88 benign ovarian tumors). Differences in median GSTP1-1 between clinicopathological subgroups were studied using Mann-Whitney and Kruskal-Wallis tests. Differences in disease free (DFS) and overall survival (OS) between groups were analyzed by applying Kaplan-Meyer estimates and log-rank tests. Univariate and multivariate analysis were performed using Cox proportional hazard model. Significantly higher levels of GSTP1-1 were found in the oCF of malignant (median: 383; range: 10-32695 ng/ml) compared to benign (median: 20; range: 0-1128 ng/ml) ovarian tumors ($p < 0.01$). Significantly higher GSTP1-1 levels were found in patients with advanced FIGO stage ($p = 0.01$), high-grade tumors ($p = 0.44$) and/or high levels of preoperative CA 125 ($p = 0.01$). Of patients who received chemotherapy (stage ≥ Ic; $n = 30$), high GSTP1-1 levels were significantly associated with a poor DFS and OS (log-rank: $p = 0.047$ and $p = 0.033$, respectively). FIGO stage was the only independent predictor for DFS. GSTP1-1 was the only independent predictor for OS.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy world-wide. Approximately 70% of the patients are diagnosed with advanced stage of disease (International Federation of Gynecology and Obstetrics (FIGO) III and IV) with 5-year survival rates of only 10-20%.[1,2] Because the introduction of platinum-based chemotherapy into clinical practice, prognosis of patients with advanced EOC has markedly improved. However, this success is limited by the phenomenon of platinum-based chemoresistance. Platinum-resistant disease includes patients who do not respond to platinum-based chemotherapy at all and those who relapse within 6 months following primary chemotherapy. These patients have a poor prognosis, with an expected median overall survival of less than 10 months.[3] Early identification of platinum-resistant disease will provide the opportunity to change usual regimens, which may improve the prognosis of this group of patients with EOC.

The mechanism of chemoresistance has been studied extensively in recent years, and today, it is generally accepted that the detoxification enzymes glutathione S-transferases (GST) play an essential role in this process.[4] GSTs are enzymes that catalyze the phase II conjugation reaction with glutathione of highly reactive compounds that are formed during phase I modification of endogenous compounds and xenobiotics, such as anticancer agents. The human GST superfamily comprises cytosolic dimeric isoenzymes, which have been assigned to at least four generic classes: Alpha (A), Mu (M), Pi (P), and Theta (T), each class consisting of one or more isoenzymes with a wide variety of substrate specificities.[5] The Pi class GSTs (GSTP1-1) are believed to interact with platinum-based compounds and are frequently found to be over-expressed in a variety of neoplastic tissues, including ovarian cancer.[5,6] It has been shown that high GSTP1-1 activity results in an increased metabolism of several anti-cancer drugs, including platinum-based compounds, which subsequently results in a diminished cytotoxic effect on tumor cells.[4,6,7] For ovarian cancer, some researchers found a relationship between over-expression of GSTP1-1 in malignant ovarian tissue and poor prognosis or bad response to chemotherapy, [8-13] whereas others could not detect such an association.[14-21]

Less attention has been paid to the analysis of GSTP1-1 in body fluids of patients with ovarian cancer. Ovarian cyst fluid (oCF) might provide a practical source of prognostic markers because it is in close contact with tumor tissue, it is readily available and its components are homogeneously distributed. The purpose of the present study was to examine the relation between important clinicopathological variables and their association with GSTP1-1 levels in oCF of patients with EOC.

Materials and Methods

Patients and cyst fluid collection

This study includes 165 patients diagnosed with primary ovarian tumors at the Radboud University Nijmegen Medical Centre in the period between 1988 and 2007. All these patients have undergone primary surgery. oCF was collected by aseptic fine needle aspiration at the Department of Pathology, immediately after surgical removal of the tumor. After cooled transport to the laboratory, the oCF samples were centrifuged at 3000 x *g* for ten minutes and the supernatant was stored at -35 °C in small portions until use. Determination of the levels of GSTP1-1 was carried out by ELISA, without prior knowledge of the histological or clinical outcome. Histopathological diagnosis was performed by a pathologist specialized in gynecologic oncology and revealed 88 benign, 21 borderline, and 56 malignant epithelial ovarian tumors. Informed consent was obtained from all participants.

ELISA procedures

ELISA for GSTP-1 was done in microtiter plates as described previously.[22,23] In short: plates were coated overnight with anti-GSTP1-1 monoclonal antibody in phosphate buffered saline (PBS) and were blocked with PBS-T supplemented with 1% bovine serum albumin. Between the incubations, plates were washed 5 times with PBS. Standards of GSTP1-1 diluted in PBS-T or diluted samples were then added to the wells. Plates were incubated overnight, washed, incubated with rabbit anti-GSTP1-1 antiserum and subsequently incubated with peroxidase-labeled swine anti-rabbit. After the final wash, plates were incubated with o-phenylenediamine, H_2O_2 in sodium citrate and Na_2HPO_4. The reaction was stopped by adding H_2SO_4. All standards and samples were measured in duplicate. A four-parameter weight logistic regression model was used to calculate standard curves and unknowns.

Clinicopathologic characteristics

From the medical and pathology reports of the patients diagnosed with EOC (n = 56), the following clinicopathologic characteristics were retrieved retrospectively: age at diagnosis, FIGO stage, histological tumor subtype, histopathologic grade, residual tumor after surgery, presence of malignant cells in ascites, tumor recurrence, preoperative CA 125 levels, and chemotherapeutic treatment. Staging was done according to the FIGO criteria.[24] Histopathological tumor type and grade were classified according to the World Health Organization (WHO) criteria.[25] Chemotherapeutic treatment was defined as complete adjuvant combination chemotherapy of six courses, always including a platinum-based agent and was started within three weeks after surgery. Additional information regarding recurrence of disease was collected for patients who received chemotherapy. Recurrence

of disease was defined as a measurable lesion detected by computed tomography, magnetic resonance imaging and/or ultrasonography.

Statistical analyses

GSTP1-1 samples were measured in duplicate and in the analysis the values were averaged. For the purpose of statistical analysis, variables regarding patient characteristics were grouped in the following manner: FIGO stage, I, II versus III, IV; tumor grade, low-grade (grade 1) versus high-grade (grade 2 and 3) [26]; histology, serous versus mucinous versus endometrioid versus other; residual disease, < 1 cm (definition of optimal cytoreductive surgery) versus ≥ 1 cm (definition of suboptimal debulking); ascites, presence of malignant cells versus no malignant cells; preoperative CA 125, ≤ 126 U/ml versus > 126 U/ml (median value). Differences in concentrations of GSTP1-1 between groups of patients were tested for statistical significance using the Mann-Whitney test in case of two groups and the Kruskal-Wallis test in case of more than two groups, respectively. Survival analysis was performed with the subgroup of patients with EOC that received chemotherapy after primary surgery (n = 30). Survival techniques were used to study the time to recurrence and to study the time to death. Disease free survival (DFS) was defined as the time interval from the date of the last course of chemotherapy to recurrence or last follow-up. Overall survival (OS) was defined as the time interval from the date of surgery to the date of either death or last follow-up. The oCF GSTP1-1 value of 160 ng/ml was used for dividing patients into two groups after a statistically significant difference in DFS and OS was found with logistic regression analysis. The Kaplan-Meyer estimates were calculated of the patients with oCF GSTP1-1 values below 160 ng/ml and above 160 ng/ml, respectively. Subsequently, the log-rank test was used to test their difference for statistical significance. Univariate proportional hazards model was used to study the influence of the clinicopathological parameters on DFS and OS separately. Histology was not studied because of the small number of patients within the different subgroups (serous n = 18, mucinous n = 3, endometrioid n = 5, other n = 4). Tumor grade was not studied because of the small number of patients with low-grade carcinomas (n = 3). The hazard ratio's (HR) with the corresponding 95% confidence interval (CI) are presented. Multivariate proportional hazards model with selection procedures was used to find the clinicopathological parameters that independently contribute to a decreased time to recurrence (or death). The adjusted HRs with the corresponding 95% CI of the final model are presented. P-values less than 0.05 were considered statistical significant. All statistical analyses were performed using the software package SPSS 14.0 for Microsoft Windows (SPSS Inc., Chicago, IL, USA).

Results

Patient's characteristics

Median age at diagnosis was 56 years (range: 31-89 years) for patients with EOC (n = 56), 53 years (range: 15-82 years) for patients with borderline tumors (n = 21), and 46 years (range: 19- 77 years) for patients with benign epithelial tumors (n = 88). Age differed significantly between patients with EOC and patients with benign ovarian tumors (p <0.01, Mann-Whitney test). No significant differences in age were found between patients with EOC and borderline tumors and between patients with benign and borderline tumors. Of the 56 patients with EOC, 22 had FIGO stage I (39%), 5 had FIGO stage II (9%), 21 had FIGO stage III (38%), and 7 FIGO had stage IV (13%). The remaining clinicopathological data are listed in Table 1. For some patients, information about clinicopathological parameters was incomplete (Table 1). Of the subgroup of EOC patients that received complete adjuvant chemotherapeutic treatment (n = 30), recurrence of disease was observed in 17 patients (57%) of whom 5 (17%) showed recurrence within 6 months after completing chemotherapeutic treatment. DFS ranged from 1 to 84 months, with a median of 13 months. OS ranged from 6 to 91 months, with a median of 26 months. Twelve patients (40%) died within the follow-up period.

Histopathological diagnosis

Figure 1 shows the boxplots of the GSTP1-1 concentrations (ng/ml) in oCF of patients by histologic subtype. Median (range) concentrations were 383 (10-32,695) ng/ml, 240 (2-1,193) ng/ml, and 20 (0-1,128) ng/ml for malignant (n = 56), borderline (n = 21) and benign (n = 88) oCF samples, respectively. Significantly higher concentrations of GSTP1-1 were found in oCF from malignant ovarian tumors compared with benign tumors and in oCF from borderline tumors compared to benign tumors (both p <0.01, Mann-Whitney test). No significant differences in GSTP1-1 level were found between patients with malignant and patients with borderline ovarian tumors (p = 0.114).

GSTP1-1 and clinicopathologic characteristics

Table 1 shows the clinicopathological outcomes and median (range) concentration of GSTP1-1 (ng/ml) for the 56 patients with EOC. Significantly higher GSTP1-1 concentrations were found in patients with FIGO stage III and IV disease compared to patients with FIGO stage I and II (p = 0.01). Patients with high-grade carcinomas had significantly higher levels of GSTP1-1 compared to patients with low-grade carcinomas (p = 0.044). High CA 125 levels were correlated with high GSTP1-1 levels (p = 0.01) (all Mann-Whitney U test).

Table 1. Median (range) GSTP1-1 cyst fluid concentrations (ng/ml) by clinicopathological parameters of patients with EOC ($n = 56$)

	n (%)	Cyst fluid median	GSTP1-1 (range)	*p*-value
FIGO stage				0.010*
I + II	27 (48)	162	(10-1,302)	
III + IV	28 (50)	703	(10-32,695)	
unknown	1 (2)			
Tumor grade				0.044*
low-grade	14 (25)	89	(10-1,302)	
high-grade	37 (66)	448	(10-32,695)	
unknown	5 (9)			
Histology				0.264**
serous	24 (43)	224	(10-25,963)	
mucinous	15 (27)	114	(10-32,695)	
endometrioid	12 (21)	447	(28-3,583)	
Other§	5 (9)	881	(362-2,384)	
Residual tumor				0.301*
< 1 cm	38 (68)	299	(10-3,583)	
≥ 1 cm	18 (32)	617	(10-32,695)	
Malignant cells in ascites				0.101*
no	25 (45)	162	(12-13,020)	
yes	28 (50)	535	(10-32,695)	
unknown	3 (5)			
Preoperative CA 125				0.010*
≤ 126 U/ml	27 (48)	114	(10-960)	
> 126 U/ml	26 (47)	594	(10-32,695)	
unknown	3 (5)			
Total	56 (100)	383	(10-32,695)	

**Mann-Whitney U test; **Kruskal-Wallis test; §clear cell, n = 4; undifferentiated, n = 1*

Disease free and overall survival

Of the 56 patients with epithelial ovarian cancer, 5 patients received neo-adjuvant chemotherapy before surgery and 21 patients did not receive chemotherapy at all or received less than 6 courses. These patients were therefore excluded and the remaining 30 patients were included for survival analysis. Surgery dates for this group of patients ranged between January 1996 and November 2007, and the follow-up period was at least 6 months after completing chemotherapeutic treatment (median: 26 months). Figure 2 shows the Kaplan-Meyer curves of the patients with GSTP1-1 values above 160 ng/ml and below 160 ng/ml of both DFS (Figure 2A) and of OS (Figure 2B). In the first group (GSTP1-1 >160 ng/ml), histological subtypes were serous ($n = 9$), mucinous ($n = 1$), endometrioid ($n = 4$), clear cell ($n = 1$), mixed ($n = 1$), and NOS ($n = 2$). Tumor grade was 1 ($n = 1$), 2 ($n = 6$), 3 ($n = 8$), and unknown ($n = 3$). In the second group

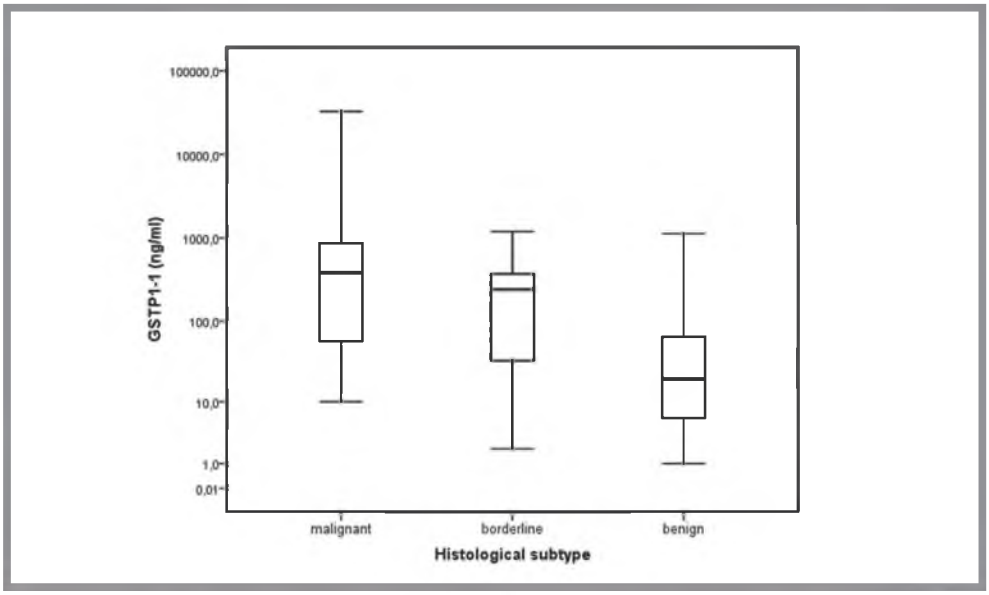

Figure 1. The boxplots of the GSTP1-1 (ng/ml) concentration in oCF by histological subtype of patients with malignant (n = 56), borderline (n = 21) and benign (n = 88) ovarian tumors .

(GSTP1-1 ≤160 ng/ml), histological subtypes were serous (n = 9), mucinous (n = 2), and endometrioid (n = 1). Tumor grade was 1 (n = 2), 2 (n = 4), 3 (n = 4), and unknown (n = 2). Higher levels of GSTP1-1 were related with both shorter DFS and shorter OS (log-rank test: p = 0.047 and p = 0.033, respectively). Of the group of patients with a high level of GSTP1-1, 45% (95% CI: 32-58) had recurrence of disease within 12 months compared to 17% (95% CI: 6-28) of the group of patients with a low level of GSTP1-1. After 24 months, this was 74% (95% CI: 60-88) and 47% (95% CI: 31-63), respectively. Similar results were found regarding the time to death. Two-year survival and 3-year survival were 82% (95% CI: 70-93) and 41% (95% CI: 25-57) for the group of patients with a high level of GSTP1-1 and 100% and 66% (95% CI: 45-87) for the group of patients with a low level of GSTP1-1, respectively.

Table 2 shows the hazard ratio with 95% CI, using the univariate proportional hazard model. For the variables "Malignant cells in ascites" and "Preoperative CA 125", in one case each, data were missing. GSTP1-1, FIGO stage, and presence of malignant cells in ascites were regarded as significant predictors of DFS. However, using the multivariate proportional hazard model with selection procedure, FIGO stage was the only independent predictor that had impact on DFS (HR = 9.8; 95% CI: 2.2 - 44.6; p < 0.01). As a result, the other factors did not contribute additionally to FIGO stage to predict the time to recurrence. Regarding the time to death we found that GSTP1-1, FIGO stage, and preoperative CA 125 level could be regarded as significant predictors of OS in univariate analysis. Now, we found, after the model selection procedure, that GSTP1-1 appeared to be the only independent prognostic factor that had impact on OS (HR = 8.3; 95% CI: 1.1-66.1; p = 0.045).

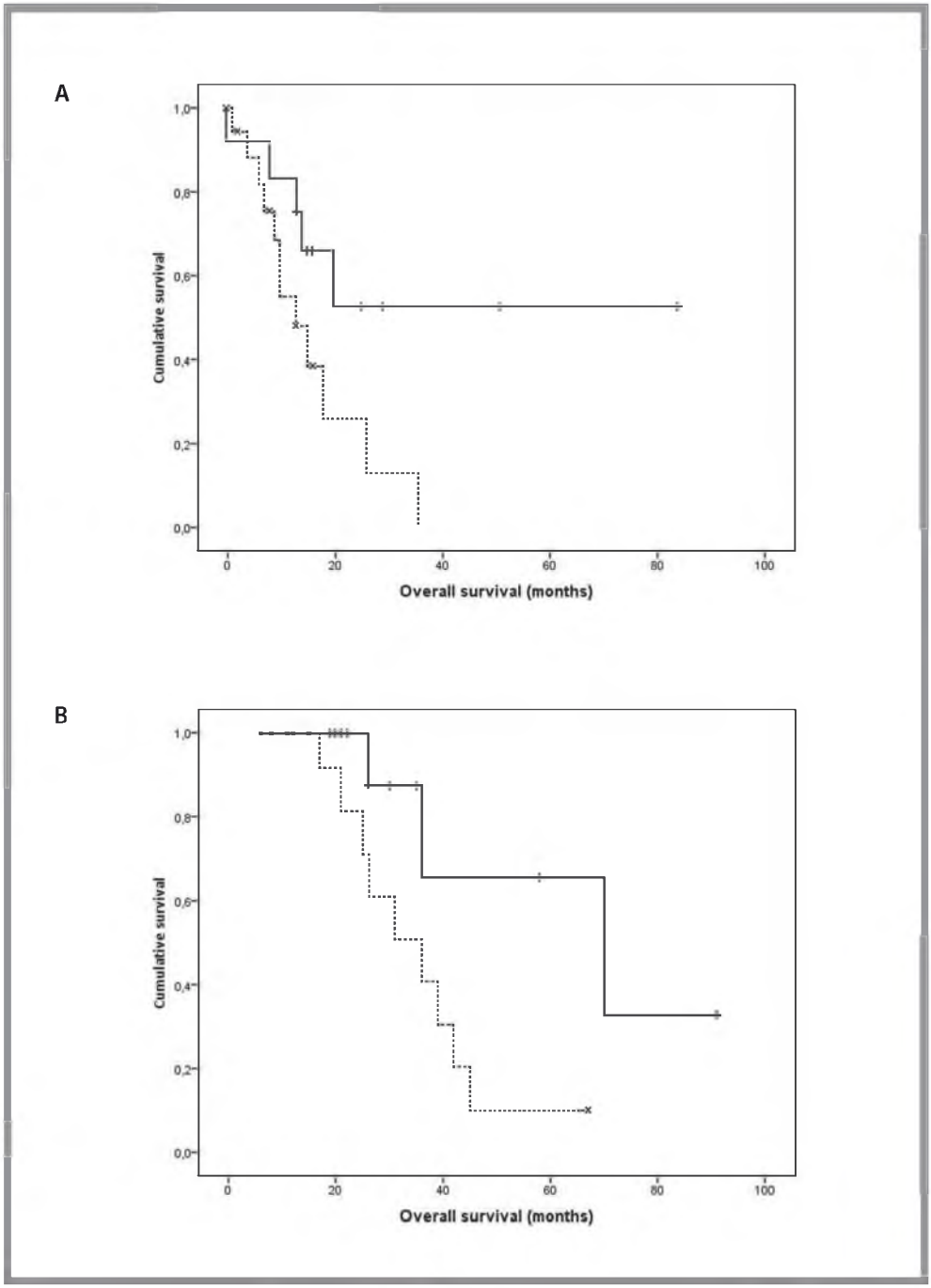

Figure 2. Kaplan-Meier estimates of disease free (A) and of overall survival (B) of patients who received chemotherapy ($n = 30$). The group with low GSTP1-1 values (≤160 ng/ml; solid line) and the group with high GSTP1-1 values (>160 ng/ml; broken line) include 12 and 18 patients, respectively. Vertical bars indicate patients with censored data.

Table 2. HR with 95% CI of DFS and of OS by clinicopathological parameters of patients with EOC who received 6 courses of adjuvant platinum-based chemotherapy ($n = 30$), using univariate Cox Regression

	n	DFS HR	(95% CI)	*p*-value	OS HR	(95% CI)	*p*-value
GSTP1-1							
low (≤ 160 ng/ml)	12	1.00	(Reference)		1.00	(Reference)	
high (> 160 ng/ml)	18	2.79	(0.97-8.04)	0.058*	4.50	(0.97-20.92)	0.055*
FIGO stage							
I + II	11	1.00	(Reference)		1.00	(Reference)	
III + IV	19	10.17	(2.25-45.99)	0.003*	3.12	(0.84-11.59)	0.089*
Residual disease							
< 1cm	18	1.00	(Reference)		1.00	(Reference)	
≥ 1cm	12	1.56	(0.52-4.67)	0.424	1.06	(0.28-4.03)	0.935
Malignant cells in ascites							
no	9	1.00	(Reference)		1.00	(Reference)	
yes	20	5.07	(1.14-22.65)	0.033*	2.30	(0.61-8.67)	0.218
Preoperative CA 125							
≤ 126 U/ml	11	1.00	(Reference)		1.00	(Reference)	
> 126 U/ml	18	2.65	(0.82-8.60)	0.104	4.97	(1.06-23.20)	0.041*

**Parameters with a p-value ≤ 0.10 were selected for multivariate analysis*

Discussion

In this study, levels of GSTP1-1 were significantly higher in patients with EOC compared with patients with benign ovarian tumors. In patients with EOC, levels of GSTP1-1 were correlated positively to FIGO stage, tumor grade and preoperative CA 125 levels. For the subgroup of patients that received 6 courses of chemotherapy, higher levels of GSTP1-1 were significantly associated with a poorer DFS. Although GSTP1-1 was not an independent predictor for DFS, it was the most important factor after FIGO stage, which was the only independent prognostic factor. Therefore, determination of GSTP1-1 in surgically obtained ovarian cyst fluid (oCF) might be of value to predict relapse of disease and response to chemotherapy at the time of diagnosis. In addition, GSTP1-1 seemed to be the only independent predictor of OS in multivariate analysis and therefore, might be of value to serve as a biomarker for survival.

