Vulvovaginal *Candida*:

a study of (a)symptomatic women

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Vulvovaginale *Candida*:
Een studie aangaande (a)symptomatische vrouwen
(met een samenvatting in het Nederlands)

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General introduction

Fungi and yeasts

Fungi are unique organisms that differ form other eukaryotes in nutritional mode, structural organization, growth, and reproduction. Since fungi lack chlorophyll, photosynthesis and CO2-assimilation can not take place. Therefore, fungi are dependent on the supply of organic material from their surroundings. Fungi digest food outside their body by secreting powerful hydrolytic (exo)enzymes into the organic material. These enzymes decompose complex molecules to simpler compounds that the fungus can absorb and use. The bodies of fungi are constructed of tiny filaments called hyphae, which can form an interwoven mat known as mycelium. Hyphae are composed of tubular walls surrounding plasma membranes and cytoplasm. These tubular walls are mainly composed of chitin, a strong but flexible nitrogen-containing polysaccharide. In general, the fungus concentrates its energy on adding hyphal length to increase overall absorptive power. Fungi reproduce by releasing spores that are produced either sexually or asexually. Carried by wind or water, these spores will germinate and produce mycelia if they land in suitable surroundings.[1]

Yeasts are unicellular fungi that inhabit moist habitats, including animal tissues. Yeasts reproduce asexually, by simple cell division or by the pinching of small 'bud cells' off a parent cell. Yeasts grow by forming spurs, which are called (pseudo)hyphae, since yeasts are composed of one cell only. Humans use yeasts to raise bread and ferment alcoholic beverages. On the other hand, the yeast *Candida* is one of the normal inhabitants of moist human epithelial tissue and therefore part of the normal flora of the vagina, mouth and gastro-intestinal tract. Certain circumstances can cause *Candida* to become pathogenic by growing too rapidly and releasing harmful substances ('yeast infections'). Therefore, *Candida* is an opportunistic pathogen: a normal inhabitant of the human body that only causes problems when changes in host conditions allow the yeast to grow. This can occur, for example, with an environmental

change such as a pH shift, or when the immune system of the human host is compromised – by AIDS for instance. [1-3]

Candidiasis

Candida species are the most frequent cause of human fungal infections. *Candida* shows a predilection for stratified squamous epithelium and grows best on warm, moist surfaces. Therefore, *Candida* frequently causes vaginitis, diaper rash and oral thrush. *Candida* infections of the oral cavity (thrush) and vagina produce superficial patches or large, almost fluffy membranes that are easily detached, leaving a reddened, irritated underlying surface. Spread of oral candidiasis as by a nasogastric tube, may lead to similar lesions in the esophagus. *Candida* can also cause cutaneous eczematoid lesions in moist areas of the skin (between fingers and toes, in inguinal creases, infra mammary folds, and the anogenital region). Severe, invasive candidiasis, associated with immunosuppression or with phagocyte depletion, involves the kidney in 90% of the cases, causing multiple micro abscesses in both the cortex and the medulla. *Candida* right-sided endocarditis resulting from direct inoculation of the fungi into the bloodstream, most often in drug addicts, gives rise to large friable vegetations that frequently break into emboli. Pneumonia, meningitis, intracerebral or hepatic abscesses, enteritis, endophtalmitis, arthritis, and osteomyelitis are some of the other presentations of disseminated candidiasis. [2,3]

Vulvovaginal candidiasis (VVC)

Vulvovaginal candidiasis (VVC) is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract. It is a frequent diagnosis in the daily practice of gynaecology and accounts for large numbers of visits to general practices in The Netherlands.[4] Around 75% of adult women will experience at least one episode of VVC during their lifetime, of which 5% will develop recurrent vulvovaginal candidiasis, with at least four symptomatic episodes of vaginitis in one year.[5] On the

other hand, *Candida* can be isolated from the genital tract of approximately 20% of asymptomatic women in their childbearing years. *Candida albicans* is the species most frequently isolated for VVC.[5]

Candida virulence factors

Candida albicans possesses the ability to survive and proliferate in physiological extremes of pH, osmolarity, availability of nutrients, and temperature.[6] This versatility may account for the successful behaviour of *C. albicans* both as a commensal colonizer of the vagina and as a pathogen.[7] The mechanism whereby *Candida* organisms induce inflammation is not yet fully established. The host immune system is the major factor balancing the transition from commensalism to pathogenicity. However, *C. albicans* expresses several virulence attributes, such as adhesion factors, phenotypic switching, dimorphic behaviour, and secretion of hydrolytic enzymes.[8-14] The ability to adhere to host tissue is considered essential in the early stages of colonisation and tissue invasion. Adherence is achieved by a combination of specific (ligand-receptor interactions) and non-specific (electrostatic charge, van der Waals forces) mechanisms which allow the yeast to attach to a wide range of tissue types and inanimate surfaces.[5,15] Furthermore, *Candida* organisms are dimorphic, in that they may be found in humans in different phenotypic phases. In general, blastospores represent the phenotypic form responsible for transmission and spread, whereas germinated yeast with production of mycelia most commonly constitute a tissue-invasive form usually identified in the presence of symptomatic disease. However, both blastoconidia and (pseudo)hyphae are capable of destroying superficial cells by direct invasion.[5,16,17] Moreover, high frequency heritable switching of colonies occurs in vitro, whereby the variant phenotypes represent a varying capacity to form mycelia spontaneously and to express other virulence factors, such as drug resistance, adherence and capacity to invade and survive in diverse body sites, as well as to cause disease.[8,18] The concept of pathogenesis may well

be the result of these switching phenotypic properties. Little is known regarding the role of candidal proteolytic enzymes, toxins and phospholipase in determining the virulence of the organisms. Secreted aspartic proteinases (Saps) are expected to fulfil different tasks during mucosal or disseminated infections. Mycotoxin may act to inhibit phagocytic activity or suppress the local immune system and has been found in vaginal secretions.[14,19,20]