Up until now, the only other study investigating GSTP1-1 levels in oCF of ovarian tumors was published by our own research group.[23] At that time, we also found higher levels of GSTP1-1 in oCF of malignant tumors compared with their benign counterparts. The present study included more patient samples, a longer follow-up and focused on the role of oCF GSTP1-1 as a prognostic biomarker for the response to chemotherapy of patients with EOC. All other studies that investigated the relationship between GSTP1-1 and clinical outcomes and/or response to chemotherapy in patients with EOC were done

Table 3. Published reports on outcomes of the relationship between GSTP1-1 levels and response to chemotherapy and survival including the methods used for tissue analysis of GSTP1-1

Reference	*n*	Method used for analysis of GSTP1-1	Correlation between GSTP1-1 and response to chemotherapy	Correlation between GSTP1-1 and survival
Van der Zee *et al.*	17	High performance liquid chromatography	No	–
Green *et al.*	78	Immunohistochemistry	Yes	Yes
Hamada *et al.*	61	Immunohistochemistry	Yes	Yes
Van der Zee *et al.*	89	Immunohistochemistry	No	No
Hirazono *et al.*	36	Immunohistochemistry	–	Yes
Wrigley *et al.*	66	Immunohistochemistry Western blotting	No No	No No
Ghazal-Aswad *et al.*	39	Immunohistochemistry	No	–
Cheng *et al.*	20	Immunohistochemistry Western blotting	Yes No	–
Tanner *et al.*	121	Western blotting	–	No
Kase *et al.*	87	Immunohistochemistry	Yes	–
Satoh *et al.*	67	Immunohistochemistry	–	Yes
Ikeda *et al.*	93	Immunohistochemistry	–	No
Howells *et al.*	77	Immunohistochemistry	No	No
Saip *et al.*	55	Immunohistochemistry	No	No

**Parameters with a p-value ≤ 0.10 were selected for multivariate analysis*

using ovarian cancer tissue. In Table 3, we summarized published studies in which GSTP1-1 was determined in ovarian cancer tissue and related to the clinical outcomes of patients with EOC. Some researchers found a relationship between overexpression of GSTP1 1 in malignant ovarian tissue and poor prognosis or bad response to chemotherapy, [8-13] whereas others could not detect such an association.[14-21] These conflicting results might be explained by differences in techniques used to quantify GSTP1-1 (Table 3). It has been demonstrated that immunohistochemical quantification of GSTP1-1 did not always correlate with the concentrations of GSTP1-1 as measured in the tissue cells by other methods, such as Western blot analysis.[9,14] Wrigley *et al.* [14] concluded that the Western blotting technique was more sensitive than immunohistochemistry because it detected GSTP1-1 that had been missed by observer examination of stained sections. In addition, different scoring systems to quantify GSTP1-1 by immunohistochemistry, observer variation in identifying positively stained cells, different cut-off points for discriminating high from low values, and different types of tissue, i.e. fresh versus formalin fixed and paraffin embedded may also be responsible for the conflicting findings. In two studies, researchers classified their results as either positive or negative, which resulted in a majority of GSTP1-1 positive samples and consequently, no correlation of GSTP1-1 expression with response to chemotherapy or survival could be achieved.[15,20] In addition, immunohistochemical GSTP1-1 expression in ovarian tumor sections was found to be heterogeneous and not uniform [16], which means that the tumor biology will probably not be optimally

reflected by analyzing one tissue biopsy. Therefore, the analysis of GSTP1-1 concentrations in body fluid samples, in which components are distributed homogeneously, might provide a valuable alternative. Ovarian cyst fluid has a direct relationship with the tumor tissue and compounds are homogeneously distributed. We believe that oCF might provide possible new prognostic biomarkers. In addition, measurement of GSTP1-1 by ELISA is an uncomplicated, sensitive and reliable method as compared to immunohistochemistry, which at best is only semiquantitative.

Our findings are consistent with our hypothesis that women with decreased levels of oCF GSTP1-1 would have a better survival due to improved response to chemotherapy. We are aware of the fact that we have only studied 30 patients who received chemotherapy and we therefore suggest that larger studies with more uniform samples and increased power are required to replicate our findings. In addition, little is known about the mechanism of release of GSTP1-1 from tumor tissue into ovarian oCF. Since GSTP1-1 enzymes are predominantly expressed in the cytosol of living cells, it might be possible that an increased release into the oCF occurs in case of tumor necrosis. Necrosis is often associated with the differentiation grade of the tumor, the latter of which was found to be a prognostic factor for survival.[26] In our study, high-grade tumors contained significantly higher oCF levels of GSTP1-1 compared to low-grade tumors. However, we believe that tumor grade was not a confounding factor for survival in the group of patients that received chemotherapy because almost all patients in this subgroup had high-grade tumors. We therefore conclude that assessment of GSTP1-1 level in ovarian oCF, obtained during primary debulking surgery, could provide information about future response to chemotherapy and therefore might be of clinical value as a prognostic biomarker.

In summary, to the best of our knowledge this is the first study in which the relationship between GSTP1-1 in oCF and treatment outcomes in patients with EOC was analyzed. We focused on the group of patients who received chemotherapy to assess if GSTP1-1 could be a marker which can be used for the early identification of platinum resistant disease. We found a significant association between high GSTP1-1 levels in oCF and poor DFS and OS. Moreover, GSTP1-1 seemed to be the only independent predictor for OS in multivariate analysis. Although GSTP1-1 did not seem to be an independent predictor of early recurrence, after FIGO stage, it was the most important factor for prediction of DFS. In addition, high GSTP1-1 values were found more often in patients with chemoresistant EOC compared to patients with chemosensitive disease. Therefore, a combination of FIGO stage and GSTP1-1 level might be of value in identifying patients with chemoresistant EOC in an early phase of the disease in order to change therapy and improve prognosis.

References

1. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43-66.
2. Quirk JT, Natarajan N. Ovarian cancer incidence in the United States, 1992-1999. Gynecol Oncol 2005;97:519-23.
3. Gonzalez-Martin A. Treatment of recurrent disease: randomized trials of monotherapy versus combination chemotherapy. Int J Gynecol Cancer 2005;15 Suppl 3:241-6.

4. Tew KD. Glutathione-associated enzymes in anticancer drug resistance. Cancer Res 1994;54:4313-20.
5. Beckett GJ, Hayes JD. Glutathione S-transferases: biomedical applications. Adv Clin Chem 1993;30:281-380.
6. Raunio H, Husgafvel-Pursiainen K, Anttila S et al. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility--a review. Gene 1995;159:113-21.
7. McIlwain CC, Townsend DM, Tew KD. Glutathione S-transferase polymorphisms: cancer incidence and therapy. Oncogene 2006;25:1639-48.
8. Green JA, Robertson LJ, Clark AH. Glutathione S-transferase expression in benign and malignant ovarian tumours. Br J Cancer 1993;68:235-9.
9. Cheng X, Kigawa J, Minagawa Y et al. Glutathione S-transferase-pi expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. Cancer 1997;79:521-7.
10. Hamada S, Kamada M, Furumoto H et al. Expression of glutathione S-transferase-pi in human ovarian cancer as an indicator of resistance to chemotherapy. Gynecol Oncol 1994;52:313-9.
11. Hirazono K, Shinozuka T, Kuroshima Y et al. Immunohistochemical expression of glutathione S-transferase pi (GST-pi) and chemotherapy response in malignant ovarian tumors. J Obstet Gynaecol 1995;21:305-12.
12. Satoh T, Nishida M, Tsunoda H et al. Expression of glutathione S-transferase pi (GST-pi) in human malignant ovarian tumors. Eur J Obstet Gynecol Reprod Biol 2001;96:202-8.
13. Kase H, Kodama S, Nagai E et al. Glutathione S-transferase pi immunostaining of cisplatin-resistant ovarian cancer cells in ascites. Acta Cytol 1998;42:1397-402.
14. Wrigley EC, McGown AT, Buckley H et al. Glutathione-S-transferase activity and isoenzyme levels measured by two methods in ovarian cancer, and their value as markers of disease outcome. Br J Cancer 1996;73:763-9.
15. Ghazal-Aswad S, Hogarth L, Hall AG et al. The relationship between tumour glutathione concentration, glutathione S-transferase isoenzyme expression and response to single agent carboplatin in epithelial ovarian cancer patients. Br J Cancer 1996;74:468-73.
16. Howells RE, Dhar KK, Hoban PR et al. Association between glutathione-S-transferase GSTP1 genotypes, GSTP1 over-expression, and outcome in epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:242-50.
17. Van der Zee AG, van Ommen B, Meijer C et al. Glutathione S-transferase activity and isoenzyme composition in benign ovarian tumours, untreated malignant ovarian tumours, and malignant ovarian tumours after platinum/cyclophosphamide chemotherapy. Br J Cancer 1992;66:930-6.
18. Tanner B, Hengstler JG, Dietrich B et al. Glutathione, glutathione S-transferase alpha and pi, and aldehyde dehydrogenase content in relationship to drug resistance in ovarian cancer. Gynecol Oncol 1997;65:54-62.
19. Ikeda K, Sakai K, Yamamoto R et al. Multivariate analysis for prognostic significance of histologic subtype, GST-pi, MDR-1, and p53 in stages II-IV ovarian cancer. Int J Gynecol Cancer 2003;13:776-84.
20. Van der Zee AG, Hollema H, Suurmeijer AJ et al. Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol 1995;13:70-8.
21. Saip P, Tuzlali S, Demir K et al. Value of glutathion-S transferase pi as a prognostic factor in epithelial ovarian carcinoma. Eur J Gynaecol Oncol 2005;26:90-4.
22. Mulder TP, Peters WH, Wobbes T et al. Measurement of glutathione S-transferase P1-1 in plasma: pitfalls and significance of screening and follow-up of patients with gastrointestinal carcinoma. Cancer 1997;80:873-80.
23. Boss EA, Peters WH, Roelofs HM et al. Glutathione S-transferases P1-1 and A1-1 in ovarian cyst fluids. Eur J Gynaecol Oncol 2001;22:427-32.
24. Pecorelli S, Benedet JL, Creasman WT et al. FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
25. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
26. Malpica A, Deavers MT, Lu K et al. Grading ovarian serous carcinoma using a two-tier system. Am J Surg Pathol 2004;28:496-504.

7

N-acetyl resonances in *in vivo* and *in vitro* ^{1}H-NMR spectroscopy of cystic ovarian tumors

Kolwijck E, Engelke UF, Van der Graaf M, Heerschap A, Blom HJ, Hadfoune M, Buurman WA , Massuger LFAG, Wevers RA

NMR in Biomed 2009;22(10):1093-9

Abstract

An unassigned and prominent resonance in the region from δ 2.0-2.1 ppm has frequently been found in the *in vivo* magnetic resonance (MR) spectra of cancer patients. We demonstrated the presence of this resonance with *in vivo* MR spectroscopy in the cyst fluid of a patient with an ovarian tumor. Proton nuclear magnetic resonance spectroscopy on the aspirated cyst fluid of this patient confirmed the observation. A complex of resonances was observed between 2.0 and 2.1 ppm. It was also present in 11 additional ovarian cyst fluid samples randomly chosen from our biobank. The resonance complex was significantly more prominent in samples from mucinous tumors than in samples from other histological subtypes. A macromolecule (>10 kDa) was found responsible for this complex of resonances. A correlation spectroscopy (COSY) experiment revealed cross peaks of two different types of bound sialic acid suggesting that N-glycans from glycoproteins and/or glycolipids cause this resonance complex. In literature, plasma alpha 1-acid glycoprotein (AGP), known for its high content of N-linked glycans, has been suggested to contribute to the δ 2.0-2.1 spectral region. The AGP cyst fluid concentration did not correlate significantly with the peak height of the δ 2.0-2.1 resonance complex in our study. AGP may be partly responsible for the resonance complex but other N-acetylated glycoproteins and/or glycolipids also contribute. After deproteinization of the cyst fluid N-acetyl aspartic acid (NAA) was found to contribute significantly to the signal in this spectral region in three of the 12 samples. GC-MS independently confirmed the presence of NAA in high concentration in the three samples, which all derived from benign serous tumors. We conclude that both NAA and N-acetyl groups from glycoproteins and/or glycolipids may contribute to the δ 2.0-2.1 ppm resonance complex in ovarian cyst fluid. This spectral region seems to contain resonances from biomarkers that provide relevant clinical information on the type of ovarian tumor.

Introduction

Ovarian cancer is the most lethal gynecological malignancy worldwide.[1] Due to the lack of specific clinical symptoms in an early stage of disease, patients are generally diagnosed with advanced stage ovarian cancer and have a 5-year overall survival of less than 20%.[2] Despite more than two decades of research, appropriate biomarkers for detection, diagnosis and prognosis of ovarian cancer are still lacking.

Ovarian tumors, benign as well as malignant, mostly present as cystic masses that contain variable amounts of fluid.[3] The cyst fluid has been shown to contain compounds released by the tumor tissue, [4,5] and therefore, might provide biomarkers for ovarian cancer. Although researchers have become increasingly interested in the analysis of the biochemical composition of cyst fluid, only a few studies have been performed investigating cyst fluid using magnetic resonance spectroscopy (MRS).[6-11] Boss *et al.* [6] were the first to investigate the overall metabolic composition of ovarian cyst fluid by *in vitro* proton nuclear magnetic resonance spectroscopy (^{1}H-NMRS). They found detectable concentrations for 36 metabolites. For several metabolites they demonstrated significant differences in concentration between malignant and benign ovarian cysts. Studies performed with *in vivo* MRS on patients with ovarian tumors focused on lipid resonances at 1.3 ppm and 5.2 ppm, choline, lactate and creatine.[8,9,11] However, in a recently published *in vivo* MRS paper, a prominent and unknown resonance at 2.07 ppm was described in cystic and solid parts of 8 of the 14 ovarian tumors.[10] Although yet unassigned, the authors suspected this peak to be a resonance of N-acetyl-L-aspartic acid (NAA) or sialic acid (N-acetylneuraminic acid). For human plasma, it has been suggested that N-acetyl groups in alpha 1-acid glycoprotein (AGP) might contribute to a resonance complex in this spectral region.[12] The carbohydrate content of AGP is very high (45% of its molecular weight) and consists of 5 or 6 highly sialylated complex type N-linked glycans.[13]

In the present study, we investigated the presence and nature of the unassigned resonance at 2.1 ppm in the magnetic resonance spectra of cystic ovarian tumors. At first, *in vivo* MRS was performed on a patient with a cystic ovarian tumor. Subsequently, the cyst fluid from this patient and 11 additional cyst fluid samples from our biobank were investigated by *in vitro* ^{1}H-NMRS and by independent techniques specific for NAA and AGP.

Materials and methods

In vivo MR spectroscopy

A 57-year-old patient (patient 1) was admitted to our Department of Obstetrics and Gynecology with an enlarged left ovary revealed by ultrasound examination. After informed consent was obtained, the patient was included in our *in vivo* MRS study. The patient was positioned inside the magnet bore of a 3.0 Tesla MR system (Magnetom Tim Trio, Siemens, Erlangen, Germany). She was positioned with her back on the supine coil matrix and a body matrix coil was placed on top of the patient. Of both coil arrangements one to two three-element coil arrays were used for resonance reception during the MR examination. Conventional MR images revealed a 28 cm x 21 cm tumor of the left ovary. Subsequently, a voxel of interest (3cm x 3cm x 3cm) was selected in the inferior cystic part of the tumor (Figure 1). MR spectra were acquired by using a point-resolved spectroscopy (PRESS) [14,15] sequence at a short echotime (TE) of 30 ms and a long TE of 136 ms to obtain optimal information of as many metabolites as possible. Metabolite spectra were acquired with WET water suppression, [16] a repetition time of 2 seconds and 64 averages. In addition, non-suppressed water spectra were recorded for referencing. Total examination time was 45 minutes.

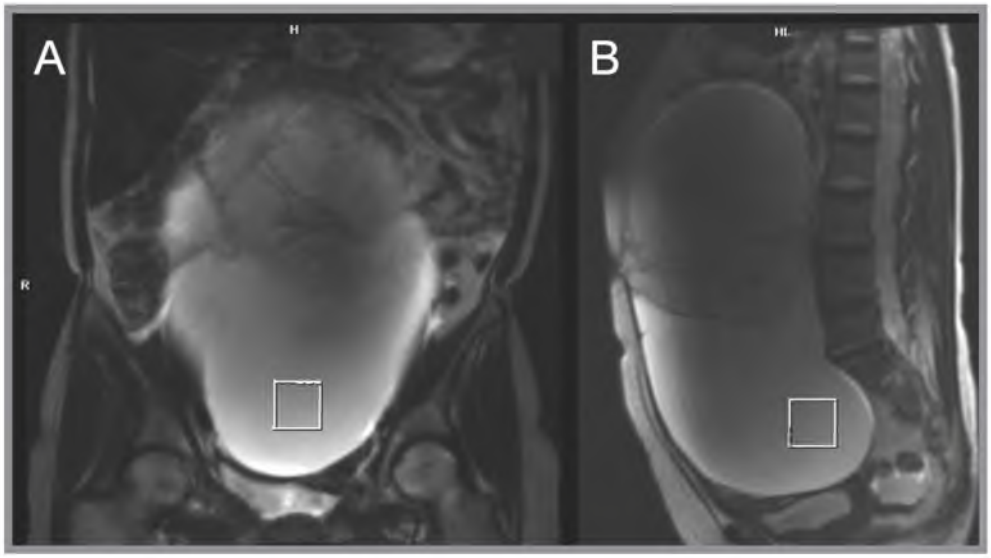

Figure 1. Frontal (A) and sagittal (B) MR image (3.0 T) of a 57-year-old patient with an ovarian tumor. The voxel of interest (30 cc) was placed in the inferior cystic part of the tumor.

Cyst fluid collection

After surgical removal, the ovarian tumor of the patient was transported to the Department of Pathology and collection of 40 ml fluid from the inferior part of the tumor (Figure 1) was performed by aseptic fine needle aspiration. Cyst fluid samples from 11 other patients (obtained from 3 malignant, 3 borderline and 6 benign epithelial ovarian tumors) were randomly selected from our cyst fluid biobank (Table 1). These samples were obtained from patients who underwent primary surgery for an adnexal mass at the Radboud University Nijmegen Medical Center in the period 2000 until 2008. After cooled transport to the laboratory, all cyst fluid samples were centrifuged at 3000 *g* for 10 minutes for cell separation and immediately stored at -70 °C in multiple small portions. Histopathological diagnosis was performed by a pathologist specialized in gynecology. Informed consent was obtained from all patients.

Table 1. Clinicopathological characteristics of 12 patients with ovarian tumors

Patient No.	Age (years)	Pathology	Histological subtype
1 (case report)	56	Borderline	Serous
2	55	Borderline	Mucinous
3	63	Borderline	Mucinous
4	66	Malignant	Mucinous/Endometrioid
5	60	Malignant	Serous
6	69	Malignant	Serous
7	29	Benign	Serous
8	56	Benign	Serous
9	20	Benign	Serous
10	62	Benign	Mucinous
11	39	Benign	Mucinous
12	55	Benign	Mucinous

**Parameters with a p-value ≤ 0.10 were selected for multivariate analysis*

Sample preparation for *in vitro* ^{1}H-NMR spectroscopy

Cyst fluid samples (n = 12) were measured in three different ways: (1) in their native state (not deproteinized or pH standardized); (2) after deproteinization over a 10 kDa cut-off filter (Sartorius, 3000 *g* for 70 min; before use, the filter was washed twice by centrifugation with water to remove glycerol); (3) as in (2) but additionally pH adjusted to 2.50 ± 0.10. For analysis 50 µl D_2O and 20 µl TSP were added to 700 µl sample.

One-dimensional *in vitro* ^{1}H-NMR spectroscopy

Single pulse ^{1}H-NMR spectra (500 MHz) were obtained on a Bruker DMX-500 spectrometer as described by Engelke *et al.*[17] Sample volume in the spectrometer was standardized at 650 µl and samples were placed in 5 mm NMR tubes. For evaluation of the NMR spectra AMIX software (Bruker, Germany) was used. Chemical shifts for the samples containing protein were referenced internally to the lactate resonance at δ 1.33. Chemical shifts for deproteinized samples were calibrated with respect to the position of the TSP resonance. Phase and baseline were corrected manually. Identification of the resonances in the NMR spectra was performed by using our ovarian cyst fluid model compound database.[6]

^{1}H-^{1}H correlation spectroscopy (COSY)

A two-dimensional ^{1}H-^{1}H COSY 500 MHz spectrum was recorded from the cyst fluid from patient 1. A spectral width of 6010 Hz in both dimensions, 256 and 2000 data points in F_1 and F_2, respectively, 16 scans per increment and a recycle delay of 6 sec were used. Prior to Fourier transformation, both time domains were apodized using a sine bell function and zero filled once.[17]

Quantitative data

Quantification of the resonance complex in the region of 2.0-2.1 ppm of NMR spectra was performed by its peak intensity normalized to the peak intensity of the lactic acid resonance (arbitrary units). Lactic acid was independently measured and enzymatically determined on a COBAS FARA centrifugal analyzer (Hoffmann La Roche, Basel, Switzerland).[18] Differences in concentration of the compound(s) contributing to the δ 2.0-2.1 ppm resonance complex between tumor type groups were determined using Mann-Whitney and Kruskal-Wallis tests. Pearson's test was used to establish the correlation between AGP and the resonance complex.

N-acetyl-L-aspartic acid (NAA) was measured using a modified stable isotope dilution GC-MS method as essentially described by Jakobs *et al.*[19]

AGP concentrations were measured using ELISA as reported previously.[20] In summary, ELISA plates were coated overnight with polyclonal anti-human AGP obtained from Dako (Glostrup, Denmark). Diluted plasma samples and a standard dilution series with human and rAGP, were added to the plate. Detection was carried out with a biotinylated polyclonal rabbit anti-human AGP IgG, followed by peroxidase-conjugated streptavidin and substrate essentially as described by De Vries *et al.*[21]

Results

In vivo MR spectroscopy

In vivo MR spectra from a voxel in the inferior cystic part of the tumor of patient 1 are shown in Figure 2A and 2B. Both spectra with echo times of 30 and 136 ms contained resonances of lactic acid (1.33 ppm), which was inverted in the MR spectrum with the longer echo time (Figure 2B). A prominent and unknown resonance was observed with a high peak intensity at 2.06 ppm similar to the observations of Stanwell *et al.*[10]

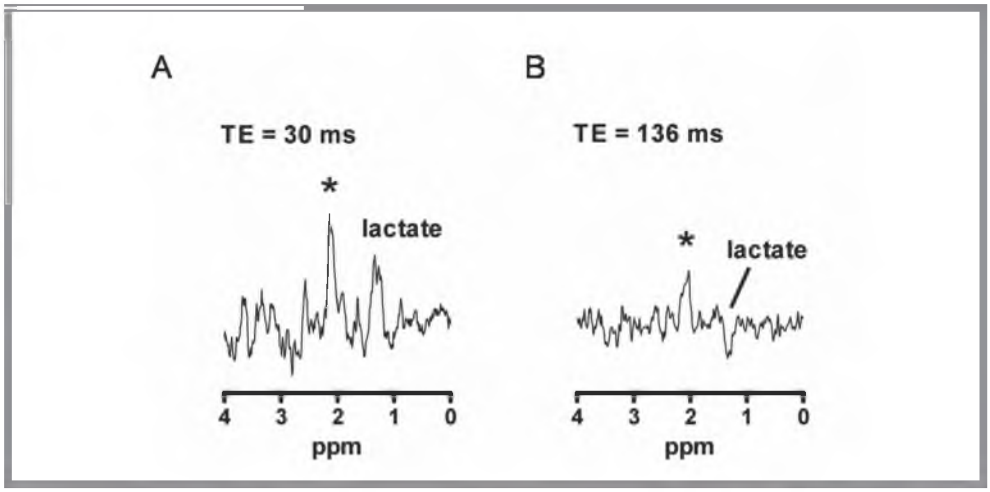

Figure 1. *In vivo* MR spectra of the cyst fluid of our patient with echo times of 30 (A) and 136 (B) ms. An abnormal resonance at 2.06 ppm is indicated by an asterisk.

In vitro ^{1}H-NMR spectroscopy

Additional information on the unknown resonance at 2.06 ppm in the *in vivo* MR spectrum was provided by *in vitro* ^{1}H-NMRS performed on the aspirated cyst fluid of 12 patients before and after deproteinization.

(1) Native cyst fluid. For the first analysis, no sample pretreatment was performed to imitate the *in vivo* spectra. For patient 1, the native cyst fluid NMR spectrum (Figure 3A) shows many resonances, including the resonances from lactic acid (1.33 ppm). Furthermore, the spectrum shows a complex of several overlapping resonances with high intensities in the region from 2.0 to 2.1 ppm, observed as one broad peak at 2.06 ppm in the *in vivo* MR spectrum (Figure 2A and 2B). In this spectral region, CH_3 protons

from N-acetyl groups characteristically give a singlet resonance.[22] A COSY experiment on the native cyst fluid of patient 1 was performed (Figure 4). Cross peaks of two different types of bound sialic (N-acetylneuraminic) acid were observed (1.83/2.79 ppm from α 2→3 bound sialic acid and 1.75/2.70 ppm from α 2→6 bound sialic acid).[23] These signals may derive from glycoproteins like AGP that carry N-glycans. Signals from N-acetyl protons in N-acetylglucosamine residues from the same N-glycans may contribute to the resonance complex between 2.0 and 2.1 ppm in Figure 3A. Subsequently, we studied the native cyst fluid of 11 additional samples and found this resonance complex in all samples. In the *in vitro* cyst fluid spectra of patient 7-9 (Table 1) an additional high singlet resonance was observed at 2.03 ppm (Figure 5A) that was identified as NAA (see below). Peak intensities of the resonance complex between δ 2.0 and 2.1 ppm for all patients are listed in Table 2.