Vaginal defence mechanisms

The normal vaginal microflora is best described as a broad spectrum of facultative organisms, dominated by presence of *Lactobacillus*.[21,22] Lactobacilli produce lactic acid from glucose, keeping the vagina at an acidic pH. Glucose is metabolized by vaginal epithelial cells from glycogen, which is deposited under estrogenic control after puberty. In addition to producing acid, some species of *Lactobacillus* produce hydrogen peroxide (H_2O_2).[21-26] H_2O_2 of microbial origin interacts with peroxidases produced by the host along with halide ion. The product of this reaction is a potent oxidant that is toxic to many bacteria.[21-30] H_2O_2-positive lactobacilli are capable to kill HIV in vitro as well as *Gardnerella vaginalis*, anaerobes and *Neisseria Gonorrhoeae*.[21,27-30] Furthermore, women with H_2O_2-producing lactobacilli are less likely to have Bacterial Vaginosis, [23,24] *Chlamydia trachomatis*[24,31] and *Trichomonas vaginalis*.[24,32] It has been claimed that some lactobacilli are also protective against *Candida* vaginitis.[33] In vitro studies have demonstrated that *Candida albicans* is inhibited by culture supernatants of *Lactobacillus acidophilus*.[34-37] Furthermore, H_2O_2 produced by lactobacilli in combination with myeloperoxidase has antifungal effects.[37] Moreover, candidal vulvovaginitis is a common occurrence after systemic use of broad spectrum antibiotics.[38] Antibiotic agents increase vaginal yeast colonization and are thought to act by eliminating lactobacilli, thereby facilitating *Candida* to grow, adhere and germinate.[39,40] The concept of interaction between lactobacilli and *Candida* includes competition for

nutrients and stearic interference of adherence to vaginal epithelial cells.[41,42] Other mechanisms comprise the elaboration of bacteriocin by lactobacilli that inhibit yeast proliferation and germination.[43] Nevertheless, clinical studies show that women with yeast vaginitis have the same frequency and concentration of *Lactobacillus* as women without recurrent infections.[44-47] Vulvovaginal candidiasis occurs more frequently in women with a lactobacilli-predominated vaginal flora, as compared with those with a flora change with a mixture of anaerobic and facultative anaerobic bacteria.[48] Moreover, the use of oral or vaginal forms of lactobacilli does not prevent post-antibiotic vulvovaginitis.[49] These findings contradict the hypothesis that presence of H2O2-producing vaginal lactobacilli protects against vaginal candidiasis.

The phagocytic system plays an important role in limiting systemic candidal infection and deep tissue invasion, but phagocytic cells are absent from vaginal secretions during vaginal candidiasis. Since patients with profound immunoglobulin deficiencies are not predisposed to vaginal yeast infections and patients with recurrent infection do not lack antibodies, it is thought that the humoral system does not protect the vaginal mucosa against *Candida* colonization either.[50] Studies from animal models, women with recurrent VVC (RVVC) and HIV-infected individuals suggest that distinct protective host defence mechanisms may function against vulvovaginal candidiasis.[14,51] However, while local and systemic cell-mediated immunity appears important for protection against oropharengeal candidiasis, there is little evidence to indicate that either local or systemic cell mediated immunity plays a role against VVC.[51,52]

Risk factors
Since *Candida* can be either a commensal or a pathogen in the vagina, changes in the host vaginal environment are usually necessary before the organism induces pathologic effects.

It is generally thought that high levels of reproductive hormones, by providing a higher glycogen content in the vaginal tissue, provide an excellent carbon source for *Candida* organisms.[53] Several studies have shown increased vaginal colonization with species of *Candida* following high-estrogen oral contraceptive use.[54,55] During pregnancy the vagina shows an increased susceptibility to infection by species of *Candida*, resulting in both a higher prevalence of vaginal colonization and a higher rate of symptomatic vaginitis.[56] Vaginal colonization is more frequent in diabetic women.[57] As described afore, broad-spectrum antibiotics may also trigger VVC and are thought to act by eliminating the protective vaginal bacterial flora.[41,42,57]

Recurrent vulvovaginal candidiasis

Five percent of all women who experienced an initial episode of VVC will develop recurrent vulvovaginal candidiasis (RVVC) with at least three symptomatic episodes of vaginitis in one year.[5] Recurrent vaginal candidiasis must be confirmed by culture, because thousands of women are diagnosed with 'RVVC', while their symptoms are in fact due to non-infectious causes such as allergic and hypersensitivity vulvitis. Evaluation of women with recurrent vaginitis usually fails to reveal a causal mechanism. Recurrent candidiasis is generally associated with the same strain of *Candida* as the initial infection. Avoiding antimicrobics, oral contraceptives, tight fitting clothing and hormone therapy is usually without success and most patients have normal glucose tolerance tests. In the past, repeated fungal reinfection of the vagina from a persistent intestinal source[58] or sexual transmission[59] was thought to contribute to the pathogenesis of RVVC. Nowadays, it is hypothesized that incomplete clearance of *Candida* from the vagina following topical antimycotic therapy permits the organisms to persist in small numbers in the vagina.[60] This results in continued carriage of the *Candida* organism, although signs of inflammation are reduced due to a drastic reduction of the numbers of *Candida* in the vaginal lumen. When host environmental

conditions change, the colonizing organisms would increase in number and undergo mycelial transformation, resulting in a new clinical episode.[61] The fact that negative vaginal *Candida* cultures once more turn positive within 30 days in 20-25% of women after systemic and topical antibiotic therapy of VVC, strongly supports the hypothesis that 'vaginal relapse' is responsible for RVVC.[55] Whatever the source of vaginal reinfection is, it is apparent that women with recurrent candidal vaginitis differ from women with infrequent episodes due to their inability to tolerate small numbers of *Candida* reintroduced or persisting in the vagina. Host factors responsible for the frequent episodes are not clearly defined and more than one mechanism may be operative. Fidel *et al.* showed that there are natural innate protective mechanisms associated with susceptibility to infection, such as variations in human vaginal epithelial cells inhibiting the growth of *Candida albicans*.[61] Recently, the use of a live challenge model in asymptomatic humans revealed that protection against VVC coincided with a non-inflammatory innate presence of the yeast, whereas symptomatic infection correlated with a neutrophil infiltrate in the vaginal lumen and increased growth of *Candida*.[63] Symptomatic recurrent vaginitis might therefore be due to an aggressive innate response.