(2) Deproteinized cyst fluid. After deproteinization the lactic acid resonance at 1.33 ppm was still visible in the same intensity in the cyst fluid of patient 1. However, the resonance complex between δ 2.0 to 2.1 ppm had fully disappeared (Figure 3B). This observation was confirmed in the other 11 cyst fluid samples from our biobank (data not shown). In 3 samples (patient numbers 7-9), the broad complex resonance disappeared but left a sharp resonance at 2.03 ppm (Figure 5B). Subsequently, the deproteinized cyst fluid samples were pH adjusted to pH 2.50 to compare with our model compound data base and assign the various metabolites (Figure 3C).[6] For patient 1, high intensities were visible for lactic acid (doublet 1.41 ppm), alanine (doublet 1.51 ppm), 3-hydroxybutyric acid (doublet 1.23 ppm) and acetic acid (singlet 2.08 ppm). Resonances deriving from NAA could not be observed. Spectra of cyst fluid samples from patients 7-9 showed the sharp resonance at 2.03 ppm (Figure 5B). This resonance derives from the N-acetyl group of NAA. Figure 5B shows the 2.03 ppm resonance and as well the 2.95 ppm multiplet resonance of the NAA methylene proton.[22] NAA could not be detected in the NMR spectra of the other 8 samples (data not shown).

NAA was measured independently by GC-MS (Table 2). In patient 1, cyst fluid NAA was 1.0 µmol/L, which is below the detection limit of *in vitro* ^{1}H-NMRS and *in vivo* MRS. In the samples of patients 7-9, NAA amounted to 28, 38 and 95 µmol/L, respectively, in line with the *in vitro* NMR spectral observation. These three cyst fluid samples all derived from benign serous ovarian tumors. In the remaining samples, NAA was or equal to or less than 1.5 µmol/L.

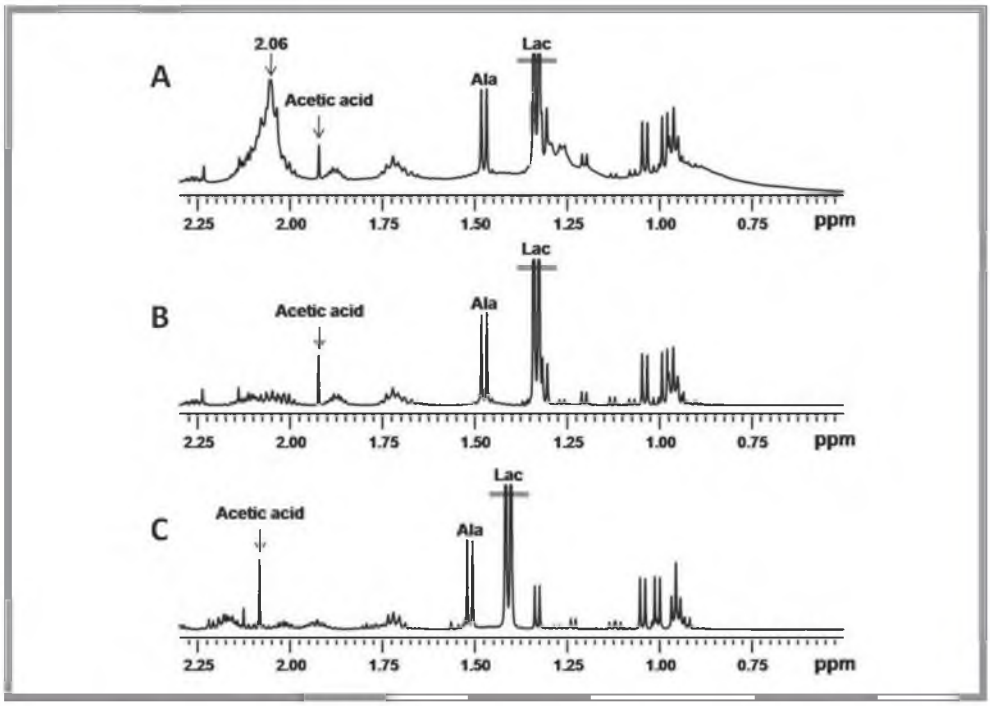

Figure 3. *In vitro* ^{1}H-NMR spectra (500 MHz) of three cyst fluid samples of our 57-year-old patient (A,B,C).
(A) Native cyst fluid (not deproteinized or pH adjusted). A broad resonance with high intensity is visible between δ 2.0-2.1 ppm.
(B) Deproteinized cyst fluid at native pH. The resonance with high intensity at 2.06 ppm has fully disappeared.
(C) Deproteinized cyst fluid adjusted to pH 2.50.

Quantitative data

The relative concentration of the compound or compounds causing the resonance complex between δ 2.0 to 2.1 ppm in the native *in vitro* NMR spectra was determined in arbitrary units by comparing the peak intensity of resonance complex to the independently measured concentration of lactic acid (Table 2). The concentration varied considerably in the 12 samples (range: 20-252). No significant difference in concentration was observed between patients with malignant, patients with borderline and patients with benign ovarian tumors ($p = 0.707$, Kruskal-Wallis test). Patients with mucinous tumors had significantly higher concentration of the compound(s) contributing to the complex (median: 217; range: 147-252) compared to patients with other histological subtypes (median: 133.4; range: 20-162) ($p = 0.016$, Mann-Whitney test).

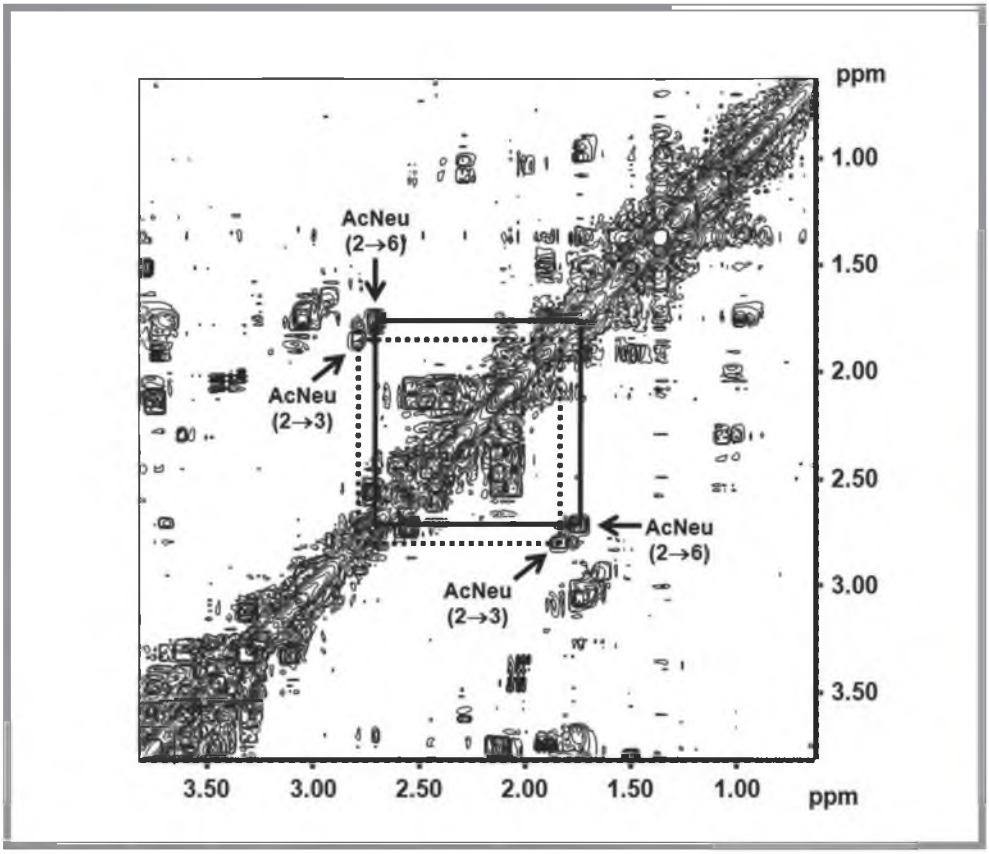

Figure 4. *In vitro* COSY spectrum (500 MHz) of the cyst fluid of patient 1. Cross peaks of two different types of bound sialic acid (N-acetylneuraminic acid) are present: 1.83/2.79 ppm from α 2→3 bound sialic acid and 1.75/2.70 ppm from α 2→6 bound sialic acid.

The AGP concentration in the 12 cyst fluid samples was measured independently by ELISA. The protein was present in detectable quantities in all samples (Table 2). The median AGP concentration in cyst fluid samples in this study was 351 µg/ml (range: 4-436 µg/ml). The AGP concentration did not correlate significantly with the concentration of the compound(s) contributing to the δ 2.0 to 2.1 ppm resonance complex (p = 0.412; R = 0.313, Pearson's test). Thus, no evidence to confirm that the presence of AGP alone can explain the detected resonance complex in the region between δ 2.0 to 2.1 ppm. on the other hand, it seems more likely that composite N-acetyl signals from the N-acetylated sugars N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) present in N-glycans of glycoproteins and/or glycolipids cause this broad resonance complex.

Discussion

In several types of cancer (including colorectal, gastric, breast, cervical, prostate and ovarian), an unassigned and prominent resonance in the region from δ 2.0 to 2.1 ppm has frequently been found by MRS, and its intensity has often been associated with tumor invasion or malignancy.[24-29] In most studies, this resonance was assigned to the $-CH_3$ moiety of sialic acid or N-acetyl groups of glycoproteins, [24-26,29] but in other studies monounsaturated fatty acyl protons of lipids were mentioned.[27,28] Recently, a broad resonance at 2.1 ppm has also been found in the cyst fluid of 8 out of 14 patients with ovarian tumors by *in vivo* MRS.[10] The authors suggested that the peak most likely derived from NAA or from the $-CH_3$ moiety of sialic acid, based on two-dimensional ^{1}H-NMRS data (not shown) of a study on ovarian tumor tissue specimens.[29] In addition, three individual cases of a cystic ovarian tumor were reported in which a prominent resonance in the region from δ 2.0 to 2.1 ppm was found by *in vivo* MRS.[9,11,30] However, in these studies, the nature of the resonance was not further confirmed by independent techniques.

The present study revealed a prominent resonance at 2.06 ppm in the *in vivo* MR spectrum of a patient with a cystic serous ovarian tumor. The presence of the resonance in the spectrum acquired with an echo time of 136 ms suggested that this resonance could originate from NAA because resonances originating from slow-tumbling molecules such as protein complexes generally disappear rapidly with increasing echo time due to a short T2 relaxation time. However, the resonance in the long TE spectrum appeared to be broadened, which is not expected for NAA. In addition, the long T2 of the 2.06 ppm resonance was most probably caused by the flexibility of the carbohydrate chains which explains why such a macromolecule resonance survives the spin-echo delay period.

Using one-dimensional and two-dimensional *in vitro* ^{1}H-NMRS on a series of 12 ovarian cyst fluid samples, we showed that both NAA and N-acetylated macromolecules may contribute to the 2.06 ppm *in vivo* MRS resonance. By *in vitro* ^{1}H-NMRS, the unknown resonance was observed as a complex of several resonances in the region from δ 2.0 to 2.1 ppm. It was seen in all cyst fluid samples in our series. We showed that resonances from N-acetyl groups from macromolecules occurred in all cyst fluid samples. An additional resonance contribution of the N-acetyl group of NAA was observed in 3 out of 12 cyst fluid samples. NAA is a neuronal marker for brain *in vivo* MRS studies. Its concentration is one of the highest of all free amino acids in the central nervous system.[31,32] Yet the functions served by this metabolite in the brain remain elusive and controversial. Outside the brain the presence of NAA has been described in the lens of the eye, in peritoneal mast cells, and in ovarian cyst fluid.[6,33,34] Our study confirms and extends the presence of NAA in ovarian cyst fluid samples. Using the sensitive GC-MS technique it could be detected in a low concentration in all ovarian cyst fluid samples (Table 2). A significantly higher concentration was found in 3 of 12 samples with two independent techniques. These three derive from benign serous tumors. Boss *et al.* [6] observed a high NAA concentration in 5 benign serous cyst fluid samples. Therefore, finding increased NAA seems a biomarker for benign serous cysts. We did not find high NAA in malignant cyst fluids in line with the findings of Boss *et al.*

Further studies with higher numbers of samples are required to decide that a high concentration of NAA does not occur in malignant cysts. The function of NAA in ovarian cysts is as yet unclear. Interestingly, Baslow proposed a supporting role for NAA as a molecular water pump in central nervous system neurons. The molecular water pump can actively pump water against its gradient.[32] Our data show that the three cyst fluid samples with significantly increased NAA had the lowest concentration of the unassigned resonance complex, the lowest concentrations of the majority of metabolites, and of AGP as well, suggesting that these samples are more diluted than the others. This may touch on a molecular water pump function of NAA in the ovarian cyst.

Besides the NAA resonance at 2.03 ppm, there was a significant resonance complex in the region between 2.0 and 2.1 ppm in the *in vitro* NMR spectrum in all 12 cyst fluid samples. This resonance complex is caused by contributions from one or more macromolecules with N-acetylgroups. Two-dimensional COSY showed that the N-acetyl groups from α 2→6 bound and α 2→3 bound sialic acid groups in the N-glycans of glycoproteins contribute. Other N-acetylated sugars such as N-acetylglucosamine from the same N-glycans may also contribute to the resonance complex between δ 2.0 and 2.1 ppm. It cannot be excluded that N-acetylgroups from glycolipids also contribute. In human plasma, a similar prominent complex of resonances has been found.[12] Nicholson *et al.* [12] suggested that N-acetyl protons from AGP might significantly contribute to this resonance. AGP is a 41-43 kDa plasma protein with a high carbohydrate content (45%) of N-linked glycans of which 12% consists of sialic acid.[13] These authors suggested that other glycoproteins and $CH_2C{=}C$ signals from plasma lipoproteins might further contribute to the broad resonance complex.[12] We found that AGP was actually present in significant concentration in all cyst fluid samples (Table 2). Its concentration does not correlate with the peak height or peak area of the 2.0-2.1 ppm resonance complex or the concentration of the compounds contributing to it. Therefore, we conclude that this resonance must be explained in a similar way as in plasma with contributions from N-acetyl protons of AGP and other glycoproteins. Ovarian cyst fluid is known to contain high levels of glycoproteins.[35,36] It cannot be excluded that glycolipids and $CH_2C{=}C$ protons from lipoproteins also contribute to the resonance complex. An increase in size and branching of N-linked glycans of serum proteins has been associated with cancer, including ovarian cancer.[37,38] In the present study, no difference in concentration of the δ 2.0-2.1 ppm resonance complex was found between patients with malignant, borderline and benign ovarian tumors. The resonance was significantly more prominent in cyst fluid of mucinous tumors than in cyst fluid of other histological subtypes. Cyst fluid from mucinous tumors is known to contain high levels of N-acetylated glycoproteins which may explain our observation.[35,39]

In this study, *in vivo* and *in vitro* MR spectroscopic techniques were used to study an unassigned and prominent resonance complex between 2.0 and 2.1 ppm in ovarian cyst fluids. It was present in all ovarian cyst fluid samples. We demonstrated that NAA and N-acetyl groups from glycoproteins and/or glycolipids may both contribute to the δ 2.0-2.1 ppm resonance complex. This spectral region seems to contain resonances from biomarkers that provide relevant clinical information on the type of ovarian tumor.

Acknowledgements

The authors thank Ms. Ivonne van Loosbroek (Department of Radiology, Radboud University Nijmegen Medical Center, Nijmegen) for performing the *in vivo* MRS measurements of our patient.

References

1. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96.
2. Colombo N, Van Gorp T, Parma G et al. Ovarian cancer. Crit Rev Oncol Hematol 2006;60:159-79.
3. Kurman RJ. Blaustein's pathology of the female genital tract. 5th ed. New York: Springer-Verlag;2002.
4. Bar JK, Harlozinska A, Sobanska E et al. Relation between ovarian carcinoma-associated antigens in tumor tissue and detached cyst fluid cells of patients with ovarian neoplasms. Tumori 1994;80:50-5.
5. Harlozinska A, Bar JK, Jothy S et al. Carcinoembryonic antigen isotypes in tissue sections and loose cyst fluid cells of ovarian neoplasms. Tumour Biol 1993;14:1-8.
6. Boss EA, Moolenaar SH, Massuger LF et al. High-resolution proton nuclear magnetic resonance spectroscopy of ovarian cyst fluid. NMR Biomed 2000;13:297-305.
7. Massuger LF, van Vierzen PB, Engelke U et al. 1H-magnetic resonance spectroscopy: a new technique to discriminate benign from malignant ovarian tumors. Cancer 1998;82:1726-30.
8. Hascalik S, Celik O, Sarac K et al. Metabolic changes in pelvic lesions: findings at proton MR spectroscopic imaging. Gynecol Obstet Invest 2005;60:121-7.
9. Cho SW, Cho SG, Lee JH et al. In-vivo proton magnetic resonance spectroscopy in adnexal lesions. Korean J Radiol 2002;3:105-12.
10. Stanwell P, Russell P, Carter J et al. Evaluation of ovarian tumors by proton magnetic resonance spectroscopy at three Tesla. Invest Radiol 2008;43:745-51.
11. Okada T, Harada M, Matsuzaki K et al. Evaluation of female intrapelvic tumors by clinical proton MR spectroscopy. J Magn Reson Imaging 2001;13:912-7.
12. Nicholson JK, Foxall PJ, Spraul M et al. 750 MHz 1H and 1H-13C NMR spectroscopy of human blood plasma. Anal Chem 1995;67:793-811.
13. Hochepied T, Berger FG, Baumann H et al. Alpha(1)-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. Cytokine Growth Factor Rev 2003;14:25-34.
14. Bottomley PA. Spatial localization in NMR spectroscopy in vivo. Ann N Y Acad Sci 1987;508:333-48.
15. Ordidge RJ, Van de Vyver FL. Re: Separate water and fat MR images. Radiology 1985;157:551-3.
16. Ogg RJ, Kingsley PB, Taylor JS. WET, a T1- and B1-insensitive water-suppression method for in vivo localized 1H NMR spectroscopy. J Magn Reson B 1994;104:1-10.
17. Engelke UF, Kremer B, Kluijtmans LA et al. NMR spectroscopic studies on the late onset form of 3-methylglutaconic aciduria type I and other defects in leucine metabolism. NMR Biomed 2006;19:271-8.
18. De Vries J, Thijssen WA, Snels SE et al. Intraoperative values of S-100 protein, myelin basic protein, lactate, and albumin in the CSF and serum of neurosurgical patients. J Neurol Neurosurg Psychiatry 2001;71:671-4.
19. Jakobs C, ten Brink HJ, Langelaar SA et al. Stable isotope dilution analysis of N-acetylaspartic acid in CSF, blood, urine and amniotic fluid: accurate postnatal diagnosis and the potential for prenatal diagnosis of Canavan disease. J Inherit Metab Dis 1991;14:653-60.
20. Van Dielen FM, Van 't Veer C, Schols AM et al. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. Int J Obes Relat Metab Disord 2001;25:1759-66.
21. De Vries B, Walter SJ, Wolfs TG et al. Exogenous alpha-1-acid glycoprotein protects against renal ischemia-reperfusion injury by inhibition of inflammation and apoptosis. Transplantation 2004;78:1116-24.
22. Engelke UF, Liebrand-Van Sambeek ML, de Jong JG et al. N-acetylated metabolites in urine: proton nuclear magnetic resonance spectroscopic study on patients with inborn errors of metabolism. Clin Chem 2004;50:58-66.
23. Dorland L, Haverkamp J, Vliegenthart JF et al. 360-MHz 1H nuclear-magnetic-resonance spectroscopy of sialyl-oligosaccharides from patients with sialidosis (mucolipidosis I and II). Eur J Biochem 1978;87:323-9.
24. Mun CW, Cho JY, Shin WJ et al. Ex vivo proton MR spectroscopy (1H-MRS) for evaluation of human gastric carcinoma. Magn Reson Imaging 2004;22:861-70.

25. Mackinnon WB, Huschtscha L, Dent K et al. Correlation of cellular differentiation in human colorectal carcinoma and adenoma cell lines with metabolite profiles determined by 1H magnetic resonance spectroscopy. Int J Cancer 1994;59:248-61.
26. Schiebler ML, Miyamoto KK, White M et al. In vitro high resolution 1H-spectroscopy of the human prostate: benign prostatic hyperplasia, normal peripheral zone and adenocarcinoma. Magn Reson Med 1993;29:285-91.
27. Mountford C, Ramadan S, Stanwell P et al. Proton MRS of the breast in the clinical setting. NMR Biomed 2009;22:54-64.
28. Mahon MM, Williams AD, Soutter WP et al. 1H magnetic resonance spectroscopy of invasive cervical cancer: an in vivo study with ex vivo corroboration. NMR Biomed 2004;17:1-9.
29. Mackinnon WB, Russell P, May GL et al. Characterization of human ovarian epithelial tumors (ex vivo) by proton magnetic resonance spectroscopy. Int J Gynecol Cancer 1995;5:211-21.
30. Hascalik S, Celik O, Sarac K et al. Clinical significance of N-acetyl-L-aspartate resonance in ovarian mucinous cystadenoma. Int J Gynecol Cancer 2006;16:423-6.
31. Sibtain NA, Howe FA, Saunders DE. The clinical value of proton magnetic resonance spectroscopy in adult brain tumours. Clin Radiol 2007;62:109-19.
32. Baslow MH. N-acetylaspartate in the vertebrate brain: metabolism and function. Neurochem Res 2003;28:941-53.
33. Baslow MH, Yamada S. Identification of N-acetylaspartate in the lens of the vertebrate eye: a new model for the investigation of the function of N-acetylated amino acids in vertebrates. Exp Eye Res 1997;64:283-6.
34. Burlina AP, Ferrari V, Facci L et al. Mast cells contain large quantities of secretagogue-sensitive N-acetylaspartate. J Neurochem 1997;69:1314-7.
35. Yang Z, Wu JH, Kuo HW et al. Expression of sialyl Lex, sialyl Lea, Lex and Ley glycotopes in secreted human ovarian cyst glycoproteins. Biochimie 2009;91:423-33.
36. Yanagi K, Ohyama K, Yamakawa T et al. Biochemical characterization of glycoprotein components in human ovarian cyst fluids by lectins. Int J Biochem 1990;22:659-63.
37. Saldova R, Wormald MR, Dwek RA et al. Glycosylation changes on serum glycoproteins in ovarian cancer may contribute to disease pathogenesis. Dis Markers 2008;25:219-32.
38. Arnold JN, Saldova R, Hamid UM et al. Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. Proteomics 2008;8:3284-93.
39. Wu AM, Khoo KH, Yu SY et al. Glycomic mapping of pseudomucinous human ovarian cyst glycoproteins: identification of Lewis and sialyl Lewis glycotopes. Proteomics 2007;7:3699-717.


8

Ovarian cyst fluid of serous ovarian tumors contains large quantities of the brain amino acid N-acetylaspartate

Kolwijck E, Wevers RA, Engelke UF, Woudenberg J, Bulten J, Blom HJ, Massuger LFAG

PLoS ONE,2010;5(4):e10293

Abstract

In humans, N-acetyl L-aspartate (NAA) has not been detected in other tissues than the brain. The physiological function of NAA is yet undefined. Recently, it has been suggested that NAA may function as a molecular water pump, responsible for the removal of large amounts of water from the human brain. Ovarian tumors typically present as large cystic masses with considerable fluid accumulation. Using Gas Chromatography-Mass Spectrometry, we demonstrated that NAA was present in a high micromolar concentration in oCF of epithelial ovarian tumors (EOTs) of serous histology, sometimes in the same range as found in the extracellular space of the human brain. In contrast, oCF of EOTs with a mucinous, endometrioid and clear cell histological subtype contained a low micromolar concentration of NAA. Serous EOTs have a cellular differentiation pattern which resembles the lining of the fallopian tube and differs from the other histological subtypes. The NAA concentration in two samples of fluid accumulation in the fallopian tube (hydrosalpinx) was in the same range as NAA found in oCF of serous EOTs. The NAA concentration in oCF of patients with serous EOTs was mostly 10 to 50 fold higher than their normal serum NAA concentration, whereas in patients with other EOT subtypes, serum and cyst fluid NAA concentration was comparable. The high concentration of NAA in cyst fluid of serous EOTs and low serum concentrations of NAA in these patients, suggest a local production of NAA in serous EOTs. Our findings provide the first identification of NAA concentrations high enough to suggest local production outside the human brain. Our findings contribute to the ongoing research understanding the physiological function of NAA in the human body.

Introduction

N-acetyl L-aspartate (NAA) is the second most abundant free amino acid in the human brain.[1] Neuronal cells contain 20 x 103 µmol/L NAA, whereas in the extracellular space of the brain, the NAA concentration ranges between 80 and 100 µmol/L.[1] In spite of these high amounts of NAA, its metabolic and neurochemical functions remain controversial. NAA is thought to function as an important intracellular osmolyte and serve as a source of acetate for lipid and myelin synthesis in glia cells. Furthermore, NAA is considered to be an intermediate in the formation of the neuropeptide N-acetylaspartylgutamate, a storage vehicle for neuronal aspartate and glutamate.[1-3] Interestingly, a patient has been described with a biosynthesis defect of NAA. This patient had a severe neurological disorder with delayed myelination but was still alive at 8 years and 6 months of age.[4]

Recently, it has been proposed that the NAA system functions as a molecular water pump (MWP) operating between neurons and oligodendrocytes.[5] In contrast to osmolytic transport, MWPs are entities that actively use intercompartmental cotransport of water against a gradient.[6] Following this hypothesis, NAA is thought to be primarily responsible for the active removal of metabolic water from myelinated neurons, of which the membranes are known to have a very low water permeability.[6]

Concentrations of NAA in body fluid outside the brain are very low. In cerebrospinal fluid (CSF), plasma, and amniotic fluid of healthy individuals, mean ± SD (range) concentrations of NAA have been reported to be 1.51 ± 0.89 (0.25-2.83) µmol/L, 0.44 ± 0.20 (0.17-0.84) µmol/L, and 1.27 ± 0.74 (0.30-2.55) µmol/L, respectively.[7] Considerable amounts of extra-neuronal NAA have been found in the lens of the eye and in peritoneal mast cells, although this has never been confirmed for humans.[8,9] Using magnetic resonance spectroscopy, NAA was shown in detectable amounts in human ovarian cyst fluid (oCF).[10,11]

Epithelial ovarian tumors (EOTs) are extremely heterogeneous entities which are mostly filled with large amounts of oCF.[12] The aim of the present study was to further investigate the presence of NAA in oCF from patients with EOTs of different histological subtypes. As the mechanism of cyst formation of EOTs is still unknown, this study was conducted to better understand the possible role of NAA in body water management.

Patients and methods

Patients and ovarian cyst fluid

Ovarian cyst fluid was retrieved from our Radboud University Nijmegen Medical Center (RUNMC) biobank. This biobank contains samples of patients with an ovarian tumor who underwent primary surgery at the RUNMC in the period between 1998 and 2008. Samples were collected by aseptic fine needle aspiration at the Department of Pathology immediately after surgical removal of the ovarian tumor. After cooled transport to the laboratory, all samples were centrifuged at 3000 x *g* for 10 minutes and the supernatant was stored at -35°C in small portions until use. For the purpose of this study, 80 oCF samples were randomly selected from our biobank. Complete histopathological reports and slides of all patients were reviewed for correct histopathological diagnosis by one pathologist (JB), specialized in gynecological pathology. Eleven oCF samples were excluded because the ovarian tumor was non-epithelial or not primary ovarian-derived. Histopathological diagnosis of the remaining 69 EOTs revealed 36 serous, 23 mucinous, 8 endometrioid and 2 clear cell tumors (Table 1). Of these 69 EOTs, 25 were malignant, 8 tumors were of borderline malignancy and 36 tumors were benign. Of 7 patients with an EOT preoperative serum was collected as well. These samples were obtained from 3 patients with serous tumors (2 malignant and one borderline), 2 patients with mucinous tumors (malignant and borderline), a patient with an endometrioid carcinoma and a patient with a clear cell carcinoma. Of 5 patients, ascites samples were obtained during primary surgery. One ascites sample was obtained without the availability of an oCF sample. Informed consent was obtained from all participants. Ethical approval for the study was obtained in advance (Radboud University Nijmegen Medical Centre AMO 09/107).

Hepatic cyst fluid

Cyst fluid from 45 patients with polycystic liver disease (PCLD) was obtained between 2002 and 2008 by percutaneous cyst aspiration or laparoscopic cyst fenestration and stored in aliquots at -20°C. The samples were centrifuged at 3000 x *g* for 10 minutes. For the purpose of this study, we randomly selected hepatic cyst fluid samples of 10 patients. None of these patients had a previous diagnosis of carcinoma.

Hydrosalpingeal fluid

Hydrosalpingeal fluid accumulates in the fallopian tube due to distal obstruction. This fluid was collected from 2 salpinges of 1 patient undergoing laparoscopic salpingectomy for presence of bilateral hydrosalpinges. Aspiration of the fluid was performed during surgery by fine needle aspiration before the fallopian tubes were excised. The samples were centrifuged at 3000 x *g* for 10 minutes and the supernatant was stored at -35°C until use.

Table 1. Overview of biological fluid samples grouped by origin

Origin of biological fluid	*n*	Histopathology		
		Malignant	Borderline	Benign
Ovarian cyst	69	25	8	36
serous	36	9	3	24
mucinous	23	6	5	12
endometrioid	8	8	0	0
clear cell	2	2	0	0
Hepatic cyst	10	0	0	10
Ascites	5	5	0	0
Hydrosalpinx	2	0	0	2
Serum	7	5	2	0

N-acetyl-L-aspartic acid (NAA)

NAA (µmol/L) was measured using a modified stable isotope dilution Gas Chromatography-Mass Spectrometry (GC-MS) method essentially as described by Jakobs *et al.*[7] In short, 1 nmol [D3]-NAA was added as internal standard to 100 µl of oCF. This was also added to aqueous standards. The samples were acidified with 30 µl HCL (6 mol/l) to pH < 2 and saturated with NaCl. Thereafter, the sample was extracted four times with 2 ml of an ethylacetate-2-propanol mixture (v/v 10:1). The collected organic fractions were dried by anhydrous Na_2SO_4 and subsequently evaporated to dryness at 40°C under a gentle stream of N_2. NAA was converted to its di-isopropyl derivative by adding 500 µl isopropanol and 10 µl 6 mol/l HCl and subsequent heating for 1 hour at 120°C. An aliquot of 1 µl of this mixture was analysed by GC-MS. GC-MS analysis of 1 µl of this mixture was performed on an Agilent Technologies GC system 6890N with a 5973 MS detector. The gas chromatographic separation was achieved on a CPSil-88 capillary fused silica column (25 m × 0.25 mm, df = 0.28 µm, Chrompack Int., Middelburg, the Netherlands). Helium was used as carrier gas. The oven temperature was kept at 80°C for 1 min and then raised to 240°C at a rate of 30°C/min. The interface and source temperatures were kept at 240°C. The inter-assay variation for this method was 8% (n = 5) and the intra-assay variation was 2% (n = 10). All samples were analysed within the linear range of the standard curve (0–20 nmol/L). If concentrations were above the standard curve, samples were diluted with water.

Clinicopathological characteristics of patients with an EOT

From the medical and pathological records of the patients with an EOTs, pathological diagnosis, histological subtype, largest tumor diameter (cm), and, in case of a malignancy, FIGO stage were scored. Staging was performed according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO).[13]

Statistical analysis

Statistical analyses were carried out using SPSS 16.0.2 software (SPSS Benelux BV, Gorinchem, the Netherlands). Values are shown as median with 25^{th}-75^{th} percentile (µmol/L). Differences between two groups were tested by Mann-Whitney, and for more than 2 groups by Kruskal Wallis tests. Correlations between oCF NAA and ovarian tumor size were analyzed by Spearman's rank correlation testing. *P*-values of <0.05 were regarded as statistically significant.

Results

NAA in cyst fluid of epithelial ovarian tumors

The median (25^{th}-75^{th} percentile) NAA concentration in oCF of serous, mucinous, endometrioid and clear cell EOTs was 5.1 (2.9-17.0), 0.6 (0.5-0.8), 1.2 (0.8-4.5), and 1.3 µmol/L, respectively. NAA in serous EOTs was significantly higher compared to the other histological subtypes ($p < 0.001$, Kruskal-Wallis test). The NAA concentration in one third ($n = 12$) of the serous EOTs was more than 10.0 µmol/L. Serous ($n = 36$) and mucinous ($n = 23$) EOTs could almost perfectly be distinguished by a cut-off value of 1.1 µmol/L (Figure 1). All, except one, oCF samples of mucinous tumors contained less than 1.1 µmol/L NAA, whereas all, except two, oCF samples of serous tumors contained more than 1.1 µmol/L NAA ($p < 0.001$, Mann-Whitney test, Figure 1).

NAA in cyst fluid of epithelial ovarian tumors of serous histology

In the group of serous EOTs ($n = 36$), median (25^{th}-75^{th} percentile) NAA concentration was 4.9 (3.8-7.3), 1.6 (0.5-14.7), and 8.2 (2.9-20.7) µmol/L for malignant ($n = 9$), borderline ($n = 3$) and benign ($n = 24$) tumors, respectively. No significant differences were found between the groups ($p = 0.343$, Kruskal-Wallis test). In addition, no significant correlation was found between the largest diameter of the serous tumors and the NAA concentration ($p = 0.246$, Pearson's correlation test, data not shown).

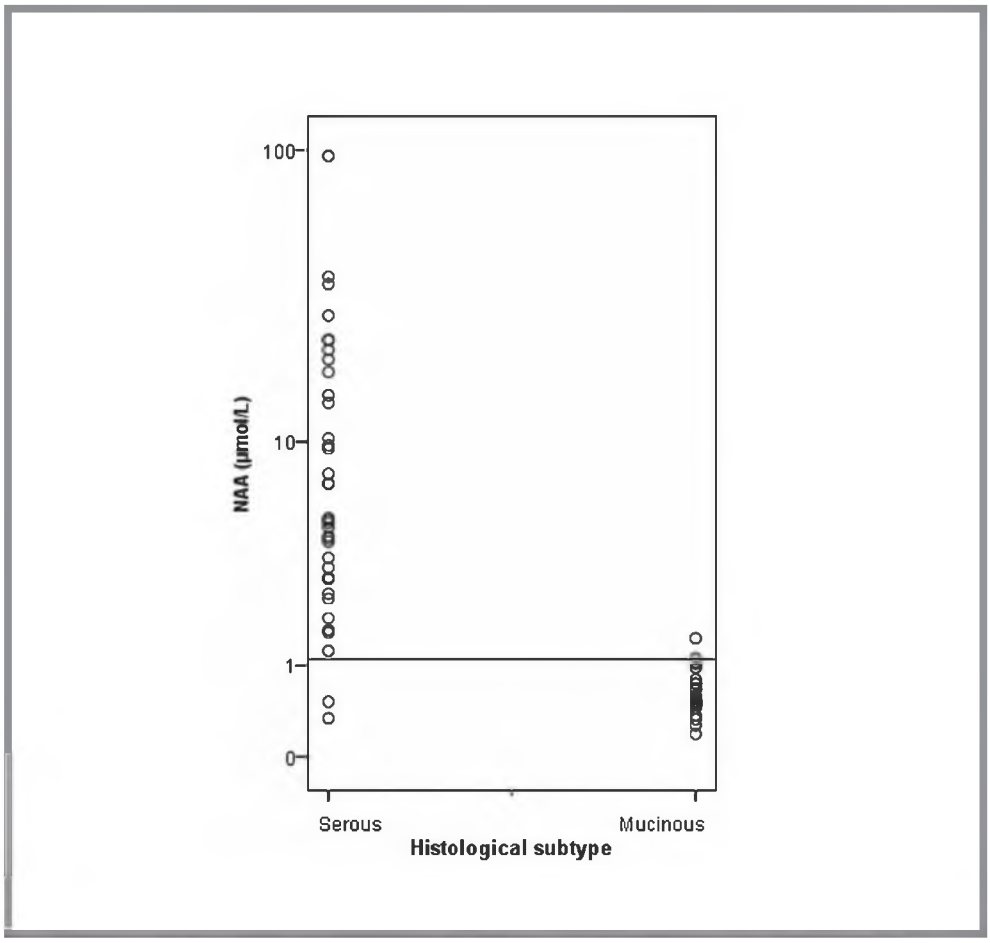

Figure 1. NAA (µmol/L) concentration of individual patients with serous (n = 36) and mucinous (n = 23) tumors. Values are presented by dots on a logarithmic scale. A horizontal line represents the cut-off value of 1.1 µmol/L NAA.

NAA in cyst fluid of malignant EOTs

Table 2 summarizes the data of patients with epithelial ovarian cancer (n = 25). Endometrioid carcinomas (n = 8) showed the largest variation in NAA concentration, ranging from 0.07 to 11.8 µmol/L (Table 2). Of the endometrioid carcinomas, 4 samples showed an NAA concentration below the arbitrary cut-off 1.1 µmol/L. Table 2 also lists the NAA concentration of serous (n = 9, range: 1.2- 22.8 µmol/L), mucinous (n = 6, range: 0.37-0.80 µmol/L) and clear cell (n = 2, range: 0.07-2.5 µmol/L) ovarian carcinomas.

When all carcinomas, regardless of subtype, were grouped by FIGO stage, NAA concentration in oCF from patients with advanced stage disease (stage III and IV, n = 14, median: 4.2, 25^{th}-75^{th} percentile: 1.1-7.2 µmol/L) was significantly higher than NAA concentration in oCF from patients with early stage disease (stage I and II, n = 11, median: 0.8, 25^{th}-75^{th} percentile: 0.5-1.5 µmol/L; p = 0.009, Mann-Whitney test). No correlation was found between tumor size and NAA concentration (p = 0.612, Pearson's correlation test).

Table 2. NAA (µmol/L) in oCF of patients with serous (n = 9), mucinous (n = 6), endometrioid (n = 8) and clear cell (n = 2) ovarian cancer

Patient no.	Histopathological diagnosis	FIGO stage	Tumor size (cm)	NAA (µmol/L)
1	Serous cystadenocarcinoma	IIIc	8	1.2
2	Serous cystadenocarcinoma	IIIb	9	4.3
3	Serous cystadenocarcinoma	IIIc	9	3.5
4	Serous cystadenocarcinoma	IIIc	9	5.1
5	Serous cystadenocarcinoma	IV	9	4.1
6	Serous cystadenocarcinoma	IV	9	7.6
7	Serous cystadenocarcinoma	IIIb	12	4.9
8	Serous cystadenocarcinoma	IIIc	18	22.8
9	Serous cystadenocarcinoma	IIIc	21	7.0
10	Mucinous cystadenocarcinoma	IIIb	10	0.68
11	Mucinous cystadenocarcinoma	IIIb	13	0.80
12	Mucinous cystadenocarcinoma	Ic	17	0.37
13	Mucinous cystadenocarcinoma	Ia	18	0.52
14	Mucinous cystadenocarcinoma	IIIa	20	0.33
15	Mucinous cystadenocarcinoma	Ic	26	0.50
16	Endometrioid cystadenocarcinoma	IIb	4	0.8
17	Endometrioid cystadenocarcinoma	Ic	9	1.5
18	Endometrioid cystadenocarcinoma	IIb	14	0.9
19	Endometrioid cystadenocarcinoma	Ia	18	5.4
20	Endometrioid cystadenocarcinoma	Ia	22	0.9
21	Endometrioid cystadenocarcinoma	IIb	22	1.6
22	Endometrioid cystadenocarcinoma	IIIb	22	11.8
23	Endometrioid cystadenocarcinoma	Ic	30	0.5
24	Clear cell cystadenocarcinoma	IIIa	14	2.5
25	Clear cell cystadenocarcinoma	Ia	23	0.1

NAA concentration in ascites fluid and serum of patients with EOTs

The NAA concentration in ascites and corresponding oCF is listed in Table 3. Ascites and oCF concentrations of NAA were comparable for each patient. Therefore, the concentration of NAA in ascites differed between patients with different histological subtypes of EOTs as well.

The NAA serum concentration ranged between 0.2 and 0.8 µmol/L for all patients with ovarian tumors (n = 7, data not shown), independent of the histological subtype of the tumor. These values are within the reported reference range.[7]

Table 3. NAA (µmol/L) in ovarian cyst fluid and ascites

Patient no.	Histopathology	Subtype	NAA (µmol/L) cyst fluid	NAA (µmol/L) ascites
1	Malignant	Serous	4.9	5.0
2	Malignant	Serous	1.2	1.0
3	Malignant	Serous	-	24.5
4	Malignant	Mucinous	0.8	0.4
5	Malignant	Clear cell	0.1	0.6

NAA concentration in hepatic cyst fluid and hydrosalpingeal fluid

Figure 2 illustrates the boxplots of NAA concentration in oCF of ovarian tumors grouped by histological subtype. In hepatic cyst fluid and in hydrosalpingeal fluid, the NAA concentration was very low in cyst fluid from patients with PCLD (n = 10). The median (25^{th}-75^{th} percentile) concentration amounted to 0.6 (0.4-0.8) µmol/L and ranged between 0.3 and 0.9 µmol/L. The NAA concentration in fluid from the two hydrosalpinges amounted to 12.3 and 16.6 µmol/L. These values are in the same range as the NAA concentration in oCF of serous EOTs.

Discussion

This study shows that NAA is present in low micromolar concentration in all oCF samples. Serous ovarian tumors however, contained a significantly higher oCF concentration of NAA than mucinous, endometrioid and clear cell tumors. The median NAA concentration in serous EOTs was 5.1 µmol/L, whereas one third of all samples contained an NAA concentration above 10.0 µmol/L. The NAA concentration in serous EOTs was 5 to 50 fold higher than the NAA concentration previously found in CSF, serum and amniotic fluid of healthy individuals.[7]

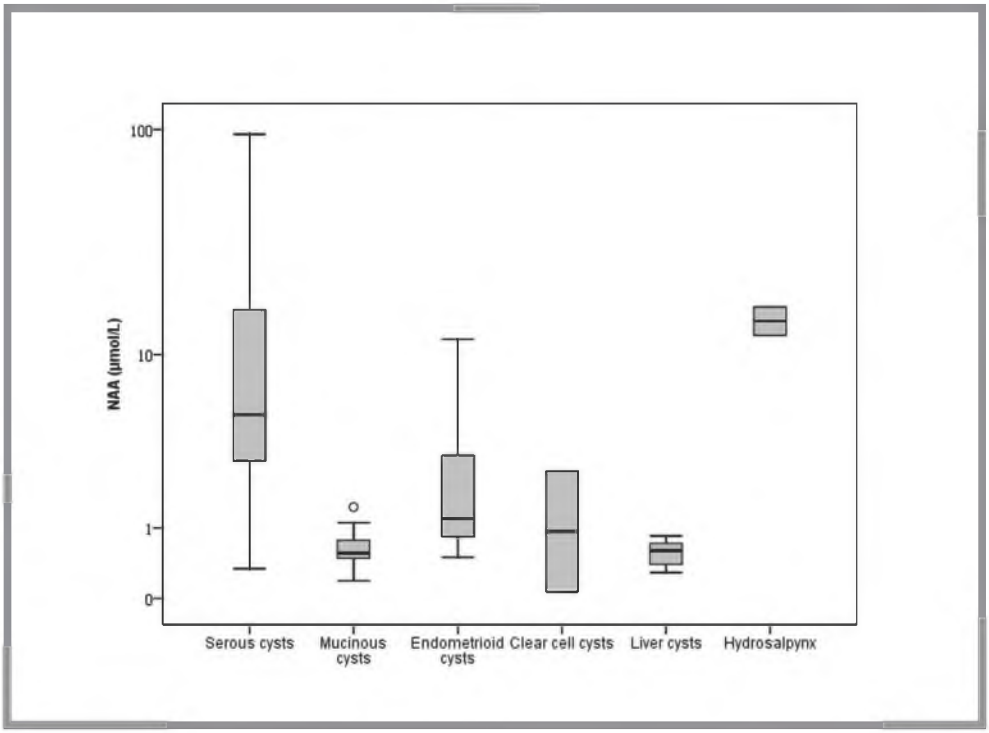

Figure 2. NAA (µmol/L) in cyst fluid of patients with serous (*n*=36), mucinous (*n*=23), endometrioid (*n*=8) and clear cell (*n*=2) EOT, patients with hepatic cysts (*n*=10) and hydrosalpinges (*n*=2), presented on a logarithmic scale.

Some serous EOTs contained an NAA oCF concentration in the same range as found in the extracellular space of the human brain and all serous tumors by far exceeded the NAA concentration in human serum.[1] Interestingly, the oCF NAA concentration in serous EOTs did not differ between benign, borderline and malignant histology, indicating that NAA expression might be specific for EOTs with a serous cell differentiation.

NAA was present in fluid of mucinous EOTs and hepatic cysts as well but this concentration was comparable to the NAA concentration in human serum.[7] Serous and mucinous EOTs could almost perfectly be

distinguished when using an NAA cut-off value of 1.1 µmol/L. Most endometrioid and clear cell carcinomas contained an NAA concentration which was comparable to the concentration of NAA in human serum or CFS. However, in some of these samples a higher NAA concentration was found. This may be explained by the common presence of cells of different ovarian cancer subtypes within a single tumor. In general, approximately 6% of the ovarian carcinomas can be classified as mixed type ovarian carcinomas (at least two histologically distinctive elements are present in at least 10% of the tumor surface).[14] However, more frequently, ovarian carcinomas are found to exist of a main subtype and a small percentage of cells representing a different histology.[14] Our findings indicate that the brain amino acid NAA is predominantly present in oCF of serous EOTs and therefore, seems to be characteristic for this specific histological subtype. NAA analysis in oCF might thus be used to diagnostically distinguish between serous EOTs and other EOT subtypes.

Although 90% of the ovarian tumors derive from the epithelial surface of the ovary, EOTs are not a single disease entity, but instead comprise a heterogeneous group of tumors.[15,16] These tumors are classified according to their pattern of histological differentiation.[17] Serous tumors bear strong resemblance to the normal cells lining the fallopian tube. Mucinous tumors show strong similarity to epithelium of the intestine and endometrioid and clear cell tumors are morphologically identical to cells of the lining of the endometrium.[18] Interestingly, the normal cellular lining of the ovary does not resemble any of these organs. The high concentration of NAA found in oCF of serous EOT, independent of their malignant potential, might be due to their specific cellular differentiation pattern, which resembles the lining of the fallopian tube and differs from the other histological subtypes. This finding is supported by our observation of similar high amounts of NAA found in hydrosalpingeal fluid, which is a fluid accumulation in the fallopian tube due to distal obstruction.[12] However, the question remains why NAA is found in such high amounts in oCF of serous EOT as its presence and function have been specifically been assigned to the human brain.

So far, the brain is the only organ where NAA synthesis has been demonstrated in humans. However, two animal studies have revealed extra-neuronal NAA synthesis.[8,9] In the first study, NAA has been demonstrated in ocular fluid, the lens and retina of fish and mammals.[9] Despite a low micromolar serum concentration of NAA, high amounts of NAA and all components required for synthesis of NAA were present in the eye of these animals. In the present study, the NAA concentration in all serum samples was within the normal range of 0.17-0.84 µmol/L [7] and did not vary between patients with different histological subtypes. The NAA concentration in oCF of serous tumors was mostly 10 to 50 fold higher than the normal serum NAA concentration. As serum and oCF samples were simultaneously taken, our data suggest a local NAA synthesis in the tumor or a pump mechanism which actively pumps NAA from the serum into the oCF. In other ovarian tumor subtypes, serum and cyst fluid concentration of NAA was comparable, which might suggest that local NAA production primarily occurs in EOTs of serous histology. Peripheral synthesis of NAA also has been described for peritoneal mast cells of the rat.[8] Release of NAA from mast cells was found to occur rapidly when degranulating agents were used.[8] Although this finding has not yet been confirmed in humans, release of NAA from mast cells might be

an alternative explanation for the high NAA concentration in oCF and ascites. It has been demonstrated that mast cells are frequently present in stroma tissue of several cancer types, including ovarian carcinomas.[19,20] A growing number of studies has shown that the mast cell count correlates with tumor stage, tumor invasiveness and prognosis.[19] In addition, mast cells are known to be present in ascites of cancer patients as well.[21] However, we found that a high concentration of NAA in oCF and ascites was characteristic for patients with serous EOTs, independent of the malignant potential of the tumor. Mast cells in ovarian stromal tissue have been demonstrated in all histological subtypes of EOTs and numbers were found to be increased in carcinomas compared to their normal tissue counterparts.[20,22] Therefore, we consider a local NAA production in serous EOTs as the most likely explanation for the higher concentration of NAA in serous oCF samples.