Symptoms

Acute vulvar pruritus and vaginal discharge are the usual presenting complaints, but neither symptom is specific to VVC.[64,65] The most frequent symptom is that of vulvar pruritus, which is present in virtually all symptomatic patients. Vaginal discharge is not invariably present and is frequently minimal. Although described as typically cottage-cheese-like in character, the discharge may vary from watery to homogeneously thick. Vaginal soreness, irritation, vulvar burning, dyspareunie and external dysuria are commonly present.

Diagnosis

Vulvovaginal examination frequently reveals epithelial erythema and swelling of the labia and vulva, together with adherent whitish discharge. However, due to lack of specificity of these signs all patients with symptomatic vaginitis should be diagnosed on the basis of microscopic examination of vaginal secretions, not only to identify yeast cells, but also to exclude the presence of clue cells or trichomonads.[64,65] A 10% potassium hydroxide (KOH) preparation can be valuable and may reach sensitivity of 50 to 60% in identifying germinated yeast.[4,64-67] If microscopy of these so-called wet-smears is negative for presence of *Candida* but VVC is suspected on the basis of symptoms and signs, a vaginal culture should be performed.[64-68] It should be noted however, that vaginal cultures can not differentiate pathogenic forms of *Candida* from harmless commensalism. A new diagnostic tool in determining VVC is PCR, which is based on DNA identification of *Candida*.[69,70] In addition, Novikova *et al.* noted that methylene blue-stained vaginal smears can also be used to determine presence of *Candida*.[71] Nevertheless, none of the diagnostic methods described above is superior in detecting VVC and reliance on microscopy, culture or PCR alone in diagnosing (R)VVC can lead to inaccurate results.[70]

Treatment

Treatment of acute VVC is available in a variety of highly effective topical azole agents.[4,72] Overall cure rates for vaginal azole treatment, defined as eradication of symptoms and *Candida*-negative cultures, are in the order of 80-90%. Oral systemic azole agents achieve comparable or marginally higher therapeutic cure rates and patients enjoy oral administration, which eliminates local side effects and messiness. On the other hand, oral azoles suffer the drawback of potential systemic toxicity, which has limited the use of ketoconazole, although constituting a lesser consideration in prescribing itraconazole and fluconazole. In RVVC treatment includes ketoconazole 100 mg daily or either 500 mg

clotrimazole suppositories or 100 mg fluconazole orally once-weekly. All three maintenance regimens are effective in preventing a recurrent episode of vaginitis.[73,74] However, when treatment is stopped, approximately half of the women suffer from symptomatic relapse shortly after cessation of therapy.[73,74] Sometimes RVVC is due to non-albicans species such as *Candida glabrata*.[75] In those cases there is often a reduced susceptibility to all azoles.[75-77] Boric acid 600 mg administered vaginally once daily in a gelatine capsule has been shown to be highly effective in this clinically resistant infection.[77,78]

Outline of the Thesis

The cervical smear

Cervical carcinoma is the second most common cancer among women worldwide.[79] In The Netherlands more than 700 cases of cervical carcinoma are diagnosed every year, which comprises approximately 2.5% of all determined female cancers.[80] Nationwide cytological screening for cervical cancer and its precursor lesions as a method to reduce cervical cancer morbidity and mortality has been introduced in The Netherlands in 1988.[81] Since 1996 all women between the ages of 30 and 60 years are invited once every 5 years to be tested for cervical cancer. The Dutch national coding system for cervical cytology (KOPAC) was introduced in 1996 to uniformly describe cytomorphological findings in order to increase the efficacy to the screening program and to decrease equivocal results.[82] The KOPAC classification interprets cervical smears by using a rating system, which includes information on specimen composition, inflammatory characteristics and adequacy of the smear. The letters K (kompositie = composition), O (ontstekingsverschijnselen = inflammation), P (plaveiselepitheel = squamous epithelium), A (andere afwijkingen endometrium = other abnormalities endometrium) and C (cylinderepitheel endocervix – endocervical cylindrical epithelium) are used to indicate the composition and morphology of the smears. Squamous, columnar and other cells are graded for presence of dyskariosis (dysplasia) and these values determine the interpretation of the smear.[82] Although it is not the main focus of cervical screening, (pseudo)hyphae and/or blastospores are often discovered during screening of the Papanicolaou-stained vaginal smear by the cytopathologist[82,83] and coded as O4 (*Candida*). The O-category comprises eight other different subgroups besides *Candida*: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Gardnerella vaginalis* (O5), no inflammatory changes (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific changes (O9). Thus, not only

cytological cervical abnormalities are established during screening, but the vaginal flora is also examined.

Furthermore, the country of birth of each woman is recorded during population screening.

This implies that large cytological databases in The Netherlands can be exploited to study possible relationships between presence of *Candida* and immigrant status, (pre)neoplasia, and the incidence of acquiring other vaginal infections in time, by the cytological identification of *Candida* and other elements of the vaginal flora in smears.

In the present studies we tried to answer the following questions concerning *Candida*:

1) How accurate is the clinician's diagnosis of *Candida*?
2) What is the prevalence of *Candida* among asymptomatic women participating in the Dutch national screening program?
3) Is there a difference in *Candida* prevalence in asymptomatic women of different ethnic background?
4) Is there an association between *Candida* and squamous (pre)neoplastic changes (in time)?
5) Are asymptomatic women carrying *Candida* at risk of receiving other vaginal pathogens in time?
6) Is it possible to establish (recurrent) symptomatic vulvovaginal candidiasis ((R)VVC) in self sampled vaginal smears?