Recently, Baslow proposed that the NAA cycle itself might serve as a neuronal mechanism to remove large amounts of water generated during action potential development.[1,5] To prevent swelling of neurons, this metabolic water has to be eliminated, which has been to occur energy driven and against a water gradient. If the NAA system would function as a so called molecular water pump, as a result of the enzymatic hydrolysis of NAA in the oligodendrocytes, its obligated water can be released. This would subsequently result in a hypoosmotic extracellular space, from which it can be removed from the brain.[5] Canavan Disease (CD) is a genetic disorder which causes NAA accumulation in the brain due to a deficiency in the NAA-degrading enzyme aspartoacylase.[2] The disease is characterized by the buildup of excessive fluid within the myelin lamellae, in swollen astrocytes and in extracellular fluid vacuoles.[5] Baslow suggests that this accumulation of NAA is responsible for the macrocephaly in CD.

EOTs, benign as well as malignant, mostly appear as large cystic masses, sometimes diagnosed with a diameter up to 50 cm. In mucinous tumors, cysts are thought to be formed by mucus producing epithelial cells. Mucus is known to contain large amounts of glycoprotein with a high carbohydrate content.[23] In contrast, serous EOT are usually filled with a clear and watery fluid, [12] but the mechanism responsible for serous cyst formation is still unknown. On the basis of our findings and taking into account the proposed role of NAA in body water management, [1-5] we hypothesize that high amounts of NAA in oCF of serous EOTs may be related to accumulation of water in the tumor and may contribute to cyst formation. In addition, this proposed mechanism might also be involved in the formation of ascites in patients with serous adenocarcinomas and in accumulation of hydrosalpingeal fluid. However, as yet there is no confirmatory study in the literature to prove the role of NAA in water management. Therefore, our findings might contribute to the understanding of the so far undefined function of NAA. More evidence is required for the putative role of serous epithelial cells in NAA synthesis and cyst formation.

References

1. Baslow MH. N-acetylaspartate in the vertebrate brain: metabolism and function. Neurochem Res 2003;28:941-53.
2. Moffett JR, Ross B, Arun P et al. N-Acetylaspartate in the CNS: from neurodiagnostics to neurobiology. Prog Neurobiol 2007;81:89-131.
3. George RL, Huang W, Naggar HA et al. Transport of N-acetyl¬aspartate via murine sodium/dicarboxylate cotransporter NaDC3 and expression of this transporter and aspartoacylase II in ocular tissues in mouse. Biochim Biophys Acta 2004;1690:63-9.
4. Burlina AP, Schmitt B, Engelke U et al. Hypoacetylaspartia: clinical and biochemical follow-up of a patient. Adv Exp Med Biol 2006;576:283-7.
5. Baslow MH. Evidence supporting a role for N-acetyl-L-aspartate as a molecular water pump in myelinated neurons in the central nervous system. An analytical review. Neurochem Int 2002;40:295-300.
6. Baslow MH. The existence of molecular water pumps in the nervous system: a review of the evidence. Neurochem Int 1999;34:77-90.
7. Jakobs C, ten Brink HJ, Langelaar SA et al. Stable isotope dilution analysis of N-acetylaspartic acid in CSF, blood, urine and amniotic fluid: accurate postnatal diagnosis and the potential for prenatal diagnosis of Canavan disease. J Inherit Metab Dis 1991;14:653-60.
8. Burlina AP, Ferrari V, Facci L et al. Mast cells contain large quantities of secretagogue-sensitive N-acetylaspartate. J Neurochem 1997;69:1314-7.
9. Baslow MH, Yamada S. Identification of N-acetylaspartate in the lens of the vertebrate eye: a new model for the investigation of the function of N-acetylated amino acids in vertebrates. Exp Eye Res 1997;64:283-6.
10. Boss EA, Moolenaar SH, Massuger LF et al. High-resolution proton nuclear magnetic resonance spectroscopy of ovarian cyst fluid. NMR Biomed 2000;13:297-305.
11. Kolwijck E, Engelke UF, van der Graaf M et al. N-acetyl resonances in in vivo and in vitro NMR spectroscopy of cystic ovarian tumors. NMR Biomed 2009;22:1093-9.
12. Kurman RJ. Blaustein's pathology of the female genital tract. 5th ed. New York: Springer-Verlag; 2002.
13. Pecorelli S, Benedet JL, Creasman WT et al. FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
14. Soslow RA. Histologic subtypes of ovarian carcinoma: an overview. Int J Gynecol Pathol 2008;27: 161-74.
15. Naora H. The heterogeneity of epithelial ovarian cancers: reconciling old and new paradigms. Expert Rev Mol Med 2007;9:1-12.
16. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 18 Suppl 2005;2:S19-S32.
17. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
18. Dubeau L. The cell of origin of ovarian epithelial tumours. Lancet Oncol 2008;9:1191-7.
19. Galinsky DS, Nechushtan H. Mast cells and cancer--no longer just basic science. Crit Rev Oncol Hematol 2008;68:115-30.
20. Samoszuk M, Kanakubo E, Chan JK. Degranulating mast cells in fibrotic regions of human tumors and evidence that mast cell heparin interferes with the growth of tumor cells through a mechanism involving fibroblasts. BMC Cancer 2005;5:121.
21. Valent P, Ashman LK, Hinterberger W et al. Mast cell typing: demonstration of a distinct hematopoietic cell type and evidence for immunophenotypic relationship to mononuclear phagocytes. Blood 1989;73:1778-85.
22. Chan JK, Magistris A, Loizzi V et al. Mast cell density, angiogenesis, blood clotting, and prognosis in women with advanced ovarian cancer. Gynecol Oncol 2005;99:20-5.
23. Schrager J. The chemical composition and function of gastrointestinal mucus. Gut 1970;11:450-6.


9

Alpha 1-acid glycoprotein is an independent predictor of survival in ovarian cancer patients treated with chemotherapy

Kolwijck E, Baurin S, Laub R, Bulten J, Buurman WA, Engelke UF, Wevers RA**, Massuger LFAG**
(** shared equal last co-authorship)

Submitted

Abstract

Increased synthesis of the drug-binding protein alpha 1-acid glycoprotein (AGP) during cancer can considerably alter the free fraction of drugs resulting in a lower efficacy of the treatment. This study aimed to identify the association between the AGP concentration in ovarian cyst fluid (oCF) and disease outcome of epithelial ovarian cancer (EOC) patients treated with chemotherapy. Additionally, the binding of paclitaxel to AGP and cisplatin to AGP was established. AGP was measured by ELISA in oCF of 86 patients with ovarian tumors (53 EOC and 33 non-malignant). Clinicopathological parameters were retrospectively retrieved. The interaction between cisplatin-AGP and paclitaxel-AGP was determined by fluorescence techniques. Significantly higher levels of AGP were found in oCF of EOC patients than in oCF of borderline and benign patients ($p < 0.001$). Of patients who received chemotherapy (stage ≥ Ic; $n = 32$), high AGP (> 600 µg/ml) was significantly associated with a poor disease free survival (DFS) and overall survival (OS)(log-rank $p = 0.010$ and $p = 0.005$, respectively). Using multivariate analysis, FIGO stage was the only independent predictor of DFS. The AGP concentration in oCF was the only independent predictor of OS. Paclitaxel was found to bind to AGP with high affinity ($K_d = 9.3\ 10^{-6}$ M). A low-affinity binding of cisplatin to AGP was found ($K_d = 1.7\ 10^{-2}$ M). AGP appears to be an independent predictor of survival for patients with EOC treated with chemotherapy. The predictive role of the oCF AGP for disease outcome is likely related to the high affinity binding of paclitaxel by AGP

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy world-wide.[1] Currently, 20% of the EOC patients still show resistance to first-line chemotherapy.[2] Biomarkers may help to early predict response to chemotherapy thereby providing possibilities for tailor-made treatment and personalized medicine.

The acute-phase protein alpha 1-acid glycoprotein (AGP) is synthesized by the liver and its plasma concentration rises during pathological conditions including cancer.[3,4] Also, AGP is the next important drug binding protein after serum albumin.[3] Increase of AGP during cancer can considerably alter the free plasma fraction of chemotherapeutics with high binding affinity for AGP, contributing to treatment failure.[5-7] This mechanism has been described for patients with lung cancer, where the serum AGP concentration appeared to be an independent predictor of response to docetaxel.[6,7] For EOC, however, AGP has only be investigated as a non-specific biomarker for monitoring response during chemotherapy of EOC patients.[8-11]

Besides hepatic synthesis of AGP and its release into the serum, local extra-hepatic synthesis of AGP has also been described.[4] Active synthesis of AGP outside the liver has been found in human breast epithelial cells [12] and many pathologic tissues.[4] Therefore, the acute-phase response also may occur in extra-hepatic cell types at the site of the initial acute-phase reaction.[4,12,13]

EOC typically presents as a large cystic mass consisting of fluid. This ovarian cyst fluid (oCF) can be easily obtained and used for the measurement of compounds derived form the ovarian epithelial tumor wall.[14] Increased local synthesis of AGP in EOC tissue might result in a decreased free fraction of the chemotherapeutics at the tumor site and thus to a diminished cytotoxic effect of the chemotherapy. We aimed to investigate the association between the AGP concentration in oCF and disease outcome of EOC patients treated with chemotherapy. Additionally, the binding between AGP and two first-line chemotherapeutic agents for EOC, paclitaxel and cisplatin, was determined.

Materials and Methods

Patients and ovarian cyst fluid (oCF) samples

In the period between January 1996 and January 2008, oCF was collected from patients who underwent surgery for an ovarian tumor at the Radboud University Nijmegen Medical Centre (RUNMC) and stored in our oCF biobank. After surgery, oCF was collected through aseptic fine needle aspiration at the Department of Pathology. After cooled transport to our laboratory, the oCF samples were centrifuged at 3000 x *g* for 10 minutes and the supernatant was aliquoted and stored at -35°C until use. The oCF was collected during primary (debulking or diagnostic) surgery and never during interval debulking surgery. Frozen section examination revealed 70 malignant ovarian tumors. Complete histopathological reports and slides of these tumors were reviewed for correct histopathological diagnosis by one pathologist (JB), specialized in gynecological pathology. Ten tumors were excluded because final paraffin examination revealed that the origin of the primary tumor was not ovarian derived. Seven were excluded because the ovarian malignancy was non-epithelial. Included were tumors of patients with histologically proven primary EOC (n = 53). As a control group, 22 and 11 oCF samples from benign and borderline ovarian tumors, respectively, were randomly selected from our oCF biobank. The informed consent was obtained verbally in presence of a witness and documented in the patient's medical record. The study was approved by the ethical board of the RUNMC (file number AMO 09/107).

Clinicopathological characteristics

Surgery and staging were always performed by a gynecologist specialized in oncology. From the medical and pathological records of the patients diagnosed with EOC (n = 53), the following clinicopathological characteristics were retrospectively retrieved: tumor grade, histological subtype, age at diagnosis, FIGO stage, residual tumor after surgery, chemotherapeutic treatment, date of tumor recurrence and date of death. For some patients, information on some clinicopathological parameters could not be retrieved (Table 1). Staging was performed according to the criteria of the International Federation of Gynecologists and Obstetricians (FIGO).[15] Histopathological tumor type and grade were classified according to the World Health Organization (WHO) criteria.[16] Chemotherapeutic treatment was defined as complete combination chemotherapy of 6 courses, always including a platinum-based agent, and was started within three weeks after surgery. Recurrence of disease was defined as a measurable lesion detected by computed tomography, magnetic resonance imaging and/or ultrasonography.

ELISA for AGP

AGP concentrations were measured using an ELISA essentially as reported previously.[17] In summary, ELISA plates were coated overnight with polyclonal anti-human AGP obtained from Dako (Glostrup, Denmark). Diluted plasma samples and a standard dilution series with human and rAGP, were added to the plate. Detection was carried out with a biotinylated polyclonal rabbit anti-human AGP IgG, followed by peroxidase-conjugated streptavidin and substrate essentially as described by De Vries *et al.*[18] Determination of the levels of AGP was carried out without knowledge of the histological or clinical outcome.

Table 1. Association of AGP in oCF (µg/ml) and clinicopathologically defined patient characteristics of all patients with EOC ($n = 53$)

Characteristics	*n* (%)	AGP (µg/ml) Median (25^{th}-75^{th} percentile)	*p*-value
Age			0.778*
< 57 years	25 (47)	650 (377-1172)	
≥ 57 years	28 (53)	615 (407-1370)	
FIGO stage			0.049*
I + II	25 (47)	438 (279-1025)	
III + IV	27 (51)	684 (436-1304)	
unknown	1 (2)		
Tumor grade			0.027**
1	15 (28)	436 (177-673)	
2	16 (30)	1172 (407-1535)	
3	19 (36)	747 (483-1287)	
unknown	3 (6)		
Histology			0.060**
serous	23 (43)	747 (420-1304)	
mucinous	14 (26)	436 (113-643)	
endometrioid	9 (17)	1143 (400-1459)	
other	7 (14)	667 (362-1191)	
Residual tumor			0.002*
≤ 1cm	43 (81)	534 (338-1143)	
> 1cm	9 (17)	1304 (803-1609)	
unknown	1 (2)		
Total	53	650 (408-1243)	

**Mann-Whitney U-test; **Kruskal-Wallis test*

Materials for the drug binding study

AGP (>99% purity) was purified using Cohn ethanol fractionation and chromatography from a plasma pool containing more than 6,000 plasma donations (CAF-DCF R&D, Brussels, Belgium). Paclitaxel was obtained from Calbiochem (Merck, Nottingham, UK), cisplatin (cis-DDP) from Acros Organics (Fisher Emergo, Landsmeer, the Netherlands), quinaldine red (QR) (2-[2-[4-(dimethylamino)-phenyl]-1-ethyquinolium iodide), phosphate buffered saline (PBS) and ethanol 99.9% from Sigma-Aldrich (St. Louis, MO,USA).

Binding of cisplatin to AGP

The binding of cisplatin to AGP was measured by quenching of the intrinsic AGP tryptophan fluorescence. A stock solution of cisplatin was freshly prepared in DMSO (33.3 mM) and stored at room temperature. Different concentrations of the drug (final concentration from 0-3.3 mM) were added to 54 µg/ml of AGP in PBS (pH 7.4). The mixture (300 µl) was transferred to black Microfluor 2 microplate (Thermo Scientific, Waltham, MA, USA). The fluorescence was measured in a multi-modale microplate reader Varioskan Flash (Thermo Scientific, Waltham, MA, USA). The emission fluorescence (300-400 nm) of the 3 tryptophan residues present in AGP was measured after excitation at 280 nm with a slit of 5 nm at room temperature.

The kinetics of the cisplatin binding to AGP was measured from 2-720 minutes. All experiments were performed in triplicate. The association constant (K_a) and binding affinity were calculated according to the equation: $\log[(F_0 - F)/F] = \log K_a + n \log[drug]$. F_0 and F are the fluorescence intensity without and with the drug, respectively. After a $\log[(F_0 - F)/F]$ versus log[drug] plot, the slope and the intercept (Y-axis) denoted the binding capacity (n) and the intercept on K_a, respectively.[19] Subsequently, the dissociation constant (K_d) was calculated using the following equation: $K_d = 1/K_a$.

Binding of paclitaxel to AGP

The binding of paclitaxel with AGP was determined by competition of a specific probe for AGP, quinaldine red (QR), [20] using a Shimadzu RF-5300 spectrofluorophotometer. A stock solution of 11.7 mM paclitaxel was prepared in DMSO and stored at -20°C. In a quartz cuvette with 1 cm optical pathway, increasing amounts of paclitaxel (final concentration from 0-30 µM) were added to a mixture of AGP (45 µg/ml) and QR (1 µM) in PBS (pH 7.4). The decline of the fluorescence of QR was measured at room temperature (excitation 495 nm, emission 480-650 nm; with excitation and emission slits of 10 and 5 nm, respectively). All experiments were carried out in duplicate. Subsequently, the fluorescence at 580 nm for each concentration of paclitaxel was transformed using the following equation: $y = 1-[(F_m-F_{min})/(F_0-F_{min})]$. F_m is the amount of fluorescence, F_0 is the fluorescence without the drug and F_{min} is the lowest value of fluorescence with the drug. After fitting, the binding parameters were determined using the equation of one binding site: $y = (n * K_d)/(K_d+[drug])$. N and K_d represent the number of binding sites (binding capacity) and the dissociation constant, respectively.

Statistical analyses

Statistical analyses were performed using SPSS 16.0.2 software (SPSS Benelux BV, Gorinchem, the Netherlands). Variables regarding patient characteristics were grouped in the following manner: FIGO stage: I-II versus III-IV; tumor grade: 1 versus 2 versus 3; histology: serous versus mucinous versus endometrioid; residual disease:

≤1cm (definition of optimal cytoreductive surgery) versus >1cm (definition of suboptimal debulking). Values are shown as median with 25^{th}-75^{th} percentile (µg/ml). Differences in AGP concentration between groups of patients were tested for statistical significance using the Mann-Whitney test in case of two groups and the Kruskal-Wallis test in case of more than two groups. Survival analysis was performed with the subgroup of patients with EOC that received complete adjuvant chemotherapy. Survival techniques were used to study the time to recurrence and to study the time to death. The disease free survival (DFS) was defined as the time interval from the date of the last course of chemotherapy to recurrence or last follow-up. The overall survival (OS) was defined as the time interval from the date of surgery to the date of either death or last follow-up. The AGP oCF value of 600 µg/ml was used for dividing patients into two groups after a statistically significant difference in DFS was found with logistic regression analysis. The Kaplan-Meyer estimates were calculated of the patients with AGP oCF values below 600 µg/ml and above 600 µg/ml, respectively. Subsequently, the log-rank test was used to test their difference for statistical significance. An univariate proportional hazards model was used to study the influence of the clinicopathological parameters on DFS and OS separately. Variables were divided into categories as described above. Only tumor grade was entered in the model as a continuous variable as 3 cases were missing and the small group of patients with grade 1. The hazard ratio's (HR) with the corresponding 95% confidence interval (CI) are presented. Multivariate proportional hazards model with selection procedures was used to find the clinicopathological parameters that independently contribute to a decreased time to recurrence or death. The adjusted HR with the corresponding 95% CI of the final model are presented. *P*-values <0.05 were considered statistically significant.

Results

Study population

The median age at diagnosis for patients with EOC, borderline and benign tumors was 57 ($n = 53$; range: 32-89), 56 ($n = 11$; range: 43-82) and 52 years ($n = 22$; range: 20-70), respectively. Age did not differ between patients groups. Borderline tumors consisted of 8 serous tumors and 3 mucinous tumors. Benign tumors consisted of 7 serous, 11 mucinous, 1 mixed type tumors and 3 simple epithelial cysts. Of patients with EOC, clinicopathological characteristics are listed in Table 1. The histological subgroup "other" consisted of 3 clear cell tumors, 1 mixed cell tumor, 1 undifferentiated adenocarcinoma and 3 adenocarcinomas not otherwise specified (NOS). Of the 53 patients with EOC, 21 had FIGO stage I (40%), 4 had FIGO stage II (8%), 22 had FIGO stage III (42%), and 5 FIGO had stage IV (10%). Of the 53 patients with EOC, 32 patients received complete adjuvant chemotherapeutic treatment with 6 courses of platinum-based combination chemotherapy and were included in survival analyses. Twenty-one patients were excluded from

survival analyses. Of these patients, 13 did not receive chemotherapy because they were diagnosed with FIGO ≤Ib, 5 received less than 6 courses of chemotherapy, and 4 (oCF obtained during diagnostic surgery) received 3 courses of chemotherapy before interval debulking. Of the subgroup of EOC patients that received complete adjuvant chemotherapeutic treatment ($n = 32$), 8 patients received a combination of cisplatin and cyclophosphamid and 24 patients received a combination of platinum (cisplatin or carboplatin) and paclitaxel. No differences in DFS or OS were found between these two groups ($p = 0.361$ and $p = 0.279$, respectively, Kaplan-Meyer log-rank test). DFS ranged from 5-117 months (median = 13 months). OS ranged from 6-120 months (median = 24 months). Eighteen patients (56%) developed recurrence and 9 patients (28%) died within the follow-up period.

Histopathological diagnosis

Figure 1 shows the boxplots of the oCF AGP concentration of malignant, borderline and benign ovarian tumors. For patients with malignant, borderline and benign ovarian tumors the median (25^{th}-75^{th} percentile) concentration amounted to 650 (408-1243), 90 (40-262), and 106 (26-311) µg/ml, respectively. Significantly higher concentrations of AGP were found in cyst fluid from malignant ovarian tumors compared to borderline and benign tumors (p <0.001, Kruskal-Wallis test). The plasma or serum AGP concentration in healthy individuals has been reported to be in the range of 500-1000 µg/ml.[3]

AGP and clinicopathological categories

Table 1 shows the clinicopathological outcome and median (25^{th}-75^{th} percentile) concentration of AGP (µg/ml) for the 53 patients with EOC. The AGP concentration differed significantly between tumor grades ($p = 0.027$). It was significantly higher for patients with FIGO stage III and IV compared to stage I and II ($p = 0.049$), and for patients with a residual tumor after surgery > 1cm compared to patients with a residual tumor of ≤ 1cm ($p = 0.002$).

Survival analysis

Thirty-two patients receiving complete adjuvant chemotherapeutic treatment were included for survival analysis (Table 2). The median follow-up period for these patients was 42 months after completing chemotherapeutic treatment. Figure 2 shows the Kaplan-Meier curves of DFS (Figure 2A) and OS (Figure 2B) for patients with an AGP concentration above ($n = 15$) and below ($n = 17$) 600 µg/ml. A high AGP concentration was correlated with both shorter DFS and shorter OS (log-rank test: $p = 0.010$ and $p = 0.005$, respectively).

Table 2 shows the HR with 95% CI, using the univariate proportional hazard model of DFS and OS. For the variable "Tumor grade" 3 cases were missing, and for "Residual tumor" one case was missing. FIGO stage, tumor grade, residual tumor, and AGP were regarded as significant predictors of DFS. However, using the multivariate proportional hazard model with selection procedure, the FIGO stage was the only independent predictor that had impact on DFS (HR = 22.15; 95% CI: 2.77-177; $p = 0.003$). Regarding the time to death we found that FIGO stage, and AGP could be regarded as significant predictors of OS in univariate analysis. Now, we found, after the model selection procedure, that the AGP concentration appeared to be the only independent prognostic factor that had impact on OS (HR = 12.13; 95% CI: 1.43-102; $p = 0.022$).

Table 2. Univariate Cox regression analyses of DFS and OS of patients that received chemotherapy ($n = 32$)

	DFS		OS	
	HR (95% CI)*	*p*-value	HR (95% CI)*	*p*-value
Age		0.502		0.655
< 57 years	1.00 (reference)		1.00 (reference)	
≥ 57 years	0.73 (0.29-1.84)		0.74 (0.20-2.77)	
FIGO stage		0.001		0.022
I-II	1.00 (reference)		1.00 (reference)	
III-IV	12.18 (2.63-56.46)		12.10 (1.43-102.52)	
Tumor grade	2.14 (1.01-4.53)	0.048	2.08 (0.83-5.18)	0.118
Histology		0.260		0.185
serous	4.96 (0.63-39.22)		2.92 (0.33-25.65)	
mucinous	1.00 (reference)		1.00 (reference)	
endometrioid	2.56 (0.22-29.30)		14.76 (1.04-210.31)	
other	2.64 (0.27-25.45)		1.42 (0.13-15.70)	
Residual tumor		0.031		0.500
≤ 1cm	1.00 (reference)		1.00 (reference)	
> 1cm	4.17 (1.35-12.93)		1.77 (0.34-9.239)	
AGP in oCF		0.019		0.022
≤ 600 µg/ml	1.00 (reference)		1.00 (reference)	
> 600 µg/ml	3.49 (1.22-9.952)		12.13 (1.43-102.69)	

Hazard Ratio (95% Confidence Interval)

Binding of cisplatin to AGP

It has been demonstrated that > 99% of cisplatin is irreversibly bound to albumin and that this covalent binding increases as a function of time.[21] Figure 3 shows the intensity of emission fluorescence of 1.2 µM of AGP in PBS at 330 nm (excitation at 280 nm) as a function of the cisplatin concentration during various incubation times.