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Chapter 2

Vulvovaginal candidiasis: diagnostic and therapeutic approaches used by Dutch general practitioners

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Abstract

OBJECTIVE: To establish how general practitioners (GPs) in The Netherlands diagnose and treat vaginal candidiasis.

METHODS: Questionnaires were sent to 1160 Dutch GPs. The GPs were asked to make an inventory of the annual number of consultations for vulvovaginal candidiasis. Furthermore, information was requested with regard to diagnostic examinations performed and preferred treatment when dealing with vulvovaginal candidiasis.

RESULTS: 380 (32.7%) GPs returned the questionnaire, of which 189 GPs worked in one-man/woman practices (n = 189). The group of 380 GPs consisted of 269 (70.8%) males and 111 (29.2%) females. On average, GPs reported 105.6 consultations concerning vaginal candidiasis per practice per year. Only 61 (16.1%) Dutch GPs always or often perform microscopy when diagnosing candidiasis, while 143 (37.6%) GPs never use a microscope to confirm their diagnosis. Furthermore, only 30 (7.9%) GPs take *Candida* cultures regularly, whereas 154 GPs (40.5%) never take a vaginal swab to diagnose acute candidiasis. Treatment of choice was mostly miconazol (50%) or clotrimazol (24%).

CONCLUSION: GPs often diagnose 'vulvovaginal candidiasis' in their practices, but often do not perform the laboratory examinations required to confirm their putative diagnosis. This may lead to wrong diagnoses and maltreatment with antimycotics, without cure of the patients' vaginal complaints.

Introduction

Vaginal complaints account for large numbers of visits to general practices in The Netherlands. Approximately 50 per 1000 female patients per year visit their general practitioner (GP) with complaints of vaginal discharge, abnormal in amount, colour and/or smell.[1] In 25 to 35%, candidiasis is the underlying cause of the vulvovaginal discomfort. Other possible causes of abnormal vaginal discharge include Bacterial Vaginosis (BV, 20%) and vaginal or cervical infections by *Trichomonas vaginalis* or *Chlamydia trachomatis* (5-10%).[1-4] Current recommendations for Dutch GPs to correctly diagnose vaginitis involve speculum examination and microscopy, since the predictive value of assessing vaginal complaints alone is low.[1,5,6] Presence of *Candida* infection is confirmed when pseudohyphae are observed during microscopic examination (wet mount): discharge mixed with a drop of 10% KOH (potassiumhydroxide).[1-11] BV is diagnosed when the pH of the usually thin and homogeneous discharge is $\geq$ 4.5, the 'whiff test' is positive, and clue cells can be seen during wet mount examination.[1-6,12] Since sensitivity of microscopy for yeasts is at best 50 to 60%[7] and the 'whiff test' is also positively associated with trichomoniasis[13], GPs can also take a vaginal swab to culture the putative pathogen causing the complaints.[5] Nevertheless, speculum examination can be uncomfortable for the patient and wet mount examination is laborious. Therefore, we hypothesize that vulvovaginal candidiasis is often not correctly diagnosed by GPs and thus remains untreated. The present study was undertaken to evaluate how GPs in The Netherlands diagnose and treat *Candida* vaginitis.

Materials and Methods

In September 2005, questionnaires were sent to 1160 general practices in The Netherlands. Standardized forms were used to record characteristics such as the age and gender of the physician and the name of the village or town where their practice is situated. Since family doctors in The

Netherlands are recommended to use the International Classification of Primary Care (ICPC) coding system[14], the general practitioners were asked to present the number of consultations given annually within ICPC-category X72 (vulvovaginal candidiasis). In addition, GPs were asked to report the percentage of patients that had suffered from recurrent vulvovaginal candidiasis (RVVC), defined as four or more episodes of candidiasis within one year. Furthermore, information was requested with regard to the methods used by GPs to diagnose acute and recurrent *Candida* infection (vaginal examination, microscopy, culture etc.) as well as the preferred medical treatment.

Results

Of the total of 1160 GPs who were addressed, 380 (32.8%) filled out and returned the questionnaire.

Information about the general practices

The group of 380 GPs consisted of 269 (70.8%) males and 111 (29.2%) females. The average age of the male physicians was 50.4 years and the average age of the female GPs 44.0 years. Of the total of 380 GPs, 189 GPs worked in a one-man/woman practice.

Incidence

The GPs reported on average 105.6 cases of vaginal candidiasis per general practice per year. A number of GPs (n = 112) did not answer this question, mainly because their database was not (yet) computerized. In addition, the GPs reported that on average one in five (19.1%) women with candidiasis suffered from recurrent vulvovaginal candidiasis (RVVC).

Diagnosis

In figures 1 and 2 (see also page 147 for full colour figures) the methods used by the 380 physicians to diagnose acute and recurrent vulvovaginal