The fluorescence of AGP was markedly quenched in the presence of increasing concentrations of cisplatin (0-3.33 mM). The quenching effect was time-dependent and more quenching was observed when the incubation time of the AGP-cisplatin solution was increased (at 3 hours: $K_d = 1.7\ 10^{-2}$ M; n = 0.62).

Binding of paclitaxel to AGP

It has been demonstrated earlier that AGP has a specific and high affinity binding for QR ($K_d = 0.76.10^{-6}$ M; n = 0.9) and that the QR-AGP fluorescence is quenched due to displacement of QR by some basic drugs. [20,22,23] We found that QR, when bound to AGP, showed similar fluorescence properties as reported previously. Figure 4 shows the relative displacement of QR bound to AGP in the presence of increasing concentrations of paclitaxel (0-30 µM). Paclitaxel strongly displaced QR from AGP ($K_d = 9.3.10^{-6} \pm 2.6.10^{-6}$ M; n = 1.23 ±0.08).

Discussion

This is the first study analyzing the association between AGP in oCF and the clinical outcome of EOC patients that received chemotherapy. AGP was present in oCF of all patients with ovarian tumors. EOC patients showed significantly higher oCF AGP than patients with borderline and benign ovarian tumors. This finding corresponds with the results of studies in serum, which demonstrated an increase of the AGP concentration in patients with EOC compared to serum reference values (500-1000 µg/ml).[8,10,11,24] For patients with EOC that received chemotherapy, oCF AGP was significantly associated with DFS and OS. The independent role of oCF AGP as a biomarker of poor OS was sustained by the results of multivariate analysis documenting the persistence of the unfavorable significance of a high oCF AGP concentration after adjustment for important clinicopathological variables.

Besides hepatic synthesis of AGP, AGP synthesis also occurs in extra-hepatic cell types, notably epithelial cells, at the site of the initial acute-phase reaction.[4,12,13] AGP synthesis has been found in the bovine ovary, [25] whereas expression of other serum proteins, such as albumin, could not be detected in this tissue.[26] In rheumatoid synovial fluid, the glycosylation patterns of AGP differed between the serum and synovial fluid in the same rheumatoid patient, suggesting local production of this glycoprotein.[27]

Although there might be passive flow of AGP between serum and oCF, we assume that AGP is, at least partly, produced by EOC cells in response to malignancy and subsequently released into the oCF. However, histochemical or genetic studies should be performed to provide direct evidence for local synthesis of AGP in EOC tissue.

Only a few studies investigated the effect of the drug binding properties of AGP on the response to chemotherapy in cancer patients, instead of using AGP as a non-specific biomarker.[6,28] In patients with lung cancer treated with docetaxel, Bruno *et al.* demonstrated that AGP correlated with the response to therapy.[6] It had been shown earlier that docetaxel binds strongly to AGP, thus limiting the free fraction of the drug.[29] Therefore, the authors stated that the effect of AGP on the pharmacokinetics and pharmacodynamics of docetaxel could contribute to the shorter survival observed for patients with lung cancer with high serum AGP.[6] As AGP was found to be synthesized by breast cancer epithelial cells, Paterson *et al.* investigated the interaction between AGP and tamoxifen.[28] They showed a weak binding between AGP and tamoxifen and therefore, concluded that in breast cancer patients treated with tamoxifen, interaction of the drug and AGP is not a factor which would decrease the efficacy of the drug.

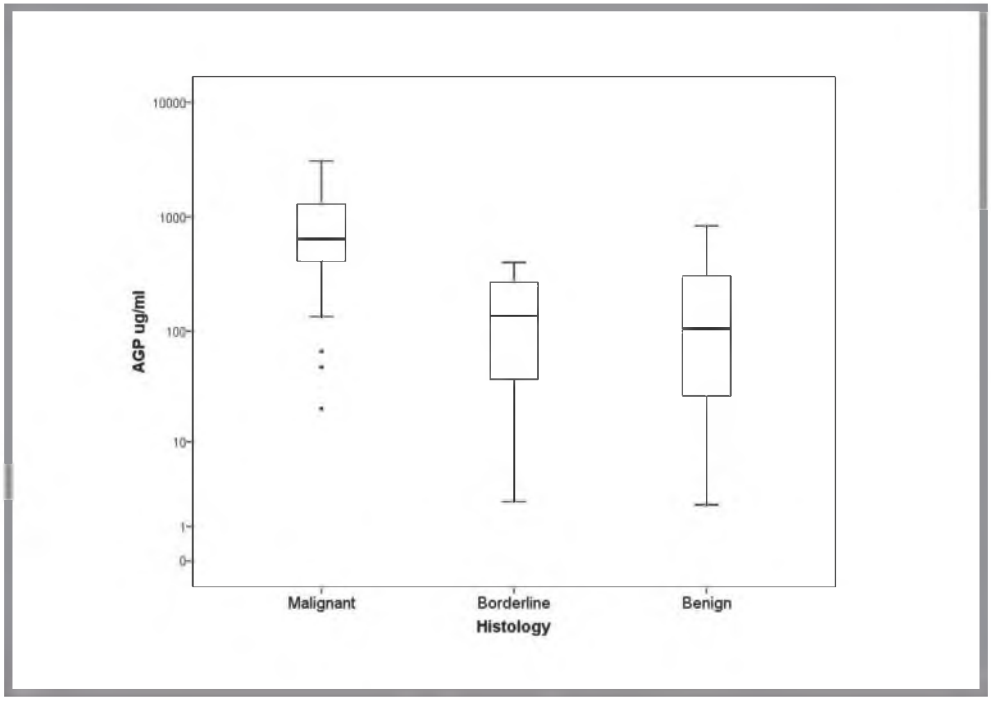

Figure 1. Boxplots of the AGP concentration in cyst fluid of patients with malignant (n = 53), borderline (n = 11) and benign (n = 22) ovarian tumors.

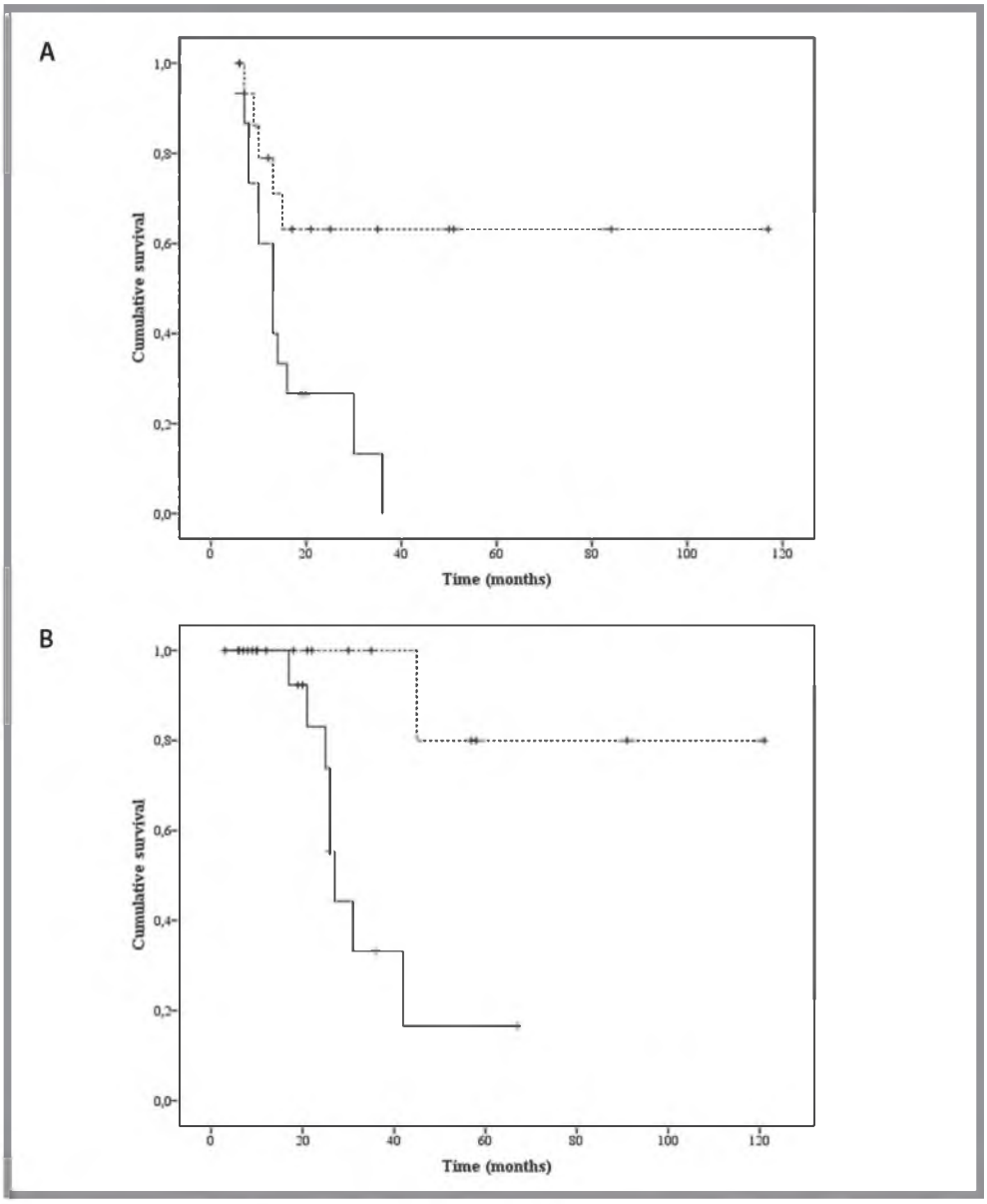

Figure 2. Kaplan-Meier estimates of disease free (A) and of overall survival (B) of patients who received chemotherapy ($n = 32$). The group with low AGP values (≤ 600 µg/ml; broken line) and the group with high AGP values (> 600 µg/ml; solid line) include 17 and 15 patients, respectively. Vertical bars indicate patients with censored data.

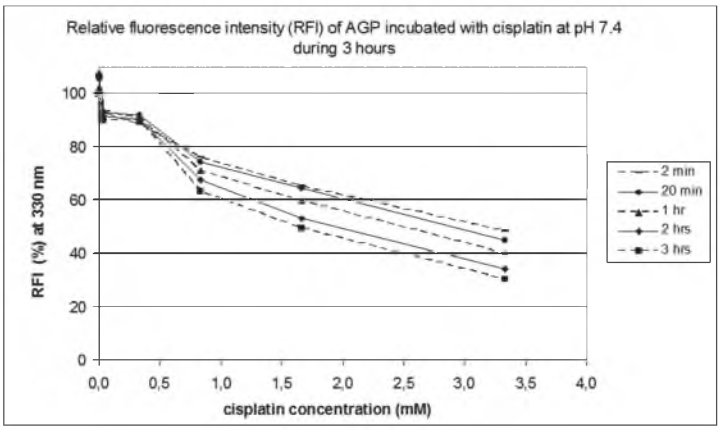

Figure 3. Maximum emission fluorescence at 330 nm of AGP in the prescence of increasing concentrations of cisplatin. Measurements were performed from 2 to 360 minutes.

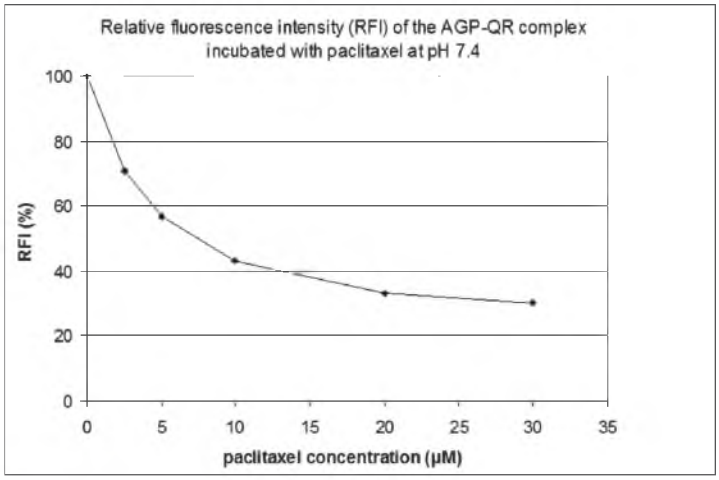

Figure 4. Displacement of QR (1µM) bound to AGP 1µM (45 µg/ml) by increasing concentrations of paclitaxel (0-30 µM).

After primary surgery, the current standard treatment for patients with advanced EOC involves the systemic administration of a paclitaxel/platinum-containing chemotherapy regimen.[30] We found a high affinity binding between AGP and paclitaxel, whereas the binding between AGP and cisplatin was rather weak. One previous study has reported the binding constant of AGP and paclitaxel (K_d = 3.45 10^{-6} M), [23] which was comparable to our findings. Another study showed that AGP has a small but statistically significant influence on the paclitaxel pharmacokinetics.[31] When AGP is synthesized by EOC cells, this effect may result in a restriction of free paclitaxel distribution in tumors with high AGP synthesis. This pharmacokinetic effect may reduce the cytotoxic influence of paclitaxel on the tumor cells and subsequently may contribute to the shorter survival observed in this study for patients with a high oCF concentration of AGP However, as AGP synthesis is increased in response to malignancy, the correlation between AGP and survival may also be related to the poor prognosis of patients with high oCF AGP. To our knowledge, no data have been published on the binding between cisplatin and AGP. For albumin, a minor cisplatin-albumin interaction has been described at low cisplatin concentrations, while at a higher drug content, cisplatin and albumin showed a weak binding (K_d = 1.2 10^{-3} M).[32] More importantly, cisplatin binding to albumin has shown to be essentially irreversible with less than 5% loss of protein-bound cisplatin after extensive dialysis.[33] Thus despite the low affinity binding, the non-covalent nature of the cisplatin-albumin complex may have significant impact on the distribution of cisplatin in the serum. The question remains whether binding between AGP and cisplatin is irreversible as well and would significantly diminish the cytotoxic effect on the tumor tissue when synthesis of AGP is increased.

We believe the independent predictive role of oCF AGP for survival is at least partly related to the high affinity protein binding effects of paclitaxel to AGP. However, further studies are needed to investigate this mechanism *in vivo*. We suggest AGP in oCF as a potential biomarker that is informative with respect to the time to disease progression, survival and response to paclitaxel/cisplatin chemotherapy. A more complete understanding of the synthesis of AGP in EOC cells as well as the pharmacokinetic and pharmacodynamic interactions between cisplatin and AGP warrants further study.

References

1. Jemal A, Siegel R, Ward E et al. Cancer statistics 2007. CA Cancer J Clin 2007;5743-66.
2. Gonzalez-Martin A. Treatment of recurrent disease: randomized trials of monotherapy versus combination chemotherapy. Int J Gynecol Cancer 2005;15 Suppl 3:241-6.
3. Israili ZH, Dayton PG. Human alpha-1-glycoprotein and its interactions with drugs. Drug Metab Rev 2001;33:161-235.
4. Fournier T, Medjoubi N, Porquet D. Alpha-1-acid glycoprotein. Biochim Biophys Acta 2000;1482:157-71.
5. Kremer JM, Wilting J, Janssen LH. Drug binding to human alpha-1-acid glycoprotein in health and disease. Pharmacol Rev 1988;40:1-47.
6. Bruno R, Olivares R, Berille J et al. Alpha-1-acid glycoprotein as an independent predictor for treatment effects and a prognostic factor of survival in patients with non-small cell lung cancer treated with docetaxel. Clin Cancer Res 2003;9:1077-1082.
7. Yildirim A, Meral M, Kaynar H et al. Relationship between serum levels of some acute-phase proteins and stage of disease and performance status in patients with lung cancer. Med Sci Monit 13 2007;CR195-200.
8. Piver MS, Moyer M, Diakun K et al. Serum alpha 1-acid glycoprotein in epithelial ovarian cancer. Gynecol Oncol 1988;29:305-8.
9. Fish RG, Gill TS, Adams M et al. Changes in serum acute phase proteins in ovarian cancer patients receiving cis-diamminedichloroplatinum (CDDP) infusion therapy. Clin Biochem 1984;17:39-41.
10. Meerwaldt JH, Halje WG, Cooper EH et al. Biochemical aids in the monitoring of patients with ovarian cancer. Gynecol Oncol 1983;16:209-18.
11. Lukomska B, Olszewski WL, Engeset A et al. Acute-phase reactant proteins and complement components and inhibitors in patients with ovarian cancer. Gynecol Oncol 1981;11:288-98.
12. Gendler SJ, Dermer GB, Silverman LM et al. Synthesis of alpha 1-antichymotrypsin and alpha 1-acid glycoprotein by human breast epithelial cells. Cancer Res 1982;42:4567-73.
13. Dube JY, Paradis G, Tetu B et al. Synthesis of alpha 1-acid glycoprotein by the human prostate. Prostate 1989;15:251-8.
14. Bar JK, Harlozinska A, Sobanska E et al. Relation between ovarian carcinoma-associated antigens in tumor tissue and detached cyst fluid cells of patients with ovarian neoplasms. Tumori 1994;80:50-5.
15. Pecorelli S, Benedet JL, Creasman WT et al. FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 1999;65:243-9.
16. Servov SF, Scully RE, Sobin LH. International histologic classification of tumors. No. 9: Histologic typing of ovarian tumors. Geneva: World Health Organization, 1973.
17. Van Dielen FM, van't Veer C, Schols AM et al. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. Int J Obes Relat Metab Disord 2001;25:1759-66.
18. De Vries B, Walter SJ, Wolfs TG et al. Exogenous alpha-1-acid glycoprotein protects against renal ischemia-reperfusion injury by inhibition of inflammation and apoptosis. Transplantation 2004;78:1116-24.
19. Varshney A, Ahmad B, Khan RH. Comparative studies of unfolding and binding of ligands to human serum albumin in the presence of fatty acid: spectroscopic approach. Int J Biol Macromol 2008;42:483-90.
20. Imamura H, Maruyama T, Otagiri M. Evaluation of quinaldine red as a fluorescent probe for studies of drug-alpha 1-acid glycoprotein interaction. Biol Pharm Bull 1993;16:926-9.
21. Bednarski PJ, Kratochwil NA, Otto AM. Reversible and irreversible interactions of a cisplatin analog bearing a 1,2-diphenylethylenediamine ligand with plasma and plasma proteins in vitro. Drug Metab Dispos 1994;22:419-27.
22. Imamura H, Maruyama T, Okabe H et al. A simple and rapid fluorometric determination method of alpha 1-acid glycoprotein in serum using quinaldine red. Pharm Res 1994;11:566-70.
23. Finlay GJ, Baguley BC. Effects of protein binding on the in vitro activity of antitumour acridine derivatives and related anticancer drugs. Cancer Chemother Pharmacol 2000;45:417-22.

24. Fish RG, Gill TS, Adams M et al. Serum haptoglobin and alpha 1-acid glycoprotein as indicators of the effectiveness of cis-diamminedichloroplatinum (CDDP) in ovarian cancer patients--a preliminary report. Eur J Cancer Clin Oncol 1984;20:625-30.
25. Lecchi C, Avallone G, Giurovich M et al. Extra hepatic expression of the acute phase protein alpha 1-acid glycoprotein in normal bovine tissues. Vet J 2009;180:256-8.
26. Shamay A, Homans R, Fuerman Y et al. Expression of albumin in nonhepatic tissues and its synthesis by the bovine mammary gland. J Dairy Sci 2005;88:569-76.
27. Smith KD, Pollacchi A, Field M et al. The heterogeneity of the glycosylation of alpha-1-acid glycoprotein between the sera and synovial fluid in rheumatoid arthritis. Biomed Chromatogr 2002;16:261-6.
28. Paterson SC, Lim CK, Smith KD. Analysis of the interaction between alpha-1-acid glycoprotein and tamoxifen and its metabolites. Biomed Chromatogr 2003;17:143-8.
29. Urien S, Barre J, Morin C et al. Docetaxel serum protein binding with high affinity to alpha 1-acid glycoprotein. Invest New Drugs 1996;14:147-151.
30. Du Bois A, Luck HJ, Meier W et al. A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. J Natl Cancer Inst 2003;95:1320-9.
31. Fruscio R, Lissoni AA, Frapolli R et al. Clindamycin-paclitaxel pharmacokinetic interaction in ovarian cancer patients. Cancer Chemother Pharmacol 2006;58:319-25.
32. Neault JF, Tajmir-Riahi HA. Interaction of cisplatin with human serum albumin. Drug binding mode and protein secondary structure. Biochim Biophys Acta 1998;1384:153-9.
33. Ivanov AI, Christodoulou J, Parkinson JA, et al: Cisplatin binding sites on human albumin. J Biol Chem 1998;273:14721-30.

10

General discussion

Introduction

This thesis describes the research conducted to identify biomarkers in ovarian cyst fluid which could be of prognostic value for patients with epithelial ovarian cancer (EOC). Blood and tissue of EOC patients have been traditionally used as starting material for biomarker research. In this thesis, for the first time ovarian cyst fluid has been used as a source of biomarkers for the prediction of survival and response to chemotherapy. Thus far, biomarker research using ovarian cyst fluid typically had an explorative design and focused on the differences in the concentration of various biomarker candidates between cyst fluid of benign and malignant ovarian tumors. As preoperative aspiration of ovarian cyst fluid might cause iatrogenic spread of tumor cells, it is advised to obtain cyst fluid only after surgical removal of the ovarian tumor. Consequently, the clinical value of cyst fluid biomarkers is limited to the prediction of prognosis and/or response to chemotherapy of EOC patients. An epidemiological analysis of the pathological data of a large series of patients with proven EOC, revealed that 84% of the ovarian carcinomas contained one or more cysts (chapter 2). Ovarian cyst fluid is thus generally available and could be used for research on prognostic and predictive biomarkers in this fluid.

Biomarkers for chemotherapy resistance

One of the main problems that may occur during the treatment of EOC is resistance to chemotherapy. Although the International Federation of Gynecology and Obstetrics (FIGO) stage remains a powerful predictor of disease outcome, ultimate survival depends on the extent of tumor debulking and response to chemotherapy. While some patients with advanced stage and bulky disease have a complete response to chemotherapy, other patients with more limited EOC can show rapid progression of disease. Contemporary chemotherapy regimens, consisting of a combination of platinum and taxol, introduced substantial improvements in disease free survival. However, the introduction of these regimens ultimately did not influence the overall survival of EOC patients. Identification of biomarkers that can serve as reliable predictors of drug response or drug activity is an area of interest to guide more effective tailor-made therapy. Chemotherapy resistance has been related to multiple mechanisms. Biomarkers predicting chemoresistance can be classified into three broad categories: biomarkers involved in drug delivery, biomarkers indicating the degree of drug penetration, and biomarkers influencing cellular response pathways.[1]

Biomarkers involved in drug delivery

Drug delivery failure is based on the hypothesis that tumor cells are exposed to insufficient doses of chemotherapeutic agents. An inadequate intratumoral drug concentration can be explained by pharmacokinetic variables such as a low bioavailability, an extensive first-pass metabolism, a high protein

binding and/or a low tissue binding.[1] Alpha 1-acid glycoprotein (AGP) is an important drug-binding and transport protein and its synthesis is induced in response to inflammation, tissue injury and cancer.[2] We assumed that if synthesis of AGP in adenocarcinoma cells of the ovary is enhanced, this would result in a higher concentration of AGP in ovarian cyst fluid (chapter 9). We indeed found a significantly higher concentration of AGP in cyst fluid of EOC patients compared to the concentration in cyst fluid of patients with benign ovarian tumors. We hypothesized that in patients with EOC, an increased cyst fluid concentration of AGP would result in a decreased free fraction of chemotherapeutics, resulting in a lower efficacy of the treatment. We first studied the *in vitro* interaction between cisplatin and AGP and paclitaxel and AGP by fluorescence techniques. The binding affinity of AGP and paclitaxel appeared to be high, whereas the binding affinity of AGP and cisplatin was rather weak. Interestingly, the concentration of AGP in ovarian cyst fluid was found to be a significant predictor of overall survival in multivariate analysis. We concluded that this independent predictive role of AGP was at least partly related to its high binding with paclitaxel. Therefore, AGP is a candidate biomarker for the prediction of paclitaxel-resistance in EOC. This finding should encourage further research on measuring AGP in body fluids of patients with EOC.

Biomarkers indicating drug penetration

Tumor hypoxia, angiogenesis and intercellular adhesion all may affect drug penetration and cell survival. The extracellular matrix (ECM) has been suggested to provide protection against chemotherapy-induced apoptosis in various types of cancers.[3] Activation of the proteolytic cascade resulting in ECM degradation, in general, is induced by the lysosomal cysteine proteases or cathepsins.[4] Previous studies have suggested that an altered ratio between proteolytic enzymes and their inhibitors might be responsible for the break-down of the ECM in cancer development.[5,6] In this thesis, we investigated whether the ratio between a number of cathepsins and their extracellular inhibitor cystatin C in ovarian cyst fluid was related to disease free survival of patients with EOC (chapter 5). No association between this ratio and disease outcome was detected, suggesting that cathepsins in ovarian cyst fluid may not be useful as prognostic biomarkers.

Biomarkers influencing cellular response pathways

The third group of biomarkers comprises compounds that influence cellular response pathways leading to cell death after exposure to cytotoxic agents.[1] Most researchers have investigated the cancer-cell specific pathway of drug inactivation via conjugation of glutathione, which can diminish the amount of free intracellular drug that is available to bind its target. This process is catalyzed by a family of glutathione S-transferases (GST), of which glutathione S-transferase Pi (GSTP1-1) especially is involved in the inactivation of alkylating agents, including platinum-based therapeutics.[7] Previous studies which examined GSTP1-1 expression in tumor cells suggested that increased expression of the enzyme is

related to platinum resistance of patients with EOC.[8-11] However, GSTP1-1 expression in EOC tissue was found to be very heterogeneous, which limits its usefulness as a prognostic biomarker. In addition, the assessment of GSTP1-1 in human plasma has proven to be unreliable and controversial because it requires critical sampling procedures.[12] As blood cells and platelets contain large amounts of GSTP1-1, release of GSTP1-1 by activation or damaging of cells during or after blood sampling may lead to a falsely high plasma concentration. It has been shown that the GSTP1-1 concentration in cyst fluid of patients with EOC was significantly higher than the concentration in fluid of patients with benign tumors.[13] This thesis shows that a high cyst fluid concentration of GSTP1-1 was an independent predictor of a poor overall survival of EOC patients treated with platinum-based chemotherapy (chapter 6), indicating that GSTP1-1 in ovarian cyst fluid might serve as a biomarker for the prediction of platinum-resistant EOC.

Targeting chemoresistance

EOC patients who relapse within 6 months after primary chemotherapy are considered chemoresistant. Information on chemoresistance mechanisms can be used to stratify patients according to their likelihood of responding to agents. In addition, this knowledge is of help to develop new drugs that aim to circumvent chemoresistance. For individual patients, elimination of chemotherapeutic drugs, for which they are or will become resistant, from the standard regimen will avoid toxic and ineffective treatment. It will also minimize delay in selection of active drugs and thus may improve their survival.

Several studies have already attempted to reverse chemoresistance by interacting with the GST-pathway. Canfosfamide (Telcyta®) is an investigational drug that was designed to make use of elevated levels of GSTP1-1. Preclinical studies suggest that activation of canfosfamide occurs when GSTP1-1 splits the drug into two active fragments: a glutathione analog fragment and an active cytotoxic fragment. The cytotoxic fragment interacts with important cell components, including DNA, RNA and proteins, ultimately leading to cell death. In addition, the glutathione analog fragment of canfosfamide may remain bound to GSTP1-1, which limits the ability of GSTP1-1 to inactivate other cancer drugs. The results of several clinical trials indeed suggest that canfosfamide is effective when combined with other chemotherapeutic drugs including platinum and taxol.[14-16] Although this novel agent has not yet been adopted for clinical use, in the future, patients with elevated ovarian cyst fluid levels of GSTP1-1 might be selected for additional treatment with canfosfamide.

Chemoresistance due to an increased AGP concentration has not yet been extensively investigated. Small molecules or drugs with a very high binding affinity for AGP (e.g. disopyramide, thioridazide, bupivacaine, trazodone) may displace paclitaxel, thereby increasing the free fraction of this chemotherapeutic agent and thus resulting in increased pharmacological efficacy.[2] Because AGP has only one drug binding site, competition between different pharmaceutics may have important clinical implications.[2]

This mechanism of competition has been investigated for patients with EOC concurrently treated with paclitaxel and clindamycin.[17] By adding clindamycin, which competes with paclitaxel in binding to AGP, the maximum concentration of paclitaxel in the plasma was enhanced. However, the changes in efficacy induced by giving a combination of the two drugs were minimal and the clinical relevance was questioned. For EOC patients with a high cyst fluid concentration of AGP, further studies with displacing agents might be valuable for the improvement of the cytotoxic effect of paclitaxel. A second possibility to circumvent paclitaxel-resistance due to an increased AGP synthesis might be first-line treatment with PEGylated liposomal doxorubicin (PLD)(Caelyx®) instead of paclitaxel. PLD has a much lower binding affinity to AGP than paclitaxel.[2] A number of studies have already shown the efficacy of PLD in relapsed ovarian cancer.[18-20] Recently, a phase III trial demonstrated the activity of PLD plus carboplatin as a first-line treatment of advanced stage EOC.[21] Patients with elevated ovarian cyst fluid levels of AGP might be selected for additional treatment with PLD.

Pitfalls in biomarker research for EOC

Chemoresistance is a complex multistep mechanism in which many genes, proteins and metabolites are involved. Therefore, a single biomarker most likely may not be able to predict the chemotherapeutic response of EOC patients in general. In addition, the extreme histological diversity of EOC severely limits the discovery of single biomarkers for response to chemotherapy. Although scientists frequently refer to EOC as a distinct disease entity, each histopathological subtype differs significantly in microscopic appearance, and in biological and genetic background as well.[22-24] Recent studies compared the expression of multiple candidate tissue-based biomarkers between the different histopathological subtypes of EOC and found profound differences.[24,25]. Also for the most common EOC biomarker, CA 125, the proportion of CA 125-producing cells in mucinous tumors is much lower than in the other histological subtypes.[26] This was confirmed in our study, as ovarian cyst fluid of mucinous tumors contained less CA 125 than cyst fluid of the other tumor subtypes (chapter 3). It might explain why CA 125 in ovarian cyst fluid failed to serve as an appropriate biomarker for disease outcome. Significant differences in concentration of cathepsins in cyst fluid were also found among histopathological subtypes of ovarian tumors (chapter 4 and 5). In addition, as described in chapter 8, serous tumors could almost entirely be distinguished from mucinous tumors using the cyst fluid concentration of N-acetylaspartate (NAA). While ovarian cyst fluid and ascites of patients with serous tumors contained high concentrations of NAA, cyst fluid of mucinous tumors contained very low concentrations of NAA, which were comparable to serum reference values.

As the incidence of primary EOC is low, the studies in this thesis were performed with relatively small sample sizes. We were not able to extend our series as ovarian cyst fluid is not collected regularly in Dutch hospitals. In addition, neo-adjuvant chemotherapy is currently offered to patients who are not suitable for primary optimal debulking surgery. Thus far, the influence of neo-adjuvant chemotherapy

on the concentration of biomarkers in ovarian cyst fluid has not been investigated. Therefore, the true prognostic value of a potential new biomarker could not be established in patients treated with neo-adjuvant chemotherapy and we had to exclude these patients from our studies. Another issue concerned the quantification of the compounds of interest in the cyst fluid. Differences in size between the cysts raised the question whether or not to correct for differences in total volume. The concentration of metabolites in urine is usually normalized on creatinine as volume correction. For ovarian cyst fluid however, there are no systematic studies that have addressed this question. In addition, differences in viscosity between the cyst fluid samples may influence the biomarker assays.

Future perspectives

It has become clear that ovarian cancer survival rates will not be improved by treating all patients uniformly according to standard guidelines. The ultimate goal for biomarker research is to define abnormalities in individual patient's cancers, permitting the choice of drug combinations that will optimize efficacy and minimize toxicity. In this thesis, we showed a stepwise discovery of potential new biomarkers using metabolomic profiling (chapter 7). By using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, NAA (chapter 8) and AGP (chapter 9) in ovarian cyst fluid were identified as new biomarkers. Advanced molecular profiling of ovarian cyst fluid may thus assist the identification of predictive biomarkers for therapy selection.

The results of this thesis have shown that that GSTP1-1 and AGP in ovarian cyst fluid may have the potential to predict the response to platinum- and paclitaxel-based chemotherapy, respectively. GSTP1-1 and AGP both were found to be independent predictors of survival, both were involved in different pathways of chemoresistance and both were found to interact with a different chemotherapeutic agent. At first, we suggest to validate the predictive value of (a combination of) these cyst fluid biomarkers for chemotherapy response in an independent cohort of EOC patients. Enlarging the sample size would improve power in statistical analyses and will enable to study the histological subtypes of EOC separately. Moreover, it would allow for stratification of patients. After successful validation, we propose to conduct an international multicenter clinical trial to investigate the advantage of an alternative first-line treatment for EOC patients with a high ovarian cyst fluid concentration of AGP and/or GSTP1-1. The alternative treatment for patients with a high cyst fluid concentration of GSTP1-1, might consist of a combination of paclitaxel and a platinum agent together with canfosfamide.[15] For patients with a high cyst fluid concentration of AGP, the alternative treatment might consist of a platinum agent and PLD.[21] The international character of such a trial in our view is mandatory in order to assure adequate accrual rates. This means that multicenter collection of cyst fluid remains a priority for the ongoing research on the predictive value of cyst fluid biomarkers for response to chemotherapy in individual patients.

References

1. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 2003;3:502-16.
2. Israili ZH, Dayton PG. Human alpha-1-glycoprotein and its interactions with drugs. Drug Metab Rev 2001;33:161-235.
3. Sethi T, Rintoul RC, Moore SM et al. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med 1999;5:662-8.
4. Skrzydlewska E, Sulkowska M, Koda M et al. Proteolytic-antiproteolytic balance and its regulation in carcinogenesis. World J Gastroenterol 2005;11:1251-66.
5. Kos J, Werle B, Lah T et al. Cysteine proteinases and their inhibitors in extracellular fluids: markers for diagnosis and prognosis in cancer. Int J Biol Markers 2000;15:84-9.
6. Nishikawa H, Ozaki Y, Nakanishi T et al. The role of cathepsin B and cystatin C in the mechanisms of invasion by ovarian cancer. Gynecol Oncol 2004;92:881-6.
7. Beckett GJ, Hayes JD. Glutathione S-transferases: biomedical applications. Adv Clin Chem 1993;30:281-380.
8. Hamada S, Kamada M, Furumoto H et al. Expression of glutathione S-transferase-pi in human ovarian cancer as an indicator of resistance to chemotherapy. Gynecol Oncol 1994;52:313-9.
9. Green JA, Robertson LJ, Clark AH. Glutathione S-transferase expression in benign and malignant ovarian tumours. Br J Cancer 1993;68:235-9.
10. Cheng X, Kigawa J, Minagawa Y et al. Glutathione S-transferase-pi expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. Cancer 1997;79:521-7.
11. Kase H, Kodama S, Nagai E et al. Glutathione S-transferase pi immunostaining of cisplatin-resistant ovarian cancer cells in ascites. Acta Cytol 1998;42:1397-402.
12. Mulder TP, Peters WH, Wobbes T et al. Measurement of glutathione S-transferase P1-1 in plasma: pitfalls and significance of screening and follow-up of patients with gastrointestinal carcinoma. Cancer 1997;80:873-80.
13. Boss EA, Peters WH, Roelofs HM et al. Glutathione S-transferases P1-1 and A1-1 in ovarian cyst fluids. Eur J Gynaecol Oncol 2001;22:427-32.
14. Dinh P, Harnett P, Piccart-Gebhart MJ et al. New therapies for ovarian cancer: cytotoxics and molecularly targeted agents. Crit Rev Oncol Hematol 2008;67:103-12.
15. Vergote I, Finkler N, del CJ et al. Phase 3 randomised study of canfosfamide (Telcyta, TLK286) versus pegylated liposomal doxorubicin or topotecan as third-line therapy in patients with platinum-refractory or -resistant ovarian cancer. Eur J Cancer 2009;45:2324-32.
16. Kavanagh JJ, Gershenson DM, Choi H et al. Multi-institutional phase 2 study of TLK286 (TELCYTA, a glutathione S-transferase P1-1 activated glutathione analog prodrug) in patients with platinum and paclitaxel refractory or resistant ovarian cancer. Int J Gynecol Cancer 2005;15:593-600.
17. Fruscio R, Lissoni AA, Frapolli R et al. Clindamycin-paclitaxel pharmacokinetic interaction in ovarian cancer patients. Cancer Chemother Pharmacol 2006;58:319-25.
18. Power P, Stuart G, Oza A et al. Efficacy of pegylated liposomal doxorubicin (PLD) plus carboplatin in ovarian cancer patients who recur within six to twelve months: a phase II study. Gynecol Oncol 2009;114:410-4.
19. Rapoport BL, Vorobiof DA, Slabber C et al. Phase II study of pegylated liposomal doxorubicin and carboplatin in patients with platinum-sensitive and partially platinum-sensitive metastatic ovarian cancer. Int J Gynecol Cancer 2009;19:1137-41.
20. Weber B, Lortholary A, Mayer F et al. Pegylated liposomal doxorubicin and carboplatin in late-relapsing ovarian cancer: a GINECO group phase II trial. Anticancer Res 2009;29:4195-200.
21. Pignata S, Scambia G, Savarese A et al. Carboplatin and pegylated liposomal doxorubicin for advanced ovarian cancer: preliminary activity results of the MITO-2 phase III trial. Oncology 2009;76:49-54.

22. Zorn KK, Bonome T, Gangi L et al. Gene expression profiles of serous, endometrioid, and clear cell subtypes of ovarian and endometrial cancer. Clin Cancer Res 2005;11:6422-30.
23. Kurman RJ, Shih I. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. Int J Gynecol Pathol 2008;27:151-60.
24. Kobel M, Kalloger SE, Boyd N et al. Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS Med 2008;5:e232.
25. Cloven NG, Kyshtoobayeva A, Burger RA et al. In vitro chemoresistance and biomarker profiles are unique for histologic subtypes of epithelial ovarian cancer. Gynecol Oncol 2004;92:160-6.
26. Høgdall EV, Christensen L, Kjaer SK et al. CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study. Gynecol Oncol 2007;104:508-15.

11

Summary | Samenvatting

Summary

Background

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy worldwide. Despite improvement in cytoreductive surgery and the introduction of platinum- and taxol-based chemotherapy, gains in survival rates over the last three decades are rather modest. Approximately 20% of the patients with primary EOC shows resistance to first-line chemotherapy. Of the EOC patients who do respond to first-line chemotherapy, the majority will relapse with drug-resistant disease within a few years. Biomarkers may help to predict drug response and survival in individual patients. Their use may lead to guide more effective individualized patient therapy. So far, biomarker research has been mainly performed using blood and tissue of EOC patients as a starting material. Up to now, these studies did not result in the discovery of (a) new prognostic biomarker(s) with both high sensitivity and specificity. The concentration of compounds in blood of patients with cancer may be influenced by drug treatment and/or physiological processes. Tissue biomarker research is severely limited by the extreme heterogeneity of EOC tissue, resulting in tumor areas with different grade and histological differentiation within the tumor. Ovarian cyst fluid offers an alternative source of biomarkers because of its homogeneous nature and proximity to the ovarian tumor. It has been demonstrated that the concentration of analytes in the fluid often reflects the biological processes taking place within the ovarian tumor surface. Ovarian cyst fluid can be easily obtained after removal of the tumor at surgery and can be used for the measurement of compounds with potential prognostic (predictive for patient survival) and/or predictive (predictive for response to therapy) value.

Chapter 1

In the introduction of this thesis, a short historical overview on the use of ovarian cyst fluid is presented, together with the current state of research about prognostic and predictive biomarkers in different biomaterials of EOC patients. A number of prognostic and predictive biomarkers which have been traditionally used in cancer studies are selected and presented in more detail. Thereafter, metabolic profiling methods for the discovery of new biomarkers are described.

Chapter 2

The first key to success in biomarker research is the collection of appropriate clinical material. Not all ovarian tumors are cystic and therefore, the presence of cysts in EOC is a prerequisite for research focused on predictive and prognostic biomarkers in ovarian cyst fluid. In chapter 2, an epidemiological analysis of the pathological data of 233 of patients with proven primary EOC is presented, showing

that cysts in EOC were present in 84% of the patients. The most common histological subtypes (serous, mucinous, endometrioid, clear cell) contained cysts in more than 85% of the cases. These findings show that ovarian cyst fluid is generally available. This enables research on prognostic and predictive biomarkers in this fluid.

Chapter 3

CA 125 is the best known biomarker for EOC and the only biomarker that is currently used in daily practice. Serum CA 125 levels measured during chemotherapy correlate well with the course of the disease. However, the clinical value of preoperative serum CA 125 for the prediction of survival of EOC patients remains controversial. In chapter 3, the prognostic value of CA 125 in ovarian cyst fluid is studied for patients with EOC. Although high levels of CA 125 in ovarian cyst fluid were significantly associated with a poor survival of EOC patients, CA 125 was not of independent value and might therefore not be useful as a prognostic biomarker for EOC.

Chapter 4

A major step in cancer growth and dissemination involves the degradation of the extracellular matrix. This process is mediated by multiple degradative actions of proteolytic enzymes or proteases. During cancer development and progression, cathepsins (lysosomal cysteine proteases) are often translocated to the cell surface or secreted into surrounding fluids. We performed the first study to explore the concentrations of cathepsins (B and L) and their inhibitor (cystatin C) in cyst fluid of patients with ovarian tumors (chapter 4). Ovarian cyst fluid contained considerable amounts of cathepsin B, cathepsin L and cystatin C. Significant differences in concentration of cathepsins in cyst fluid were found among histopathological subtypes of ovarian tumors. Cathepsin B seemed to be useful for discriminating between malignant and benign tumors of serous histology, whereas cathepsin L appeared to be useful for discriminating between malignant and benign tumors of mucinous histology. These results contribute to findings of recent studies that detected profound differences in expression of multiple candidate tissue-based biomarkers between the histopathological subtypes of EOC. In addition, cathepsin B and cystatin C were strongly correlated in the group of patients with malignant serous tumors. This suggests that an increase in cathepsin B might be balanced by a corresponding increase in cystatin C. Therefore, the ratio between extracellularly released cathepsins and cystatin C seems to be of importance for the progression and metastatic potential of EOC.

Chapter 5

In chapter 5, the ratio between the concentration of cathepsins (B, H, L, X) and their extracellular inhibitor cystatin C in ovarian cyst fluid was found to be related to disease free survival of patients with EOC. Ratio's between cystatin C and cathepsin B were significantly lower for patients with metastatic compared to localized EOC. Ratio's between cystatin C and cathepsin H and between cystatin C and cathepsin X differed significantly between histological subtypes and were significantly higher for high-grade tumors compared to low-grade tumors. Ratio's between cystatin C and cathepsins did not significantly predict survival for EOC patients. However, the interaction between cathepsins and cystatin C seems to be of importance for the development and progression of EOC.

Chapter 6

An important mechanism of chemoresistance that has been studied includes the drug inactivation via conjugation of glutathione, which can diminish the amount of free intracellular drug that is available to bind its target. This process is catalyzed by a family of glutathione S-transferases, of which glutathione S-transferase Pi (GSTP1-1) is especially involved in the inactivation of alkylating agents, including platinum-based therapeutics. In chapter 6, we showed that a high cyst fluid concentration of GSTP1-1 was an independent predictor of a poor overall survival of EOC patients treated with platinum-based chemotherapy. This indicates that GSTP1-1 in ovarian cyst fluid might serve as a biomarker for the prediction of platinum-resistant EOC. Canfosfamide (Telcyta) is an investigational drug that was designed to make use of elevated levels of GSTP1-1. Although this novel agent has not yet been adopted for clinical use, in the future patients with elevated ovarian cyst fluid levels of GSTP1-1 might be selected for additional treatment with canfosfamide.

Chapter 7

In recent years, the advent of high-throughput technologies has facilitated the molecular profiling of tumors. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy of body fluids shows the majority of proton-containing compounds and therefore provides an overall view of metabolism. For the discovery of new biomarkers, such "holistic" techniques are a great advantage compared with conventional techniques. In chapter 7, we demonstrated the stepwise discovery of potential new biomarkers using metabolomic profiling. Cyst fluid samples of malignant, borderline and benign ovarian tumors were screened by ^{1}H-NMR spectroscopy. The presence of high concentrations of NAA in cyst fluid samples of some ovarian tumors was remarkable as to this point, NAA had exclusively been attributed to neurons of the human brain. In addition, the presence of high concentrations of alpha 1-acid glycoprotein (AGP) in ovarian cyst fluid is interesting as AGP is the major binding protein for a number of chemotherapeutics. NAA and AGP are further investigated in chapter 8 and 9, respectively.

Chapter 8

N-acetyl L-aspartate (NAA) has not been detected in human tissues other than brain. Recently, it has been suggested that NAA may function as a molecular water pump, responsible for the removal of large amounts of water from the human brain. As the mechanism of ovarian cyst formation is still unknown, in chapter 8, the possible role of NAA in body water management is investigated. We demonstrated that NAA is present in a high micromolar concentration in ovarian cyst fluid of ovarian tumors of serous histology, sometimes in the same concentration range as found in the extracellular space of the human brain. The NAA concentration in cyst fluid was mostly 10 to 50 fold higher than the normal serum NAA concentration, suggesting a local production of NAA in serous ovarian tumors. In contrast, ovarian cyst fluid of ovarian tumors with a mucinous, endometrioid and clear cell histological subtype contained low micromolar concentrations of NAA. Serous ovarian tumors have an epithelial differentiation pattern which resembles the lining of the fallopian tube and differs from the other histological subtypes. Interestingly, in fluid samples from enlarged fallopian tubes (hydrosalpinx) the NAA concentration was in the same range as the NAA concentration found in ovarian cyst fluid of serous tumors. Our findings provide the first evidence of NAA production outside the human brain and contribute to the ongoing research understanding the physiological function of NAA in the human body.

Chapter 9

AGP is an important drug-binding protein and acute-phase protein of which the synthesis is increased in response to cancer. Although most research has focused on the hepatic synthesis of AGP and its release into the serum, local extra-hepatic synthesis of AGP in cancer tissue has also been described. We hypothesized that an increased cyst fluid concentration of AGP, derived from adenocarcinoma cells of the ovary, would result in an increased binding of chemotherapeutics with a high binding affinity to AGP and consequently, in a decreased free fraction of chemotherapeutics. In chapter 9, the concentration of AGP in ovarian cyst fluid was found to be an independent significant predictor of survival of EOC patients. In addition, we demonstrated that AGP binding affinity with paclitaxel was high, whereas the binding affinity of AGP and cisplatin was rather weak. We concluded that the independent predictive role of AGP was at least partly related to its high binding affinity with paclitaxel. Therefore, AGP might be a candidate biomarker for the prediction of paclitaxel-resistance in EOC. This knowledge is of help to develop new drugs that aim to target chemoresistance. For EOC patients with a high cyst fluid concentration of AGP, it would be interesting to investigate whether drugs with a very high binding affinity for AGP may displace paclitaxel, thereby increasing the free fraction of this chemotherapeutic agent and thus resulting in increased pharmacological efficacy.

Chapter 10

In the general discussion of this thesis, we primarily focus on ovarian cyst fluid biomarkers that may be useful for the prediction of drug response or drug activity. These biomarkers can be clinically of value to guide more effective, tailor-made therapy. Early identification of chemoresistance will offer the possibility to eliminate non-effective chemotherapeutic agents from the standard regimen. This will avoid toxic and ineffective treatment, and will also minimize the delay in selection of alternative drugs. In addition, this knowledge is of help to develop new drugs that aim to circumvent chemoresistance. In chapter 10, we discuss three biomarkers studied earlier in this thesis in more detail. These biomarkers were involved in different mechanisms of chemoresistance: AGP as a biomarker involved in drug delivery to the tumor, cathepsins as biomarkers indicating the degree of drug penetration, and GSTP1-1 as a cell-specific biomarker involved in the inactivation of drugs. Suggestions for targeted-therapies are offered as well. We suggest that a combination of AGP and GSTP1-1 might be useful for the prediction of response to paclitaxel- and platinum-based chemotherapy. GSTP1-1 and AGP both were found to be independent predictors of survival, both were involved in different pathways of chemoresistance, and both were found to interact with a different chemotherapeutic agent. Further prospective studies with larger series of patients are warranted to validate whether a combination measurement of AGP and GSTP1-1 in ovarian cyst fluid can predict chemotherapy resistance of EOC patients and could be useful for clinical practice.