candidiasis are shown. Both figures reveal that a substantial part of the general practitioners never performs microscopy nor takes a *Candida* culture while 'diagnosing' vaginal candidiasis. However, GPs obtain *Candida* cultures more often when recurrent vulvovaginal candidiasis is suspected.
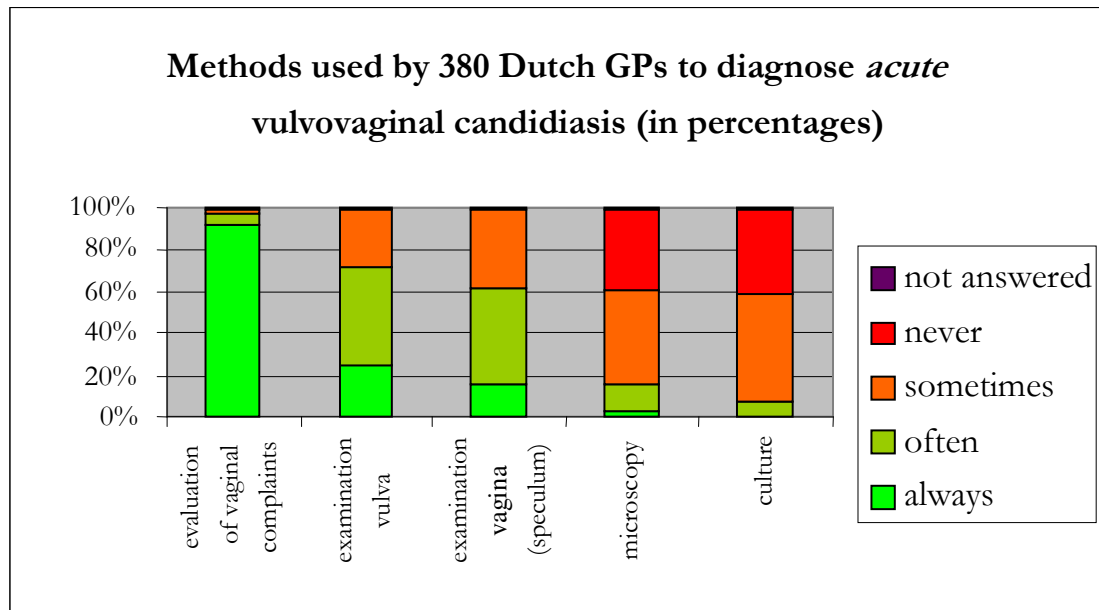

Figure 1 Methods used by 380 Dutch GPs to diagnose *acute* vulvovaginal candidiasis.
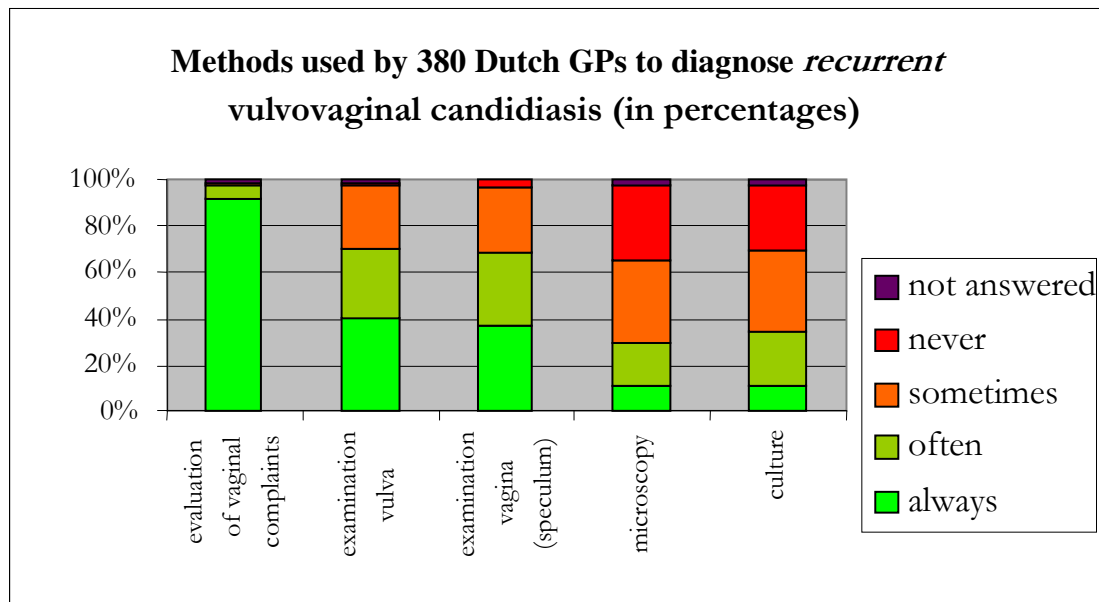
Figure 2 Methods used by 380 Dutch GPs to diagnose *recurrent* vulvovaginal candidiasis.

Treatment

Most physicians (n = 310, 81.6%) prefer local (vaginal) treatment of vaginal candidiasis, while 37 GPs (9.7%) start systemic treatment and 30 GPs (7.9%) prescribe a combination of vaginal and oral treatment. Only three GPs did not answer this question. Almost all the respondents prescribe medication for one to three days. There is a lot of variation in the medication prescribed, but when converted to generic names a clear picture appears (figure 3). Most GPs prescribe miconazol (50%) or clotrimazol (24%) when a *Candida* vaginitis is suspected.

Prescribed *Candida* medication by Dutch GPs (in percentages)