Samenvatting

Eierstokkanker is de meest dodelijke gynaecologische maligniteit wereldwijd. Ondanks de optimalisering van cytoreductieve chirurgie en het invoeren van agressieve chemotherapie zijn de overlevingskansen voor patiënten met eierstokkanker de afgelopen drie decennia nauwelijks verbeterd. Ongeveer 20% van de patiënten blijkt primair niet te reageren op eerstelijns chemotherapie. Van de patiënten die in eerste instantie wel reageren, krijgt de meerderheid binnen enkele jaren toch nog een recidief van de tumor. Biomarkers die in een vroege fase van de ziekte de respons op chemotherapie kunnen voorspellen bij individuele patiënten zouden theoretisch kunnen bijdragen aan een effectievere therapie en dus aan de overleving. Tot dusver zijn biomarkers hoofdzakelijk bestudeerd in het bloed en tumorweefsel van patiënten met eierstokkanker. Deze studies hebben echter niet geresulteerd in de ontdekking van biomarkers met voldoende sensitiviteit en specificiteit om van voorspellende waarde te kunnen zijn. Biomarker concentraties in bloed van patiënten met eierstokkanker worden mede beïnvloed door systemische processen elders in het lichaam. Biomarkers in tumorweefsel zijn moeilijk te meten door de extreme heterogeniteit van het weefsel. Dit resulteert in gebieden met verschillende tumor graad en histologische differentiatie binnen één tumor. Het aanwezige cystevocht in de tumor van de eierstok is een alternatieve bron om potentiële biomarkers te bestuderen. Men veronderstelt dat de componenten in het cystevocht een directe afspiegeling vormen van de biochemische processen die zich in de tumor afspelen. Tegelijkertijd is de distributie van componenten in het cystevocht naar verwachting homogener dan in het tumorweefsel. Het ovariële cystevocht kan eenvoudig worden verkregen na chirurgische verwijdering van de tumor. Vervolgens kan het worden gebruikt voor het bestuderen van biomarkers die de overleving (prognostische biomarkers) en/of de respons op chemotherapie (predictieve biomarkers) van patiënten met eierstokkanker zouden kunnen voorspellen.

In **hoofdstuk 1** van dit proefschrift is een kort overzicht gegeven van de toepassing van ovariëel cystevocht door de jaren heen. Vervolgens zijn studies vergeleken die prognostische en/of predictieve biomarkers hebben onderzocht in respectievelijk bloed, weefsel en cystevocht van patiënten met eierstokkanker. Daarna is er een overzicht gepresenteerd van een aantal veelbelovende prognostische en predictieve biomarkers uit eerder verschenen oncologische studies. Tot slot zijn innovatieve methoden beschreven die kunnen bijdragen aan de ontdekking van nieuwe biomarkers voor patiënten met eierstokkanker.

Voor succesvol onderzoek naar biomarkers is het verzamelen van geschikt patiënten materiaal een eerste vereiste. Het is bekend dat eierstokkanker meestal cysteus is, maar exacte prevalentie cijfers zijn niet gedocumenteerd. **Hoofdstuk 2** omvat een epidemiologische analyse van de pathologische gegevens van een groot aantal patiënten met eierstokkanker. Hieruit blijkt dat 84% van de onderzochte tumoren tenminste één cysteuze component bevat. Tevens bevatten tumoren van de meest voorkomende histologische subtypen (sereus, mucineus, endometrioid en clear cell) in meer dan 85% van de gevallen cysten. Deze bevindingen tonen aan dat ovariëel cystevocht bijna altijd beschikbaar is, wat zowel onderzoek als klinische toepasbaarheid mogelijk maakt.

CA 125 is de meest bekende biomarker voor eierstokkanker en de enige biomarker die momenteel wereldwijd in de kliniek wordt toegepast voor het monitoren van de ziekte tijdens chemotherapie. Echter, de prognostische waarde van CA 125 gemeten in preoperatief bloed van patiënten met eierstokkanker voor de overleving of respons op chemotherapie is onduidelijk. In **hoofdstuk 3** is de prognostische waarde van CA 125 in ovariëel cystevocht van patiënten met eierstokkanker onderzocht. Hoewel een hoge concentratie van CA 125 in ovariëel cystevocht correleert met een slechte overleving, blijkt CA 125 geen onafhankelijke voorspeller van de prognose. Daarom lijkt CA 125 in ovariëel cystevocht niet bruikbaar als een prognostische biomarker voor patiënten met eierstokkanker.

Een belangrijke stap in de groei en metastasering van kanker is de afbraak van de extracellulaire matrix. Dit proces wordt bewerkstelligd door proteases. Cathepsines zijn cysteine proteases die onder fysiologische omstandigheden in lysosomen van cellen zijn gelokaliseerd. Echter, tijdens de ontwikkeling van kanker worden cathepsines naar het celoppervlak getransporteerd en vervolgens uitgescheiden. In **hoofdstuk 4** is onderzocht of cathepsines (B en L) en hun belangrijkste extracellulaire remmer (cystatine C) worden uitgescheiden in cystevocht van ovariële tumoren. Het blijkt dat ovariëel cystevocht hoge concentraties cathepsine B, L en cystatine C bevat. Deze waarden zijn 6 tot 10 keer hoger dan eerder gerapporteerde concentraties in bloed. Er worden significante verschillen in concentratie van cathepsines gevonden tussen de diverse histopathologische subtypen van ovariële tumoren. Bovendien blijken cathepsine B en cystatine C sterk met elkaar gecorreleerd te zijn bij patiënten met een sereus type eierstokkanker. Mogelijkerwijs wordt bij deze patiënten een stijging van de cathepsine B concentratie gecompenseerd door een overeenkomstige stijging van cystatine C. De ratio van cathepsines en cystatine C lijkt daarom interessant voor de progressie van eierstokkanker en is verder bestudeerd in het volgende hoofdstuk van dit proefschrift.

In **hoofdstuk 5** is onderzocht of de verhouding tussen de concentratie van cathepsines (B, H, L, X) en hun extracellulaire remmer, cystatine C, in ovariëel cystevocht van patiënten met eierstokkanker geassocieerd is met de ziektevrije overleving. De ratio van cystatine C en cathepsine B blijkt significant lager te zijn voor patiënten met gemetastaseerde eierstokkanker dan voor patiënten met een locale vorm van de ziekte. Echter, de ratio van cystatine C en cathepsines (B, H, L, X) gemeten in cystevocht blijkt geen significante voorspeller te zijn voor de ziektevrije overleving van patiënten met eierstokkanker. Hoewel de interactie tussen cystatine C en (met name) cathepsine B een mogelijke rol speelt bij de ontwikkeling en progressie van eierstokkanker, lijken cathepsines niet bruikbaar als prognostische biomarkers.

Een belangrijk mechanisme van chemoresistentie omvat de inactivatie van chemotherapeutica via de conjugatie van glutathion. Dit proces wordt gekatalyseerd door de familie van de glutathion S-transferases, waarvan glutathion S-transferase Pi (GSTP1-1) in het bijzonder betrokken is bij de inactivering van platinum bevattende chemotherapie. In **hoofdstuk 6** is aangetoond dat een hoge concentratie van GSTP1-1 in ovariëel cystevocht een onafhankelijke voorspeller is van een slechte

overleving van patiënten met eierstokkanker die behandeld zijn met platinum bevattende chemotherapie. GSTP1-1 in ovariëel cystevocht zou dus een kandidaat biomarker kunnen zijn voor de voorspelling van chemoresistentie. Canfosfamide (Telcyta®) is een experimenteel geneesmiddel dat is ontworpen om de hoge concentratie van GSTP1-1 juist te gebruiken voor de behandeling van eierstokkanker. Hoewel dit nieuwe geneesmiddel nog niet op de markt is, zouden in de toekomst patiënten met verhoogde GSTP1-1 concentraties in ovariëel cystevocht kunnen worden geselecteerd voor een additionele behandeling met canfosfamide.

De afgelopen jaren hebben nieuwe diagnostische technologieën het mogelijk gemaakt om moleculaire en genetische profielen van tumoren in kaart te brengen. Met proton nucleaire magnetische resonantie (^{1}H-NMR) spectroscopie kan een compleet overzicht worden verkregen van de aanwezige metabolieten in een specifieke lichaamsvloeistof. Dergelijke "holistische" technieken zijn een groot voordeel bij de detectie van nieuwe biomarkers in vergelijking met de meer conventionele technieken. In **hoofdstuk 7** is de stapsgewijze ontdekking van nieuwe biomarkers in ovariëel cystevocht met behulp van ^{1}H-NMR spectroscopie beschreven. Er worden twee potentiële biomarkers voor eierstokkanker gevonden in ovariëel cystevocht. N-acetylaspartaat (NAA), tot dusver uitsluitend aangetoond in menselijke hersenen, blijkt in hoge concentraties voor te komen in ovariëel cystevocht. Van het alfa 1-zure glycoproteïne (AGP), een belangrijk transporteiwit voor geneesmiddelen, worden tevens hoge concentraties aangetoond in ovariëel cystevocht. NAA en AGP zijn verder onderzocht in de volgende twee hoofdstukken van dit proefschrift.

Hoewel de fysiologische rol van NAA vooralsnog niet geheel bekend is, is geponeerd dat het functioneert als moleculaire waterpomp, verantwoordelijk voor de actieve eliminatie van grote hoeveelheden water uit de menselijke hersenen. Deze waterpomp functie zou mogelijk ook een rol kunnen spelen bij het ontstaan van cysteuze ovariële tumoren. In **hoofdstuk 8** is aangetoond dat NAA in een hoge concentratie aanwezig is in cystevocht van sereuze ovariële tumoren, soms in dezelfde concentratie als in de extracellulaire ruimte van de hersenen. Ovariële tumoren met een ander histologisch subtype (mucineus, endometrioid en clear cell) bevatten daarentegen verwaarloosbaar lage concentraties NAA. Onze bevindingen leveren het eerste bewijs dat NAA wordt aangemaakt buiten de menselijke hersenen en dragen bij aan de kennis over de fysiologische functie van NAA in het lichaam.

AGP is zowel een belangrijk transporteiwit voor geneesmiddelen als een acute fase eiwit. Als reactie op kanker wordt AGP in verhoogde mate aangemaakt in de lever. Echter, lokale extra-hepatische synthese van AGP op de plaats van de tumor is eerder in de literatuur beschreven. Een verhoogde ovariële cystevocht AGP concentratie, afkomstig van ovariële tumorcellen, zou theoretisch kunnen leiden tot een verhoogde binding van chemotherapeutica, met als gevolg een verminderde cytotoxische werking op de tumor. In **hoofdstuk 9** blijkt de concentratie van AGP in ovariëel cystevocht een onafhankelijke voorspeller van de overleving van patiënten met eierstokkanker die behandeld zijn met chemotherapie. In een *in vitro* studie tonen we aan dat de bindingsaffiniteit van AGP met paclitaxel hoog is, terwijl de bindingsaffiniteit van AGP met cisplatin laag is. De voorspellende

waarde van AGP voor de overleving van patiënten met een EOC lijkt verband te houden met de hoge bindingsaffiniteit van AGP met paclitaxel. AGP zou dus een potentiële biomarker kunnen zijn voor de voorspelling van paclitaxel resistentie. In de toekomst zou PEGylated liposomaal doxorubicine (PLD) (Caelyx®) in plaats van paclitaxel als eerstelijns chemotherapie kunnen worden gegeven aan patiënten met een hoge AGP cystevocht concentratie. PLD heeft een veel lagere bindingsaffiniteit voor AGP dan paclitaxel. Uit een recente fase III studie blijkt dat een combinatie van PLD en carboplatin succesvol is als therapie bij patiënten met een vergevorderde vorm van eierstokkanker.

Hoofdstuk 10 vormt de algemene discussie van dit proefschrift. De nadrukt ligt op biomarkers in ovariëel cystevocht die zouden kunnen bijdragen aan de voorspelling van de respons op chemotherapie bij patiënten met eierstokkanker. Bevindingen uit dit proefschrift worden geïntegreerd met recente ontwikkelingen om zo een kader te scheppen voor de klinische toepasbaarheid van biomarkers in cystevocht en hun waarde voor de individuele patiënt.

Bibliography

Kolwijck E, Boss EA, van Altena AM, Beex LV, Massuger LF. Stage IV epithelial ovarian carcinoma in an 18 year old patient presenting with a Sister Mary Joseph's nodule and metastasis in both breasts: a case report and review of the literature. Gynecol Oncol 2007;107(3):583-5.

Van Altena AM, Wijnberg GJ, **Kolwijck E**, de Hullu JA, Massuger LF. A patient with bilateral immature ovarian teratoma presenting with paraneoplastic encephalitis. Gynecol Oncol 2008;108(2):445-8.

Kolwijck E, Kruitwagen RF, Massuger LF. Sister Mary Joseph nodule as a first and only sign of extraovarian carcinoma: a case report and review of the literature. Arch Pathol Lab Med 2008;132(12):1943-5.

Kolwijck E, Engelke UF, van der Graaf M, Heerschap A, Blom HJ, Hadfoune M, Buurman WA, Massuger LF, Wevers RA. N-acetyl resonances in in vivo and in vitro NMR spectroscopy of cystic ovarian tumors. NMR Biomed 2009;22(10):1093-9.

Kolwijck E, Zusterzeel PL, Roelofs HM, Hendriks JC, Peters WH, Massuger LF. GSTP1-1 in ovarian cyst fluid and disease outcome of patients with ovarian cancer. Cancer Epidemiol Biomarkers Prev 2009;18(8):2176-81.

Kolwijck E, Thomas CM, Bulten J, Massuger LF. Preoperative CA 125 levels in 123 patients with borderline ovarian tumors: a retrospective analysis and review of the literature. Int J Gynecol Cancer. 2009;19(8):1335-8.

Kolwijck E, Span PN, Thomas CM, Bulten J, Sweep FC, Massuger LF. Prognostic value of CA 125 in ovarian cyst fluid of patients with epithelial ovarian cancer. Oncol Rep 2010;23(2):579-84.

Kolwijck E, Massuger LF, Thomas CM, Span PN, Krasovec M, Kos J, Sweep FC. Cathepsins B, L and cystatin C in cyst fluid of ovarian tumors. J Cancer Res Clin Oncol 2010;136(5):771-8

Kolwijck E, Wevers RA, Engelke UF, Woudenberg J, Bulten J, Blom HJ, Massuger LF. Ovarian cyst fluid of serous ovarian tumors contains large quantities of the brain amino acid N-acetylaspartate. PLoS ONE 2010;5(4):e10293

Kolwijck E, Lybøl C, Bulten J, Vollebergh JH, Wevers RA, Massuger LF. Prevalence of cysts in epithelial ovarian cancer. Eur J Obst Gyn Rep Biol 2010; in press

Kolwijck E, Kos J, Obermajer N, Span PN , Thomas CM, Massuger LF, Sweep FC. The balance between cathepsins and cystatin C is of importance for ovarian cancer. Eur J Clin Invest 2010; in press

Kolwijck E, Baurin S, Laub R, Bulten J, Buurman WA, Engelke UF, Wevers RA, Massuger LF. Alpha 1-acid glycoprotein as an independent predictor of survival in ovarian cancer patients treated with chemotherapy. Submitted

Van Altena AM, **Kolwijck E**, Spanjer MJ, Hendriks JC, Massuger LF, De Hullu JA. CA 125 nadir concentration is an independent predictor of tumor recurrence in patients with ovarian cancer. Submitted

Curriculum Vitae

Eva Kolwijck werd geboren op 12 april 1980 te Nijmegen. In 1998 behaalde zij haar VWO diploma aan het Kandinsky College te Nijmegen. Na te zijn uitgeloot voor de studie geneeskunde, startte zij in 1998 met de opleiding gezondheidswetenschappen aan de Universiteit van Maastricht. In 2000 kon zij uiteindelijk beginnen met de studie geneeskunde aan de Radboud Universiteit Nijmegen. In 2005 werd een wetenschappelijke stage verricht bij de afdeling Medische Microbiologie van het UMC st. Radboud (prof. dr. J.M. Galama). Hier werd haar interesse gewekt voor het wetenschappelijk onderzoek en het schrijven van medische artikelen. Tijdens haar co-schappen was zij redactielid van het co-assistenten tijdschrift "Status Co". Na het behalen van haar artsexamen in 2007 begon zij met wetenschappelijk onderzoek bij de afdeling Gynaecologische Oncologie (prof. dr. L.F.A.G. Massuger), de afdeling Chemische Endocrinologie (prof. dr. C.G.J. Sweep) en het Laboratorium voor Kindergeneeskunde en Neurologie (prof. R.A. Wevers) van het UMC st. Radboud, wat tot dit proefschrift heeft geleid. In december 2009 startte zij met de opleiding tot arts-microbioloog in het Canisius Wilhelmina ziekenhuis te Nijmegen.

Dankwoord

Toen ik direct na mijn co-schappen begon aan dit project had ik nooit verwacht dat ik ook daadwerkelijk zou gaan promoveren. Ik wilde in eerste instantie gewoon heel erg graag onderzoek doen. Gaandeweg merkte ik dat ik naar iets toewerkte en dat het ook nog een naam kreeg: promotieonderzoek. Wat ik ook niet had verwacht is dat ik naast het doen van onderzoek ontzettend veel andere dingen zou leren. Van mensen, over mensen en door mensen over mezelf. Zo zie je maar.

Naast mijn mede auteurs wil ik iedereen bedanken die mij de afgelopen jaren heeft geholpen met en tijdens mijn onderzoek. Een aantal personen wil ik hier heel graag met name noemen.

Allereerst mijn promotores. Beste Leon, jouw open houding maakt het dat het heel prettig is om met je samen te werken. Je hebt altijd al mijn opmerkingen, vragen en suggesties serieus genomen, waardoor het makkelijk is te groeien en zelfstandigheid te creëren. Je hebt me de vrijheid gegeven om zelf na te denken en voor een groot deel zelf mijn eigen onderzoek te bepalen. Ik ben je hier ontzettend dankbaar voor. Daarnaast heb ik veel geleerd van jouw wijze lessen, je manier van denken en positiviteit. Ik kijk terug op een hele fijne samenwerking. Beste Ron, je bent een echte professor. Jouw kennis, nauwkeurigheid, kritische blik en didactisch vermogen bewonder ik. Je hulp bij het biochemische deel van mijn onderzoek is van grote waarde geweest. Ik heb veel van je geleerd. Beste Fred, aan jouw snelheid valt niet te tippen. Je stelde iedere keer strakke deadlines waardoor het tempo ineens ernstig werd opgeschroefd. Daarvoor in de plaats werden mijn artikelen door jou altijd binnen een dag nagekeken en bekritiseerd. Beste heren, ik wil jullie hartelijk danken voor jullie vertrouwen en voor jullie hulp.

Ten tweede wil ik de medewerkers van het lab Kindergeneeskunde en Neurologie bedanken voor de samenwerking. In het bijzonder wil ik Udo bedanken. Jij hebt mij wegwijs gemaakt in de wereld van de metabolieten en de NMRS vaardigheden. Onze uitgebreide gesprekken over hardlopen en voetbal maakte dat ik me direct thuis voelde op jullie lab. Dank voor de leuke en leerzame tijd.

Mijn dank gaat tevens uit naar de medewerkers van de afdeling Chemische Endocrinologie. In het bijzonder wil ik Chris en Paul bedanken. Chris, jouw jarenlange ervaring, nauwkeurige werkwijze en kritisch commentaar op de studies heb ik erg gewaardeerd. Paul, je hebt mij erg geholpen om statistische methoden te begrijpen en toe te passen. Heel veel dank daarvoor, want het heeft me later in mijn traject erg veel tijd bespaard.

Beste Wilbert, jij hebt mijn eerste publicatie mogelijk gemaakt. Dank voor de prettige samenwerking.
Beste Hans, ik waardeer het zeer dat je altijd de tijd hebt genomen om mij alles uit te leggen over de mysteriën van pathologie.
Beste Jan, hartelijk dank voor je hulp bij de statistiek van mijn studies.

A special thanks to all the people from the R&D department of CAF-DCF in Brussels. Stéphane, you have taught me a lot about fluorescence spectroscopy and protein binding. At first, I felt like a complete fool, but with your patience and confidence, I was able to perform the measurements by myself and complete the experiments. Ruth, thank you for your hospitality and kindness. I really enjoyed my stay at your lab.

Graag wil ik alle medewerkers van de afdeling Verloskunde en Gynaecologie bedanken voor de gezellige tijd en fijne samenwerking. In het bijzonder mijn naaste collega's. Anne, mijn eerste buurvrouw. Onze cynische humor en soepele werkmodus maakten dat we direct al een perfecte klik hadden. Zelfs in Den Haag en Berlijn bleven we tot 's morgens vroeg doorpraten. Anne, ik word altijd blij als ik je zie! Charlotte, mijn tweede buurvrouw. Over jou kan ik (nog) een boek schrijven. Je heerlijke gedetailleerde verhalen, onmiskenbare aanwezigheid en precieze manier van werken en doen zijn uniek. Onze (valse) slappe lach momenten achter de compu en gespreken thuis met goede wijn blijven me bij. Wat fijn dat je mijn paranimf wilt zijn! Petra, dank voor heel veel leuke momenten, je gezelschap tijdens congressen in Berlijn en Bangkok en voor je betrokkenheid tijdens mijn persoonlijke en werkgerelateerde zeikmomenten. De (ex-) kantoortuin bewoners: Annemijn, Annemarie, Arno, Bea, Bertho, Charlotte, Channa, Elvira, Gwendolien, Hedwig, Ineke, Inge, Irene, Linda, Marjan, Nel, Sabine, Sabrina, Sanne, Selma, Suzan, Thijs, Ralph, Refika, Roos en Willianne. Dank voor de koffiemomenten, de dagelijkse traktaties, de onderzoekersweekenden, afscheidsetentjes en borrels. Jullie betrokkenheid bij mijn sollicitatie en blijdschap toen ik werd aangenomen als AIOS heb ik enorm gewaardeerd. De stafleden van de gynaecologische oncologie: Leon, Ruud, Charles, Joanne, Maaike, Petra en Eva-Maria, dank voor de leuke tijd, de congressen samen en de gezellige momenten op jullie kamers. Ik heb met ieder van jullie een erg leuke tijd gehad. Ik denk dat de laagdrempelige manier van samenwerken tussen onderzoekers en stafleden bijdraagt aan de goede sfeer die ik heb mogen ervaren.

Lieve vrienden, in het bijzonder de ladies van het dispuut, ik wil jullie bedanken voor de fijne avonden, weekenden, vakanties en alle andere momenten samen. De oprechte interesse in elkaar, goede gesprekken, onzin verhalen en nare humor maken dat ik altijd weer thuis kom bij jullie. Lieve Floor, dank dat we na mijn promotie lekker mogen gaan borrelen in jullie restaurant! Lieve Ak, wat een feest dat ik jou heb leren kennen! Onze co-schappen hebben we opgeleukt met elkaars aanwezigheid. Ik vind het heel bijzonder dat we nog steeds vriendinnen zijn en geweldig dat jij nu mijn paranimf bent. Dank voor je eeuwige interesse en vrolijkheid.

Lieve familie, dank voor jullie steun. Irene, dank voor al het werk dat je heb verricht om dit proefschrift mooi en leesbaar te maken. Lief broertje, wat ben ik blij met de band die wij samen hebben. Daar zijn geen woorden voor. Ik vind het echt bijzonder dat we zulke goede maatjes zijn. Lieve mama, de afgelopen jaren waren heel fijn, dank voor je gezelligheid, hulp en natuurlijk voor je uitmuntende oppaskwaliteiten. Lieve papa, dank voor je interesse, de prettige gesprekken en al je steun. Onze band is heel speciaal, ik zal je straks gaan missen. Lieve opa, je was zo trots en wilde zo graag bij mijn promotie zijn. Het doet me veel verdriet dat je het niet meer mee kan maken. Allerliefste oma, je bent mijn grote voorbeeld. Dank je wel voor al je lieve woorden, oneindige interesse en de fijne momenten samen.