- miconazol
- clotrimazol
- fluconazol
- butoconazol
- itraconazol/other
- not answered
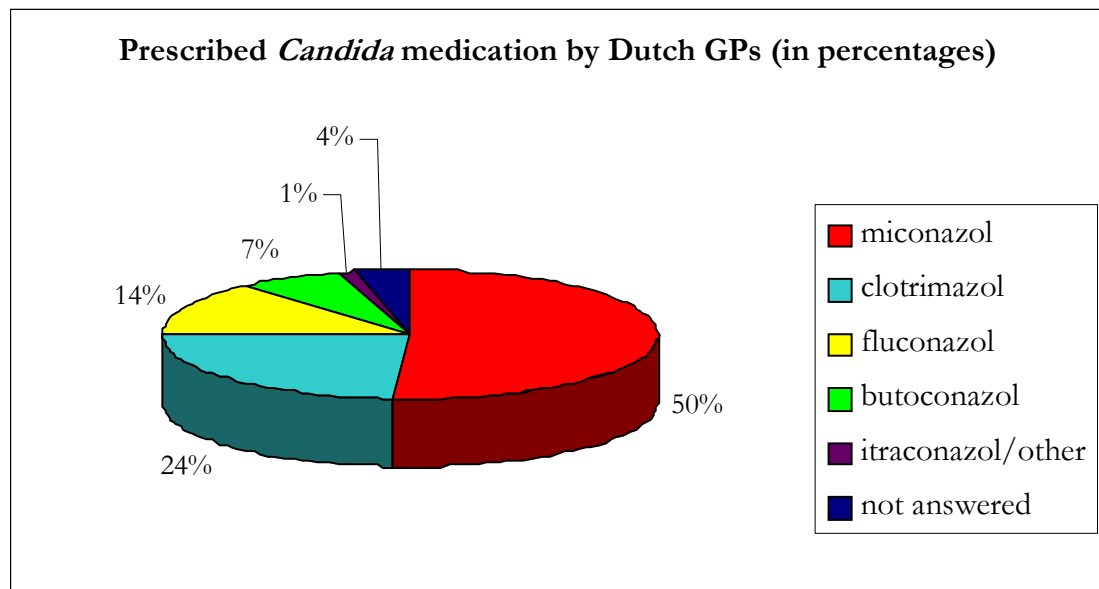
Figure 3 Prescribed *Candida* medication by Dutch GPs.

Discussion

We examined how GPs in The Netherlands diagnose and treat *Candida* vaginitis, by sending questionnaires to 1160 Dutch general practitioners. Although Dutch GPs encounter large numbers of patients complaining of vaginal discomfort annually, they scarcely perform vaginal examination, microscopy and culture-sampling. Women are frequently diagnosed with vulvovaginal candidiasis and treated with antimycotics solely based on symptoms and inspection only. This finding conflicts

with the advice given by the Dutch General Practitioners Guideline, which recommends performing office-based tests when confronted with women with vaginal complaints.[1] Mårdh *et al.*[5] and Schaaf *et al.*[6] confirmed that the predictive value of vaginal complaints such as itching, burning and white, curdy discharge is low. Therefore, taking a vaginal specimen to culture pathogens is by some authors considered mandatory in order to be able to rule out other causes of vaginitis, especially when dealing with patients with recurrent vaginal complaints.[15-17] Other studies have shown that without the benefit of microscopy or culture as many as half of the women routinely diagnosed with vulvovaginal candidiasis may suffer from other conditions.[7,18-20] Not employing microscopy or culture could therefore lead to an overestimated prevalence and incidence of 'vulvovaginal candidiasis', thereby encouraging the widespread abuse of antimycotics.[21-23] Unfortunately, the GPs in our study did not indicate *why* certain clinical or microbiological evaluations were not performed. We can only assume that microscopy was not carried out due to lack of experience or because of time constraints. However, efforts are clearly needed to improve the quality of the clinical diagnosis of vaginal complaints.

In our study, the preferred therapy of vulvovaginal candidiasis differed strongly. Most of the GPs prescribe topical clotrimazol or miconazol for one to three days when a *Candida* vaginitis is suspected. Some physicians choose another form of local treatment, and only few prescribe oral medication. This finding is in concordance with The Dutch General Practitioners Guideline, which recommends treating *Candida* vaginitis with clotrimazol or miconazol (vaginal) tablets once only. In case of recurrent vulvovaginal candidiasis, the duration of treatment can be prolonged.[1,24]

As is always the case with questionnaire-based investigations, results may be hampered by response bias. However, we presume that most of our current respondents overestimated their use of speculum examination, microscopy and culture sampling. This assumption strengthens our

conclusion that office-based testing to confirm vulvovaginal candidiasis should be stimulated.

In this study, we noted that Dutch GPs diagnose (recurrent) vulvovaginal candidiasis on the basis of gut feelings rather than adequate diagnostic procedures. Although the current clinical guidelines are advocating correct procedures, much is to be gained by more frequent implementation of these guidelines by the average Dutch GP.

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Chapter 3

The microscopic diagnosis of vulvovaginal candidiasis in stained vaginal smears by Dutch general practitioners

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Abstract

OBJECTIVE: In women with vulvovaginal candidiasis (pseudo)hyphae and blastospores can be seen in the stained vaginal smear. The aim of this study was to determine the accuracy of this microscopic diagnosis in clinical practice.

METHODS: General practitioners trained in diagnosing vulvovaginal candidiasis performed microscopy of 324 stained vaginal smears. These smears were sent to the pathologist for confirmation of the microscopic diagnosis of the clinician, whereby cytological diagnoses of the pathologist was considered the 'gold' standard.

RESULTS: In 104 of the 342 cases *Candida* was established by the pathologist. The clinicians made 24 false positive and 50 false negative diagnoses of *Candida*. Therefore, sensitivity and specificity of the microscopic diagnoses of the clinicians were 52% and 89% respectively. The most frequent reason for a false positive diagnosis was presence of hairs, whereas the most frequent reason for a false negative diagnosis was understaining of the smear.

CONCLUSION: This study shows that even in stained smears it is difficult for clinicians to recognize blastospores and/or (pseudo)hyphae. Efforts are clearly needed to improve the quality of the clinical diagnosis of vulvovaginal candidiasis.

Introduction

Approximately 75% of all sexually active women will experience one episode of vaginal candidiasis during their lives. The infection is caused by growth of yeasts in the mucosa of the female genital tract and will disappear when antimycotic therapy is started.[1-9] Current recommendations for general practitioners (GPs) to diagnose vulvovaginal candidiasis involve vaginal examination and microscopy, because the predictive value of vaginal complaints alone is low.[10,11] Presence of yeast infection is confirmed when (pseudo)hyphae are visible during microscopic examination of the discharge (wet-mount or unstained slides) mixed with a drop of 10% potassiumhydroxide (KOH).[1-9] However, sensitivity of microscopy for yeasts when examined by trained microscopists is at best 50 to 60%.[3,6,11,12] Moreover, unstained slides cannot be preserved and are thrown away when used, making it impossible to check whether the microscopic diagnosis made by the clinician was correct. In 2001 the Leiden Cytology and Pathology Laboratory (LCPL) introduced the distribution of standardized staining sets to primary care clinics. In stained slides the flora and fauna are always well visible so diagnoses may be expected to become more simple, objective and reproducible.[13] Furthermore, staining makes it possible to preserve diagnostic slides for purposes of quality control and confirmation of the diagnosis made by the clinician. In 2002 and 2003 clinicians used the expertise of the pathologists of the LCPL by sending stained slides for analysis. In this article we compare the microscopic diagnoses made by clinicians based on stained slides, with those made by the pathologist, whereby the latter was considered the 'gold standard'.

Materials and Methods

28 General practitioners collaborating with the LCPL were trained to prepare and stain vaginal smears of women visiting their practice with vaginal complaints. In addition, the clinicians were taught to recognize yeasts by small blastospores and/or by presence of (pseudo)hyphae. The

staining solution (BioClin, Delft, The Netherlands) was provided by the LCPL and comprised a mixture of methylene blue and eosin. In the Bioclin-method, DNA of bacteria, blastospores and (pseudo)hyphae stains blue due to methylene blue, whereas proteins stain pink due to eosin. The GPs systemically recorded their microscopic findings on a standardized form. All vaginal smears sent by the 28 clinicians to the LCPL in 2002 and 2003 were included in this study. The Bioclin-stained smears were restained with the PAS-method at the LCPL. In the PAS-method, the external surface of blastospores and (pseudo) hyphae appears bright red due to staining of mucopolysaccharides. These PAS-stained smears were examined by the pathologist. Thereafter, the microscopic findings described by the GPs were compared to the diagnoses made by the pathologist. Ethical approval was received from the Institutional Ethical and Scientific Review Committee.

Results

In total, 342 stained vaginal smears made by 28 clinicians were sent to the LCPL. In 104 of the 342 cases *Candida vaginalis* was unequivocally established by the pathologist (see figure 1: page 148 and Table 1). Sensitivity and specificity of the microscopical diagnoses of the clinicians were 52% and 89% respectively. The clinicians made 24 false positive and 50 false negative diagnoses of *Candida.*

	Candida (diagnosed by GPs)	No *Candida* (diagnosed by GPs)	Total
Candida (diagnosed by pathologist)	54	50	**104**
No *Candida* (diagnosed by pathologist)	24	196	**220**
Total	78	246	324

Table 1 Cross table cytological *Candida spp* diagnoses.[*]

[*]Cytological diagnosis of the pathologist is the 'gold standard'

Reasons for a false positive cytological GP diagnosis	n
Presence of contaminants (e.g. hairs)	10
Damaged nuclei	6
Dense inflammatory infiltrate	1
No obvious reason	7
Total	**24**

Table 2 Reasons for a false positive cytological GP diagnosis.[*]
[*]Cytological diagnosis of absence of *Candida spp* by the pathologist is the 'gold standard'

Reasons for a false negative cytological GP diagnosis	n
Only few (pseudo)hyphae	8
Only spores	3
Smear too thick	12
Understaining	13
Overstaining	2
No obvious reason	12
Total	**50**

Table 3 Reasons for a false negative cytological GP diagnosis.[*]
[*]Cytological diagnosis of presence of *Candida spp* by the pathologist is the 'gold standard'

The most frequent reason for a false positive diagnosis was presence of hairs from the genital area (see figure 2: page 148 and Table 2), which appeared blue due to methylene blue in the Bioclin-stained vaginal smears. The most frequent reason for a false negative diagnosis was understaining of the smear (see Table 3). In one case, the female patient was convinced that she suffered from vulvovaginal candidiasis. However, the GP could not find any evidence of *Candida* vaginitis in the Bioclin-stained vaginal smear. Before the smear was restained with the PAS-method, the pathologist examined the smear out of curiosity and noticed a few small blastospores (figure 3: page 149) and some (pseudo) hyphae (figure 4: page 149), but only on the upper side of the smear (figure 5: page 150).

Discussion

We compared the microscopic diagnoses made by Dutch GPs based on stained slides with those made by the pathologist, whereby the latter was considered a 'gold standard'. In only about half (54) of the 104 vulvovaginal candidiasis cases the clinician agreed with the pathologist that the smear was positive for *Candida*. The GPs frequently confused hairs with (pseudo)hyphae and had great difficulty noting the small fungal blastospores. This suggests that our disappointing results are probably mainly due to lack of experience in microscopy. Wiesenfeld *et al.*[14] confirmed that a majority of primary care clinicians do not commonly perform microscopy. However, current recommendations for Dutch GPs to diagnose vaginitis involve vaginal examination and microscopy, since the predictive value of vaginal complaints alone is low.[1,10,11] Besides, vulvovaginal complaints account for more outpatient visits than any other reason that women seek health care in The Netherlands.[1] Lack of experience in microscopy could therefore lead to incorrect diagnoses and maltreatment in numerous cases. In order to prevent maltreatment, yeast cultures should be performed in those instances when there is a clinical suspicion of vulvovaginal candidiasis

and wet-mount microscopy is negative.[15] Moreover, office-based techniques to diagnose vaginitis should be properly trained during medical and postgraduate education. The use of standardized staining sets to simplify the analysis of vaginal discharge and to preserve the slides for training purposes should be further evaluated.

This study shows that even in stained smears it is difficult for clinicians to recognize blastospores and/or (pseudo)hyphae. Efforts are clearly needed to improve the quality of the clinical diagnosis of vulvovaginal candidiasis.

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Chapter 4

Candida and squamous (pre)neoplasia of immigrants and Dutch women as established in population-based cervical screening

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Abstract

OBJECTIVE: To establish the relationship between *Candida vaginalis* and (pre)neoplasia, and the prevalence of *Candida* and (pre)neoplasia related to age and ethnicity.

METHODS: Data were collected from 445,671 asymptomatic women invited for mass screening between 1995 and 2002 and coded according to the Dutch cervical smear coding system (KOPAC) with 6 grades for (pre)neoplastic changes. Prevalence and relative risks were established for *Candida* and squamous abnormalities in Dutch women and four groups of immigrants.

RESULTS: The prevalence of *Candida* was significantly higher in the cohort of 30 year old women and lower in the cohorts of 45, 50, 55 and 60 year old women. The relative risk (RR) of having *Candida* was higher for Surinamese women (1.24; CI 1.08-1.42). Furthermore, the RR of having mild dysplasia (P4) was higher for Surinamese women (1.47; CI 1.14-1.89) and for women born in other countries than in The Netherlands, Turkey, and Morocco (1.36; CI 1.13-1.62). No statistically significant relationship between (pre)neoplasia and *Candida* was observed.

CONCLUSION: *Candida vaginalis* is more frequent among Surinamese women. Presence of *Candida* is not associated with an increased risk for squamous abnormalities; therefore, women carrying *Candida* are not at an increased risk of developing cervical cancer.

Introduction

Vulvovaginal candidiasis is caused by growth of yeasts in the mucosa of the female genital tract.[1-7] Approximately 75% of all sexually active women will experience one episode of symptomatic *Candida* during their lives.[2-7] Furthermore, *Candida* can be isolated from the genital tract of approximately 20% (range 10-55%) of asymptomatic healthy women in their childbearing years.[2,3,7,8] The natural history of asymptomatic colonization is uncertain, but it is known that a number of factors (for example pregnancy, use of high-estrogen oral contraceptives, and uncontrolled diabetes mellitus) are associated with increased rates of (asymptomatic) vaginal colonization with *Candida*.[2-8] In addition, an increased frequency of vaginal *Candida* has been reported among certain ethnic groups.[9]

Cervical carcinoma is the second most common cancer among women worldwide.[10] It is now well established that infection with putative oncogenic human papillomaviruses plays a central role in cervical carcinogenesis.[11-14] Cervical inflammation has been proposed as etiological cofactor on the development of cervical cancer.[15] Although bacterial infections such as Bacterial Vaginosis (BV) might influence the risk for squamous abnormalities, this role has not yet been established for vulvovaginal *Candida*.[16-26]

One way to study the relationship between *Candida vaginalis* and squamous (pre)neoplasia is to use large datasets of systematically coded, Papanicolaou-stained smears. Certain cytological changes may be detected in cervical smears, such as those caused by HPV, *Trichomonas*, and *Candida*.[17,21] The Dutch national coding system for pathology findings in cervical cytology, KOPAC, was introduced in the 1980s and especially designed to store the cytopathological findings including the presence or absence of *Candida*.[27] The Dutch screeners are taught how to recognize *Candida* (pseudohyphae or spores) and to classify the smears as 'O4'. The squamous epithelial changes are coded from P2-3 (ASCUS or borderline) to P9 (clearly invasive squamous cell carcinoma), allowing the

recording of the occurrence of *Candida* in each category. In the western region of The Netherlands population-based, cervical screening is carried out by six laboratories. All KOPAC cytology reports are centrally stored in the SBBW (Stichting Bevolkingsonderzoek Baarmoederhalskanker region West) database. This database contains information on almost 500,000 smears in the period of 1995-2002. Smears entered into the cervical cancer detection program are, in principle, from asymptomatic women only.[17] The country of birth of each woman is recorded during population screening. As a result, the relationship between immigrant status, presence of vaginal *Candida*, and the risk of developing cervical cancer can be examined in a dataset of a substantial size.[16,28,29]

Materials and Methods

Data of 445,671 smears taken between 1995 and 2002 in the western region of The Netherlands were used in this study. As they reach the age of 30, 35, 40, 45, 50, 55 and 60, women living in the western region (population of around 2 million) receive a letter of invitation to have a smear taken, usually made by their own general practitioner. This results in seven age cohorts, which are screened at 5 year intervals. Over the study period, response was almost 70%. For all women, the age was entered into the database. A woman was coded as Dutch, when born in The Netherlands. Four groups of immigrants were identified by their country of birth: Morocco, Turkey, Suriname and Other (all other countries, consisting mainly of women born in the Far East, the Antilles, the Western part of Africa, in Western Europe, Canada or USA).

All smears, screened by six pathology laboratories, were coded according to KOPAC (the Dutch national coding system for cervical cytology[27]). The O stands for Ontsteking (inflammation) and within this category, *Candida* is coded as O4. The P stands for Plaveiselepitheel (squamous epithelium). Within this category P1 stands for normal or benign, P2-3 for ASCUS (borderline changes), P4 for mild dysplasia, P5 for moderate dysplasia, P6 for severe dysplasia, P7 for carcinoma in situ, P8 for

microinvasive carcinoma and P9 for macroinvasive squamous cell carcinoma. All diagnoses of P5 or higher generate to a referral to the hospital for a colposcopic examination and if required a biopsy. For the purpose of the study we grouped smears with P5 to P9 into a single category P5-P9. For each smear there is an O code (O4 or not O4) and a P code (P1, P2-3, P4 and P5-P9), allowing for study of prevalence of both inflammation and (pre)neoplastic changes. The relationship of the KOPAC P-codes with the Bethesda system is shown in Table 1.

Codes of the KOPAC system	Description other systems	Bethesda system
P1	Normal	WNL
P2-P3	Borderline changes	ASCUS
P4	Mild dysplasia	LSIL
P5	Moderate dysplasia	HSIL
P6	Severe dysplasia	HSIL
P7	Carcinoma in situ	HSIL
P8	Micro invasive carcinoma	Carcinoma
P9	Squamous cell carcinoma	Carcinoma

Table 1 Relationship of the P-codes of the Dutch KOPAC and other classification systems.
(WNL: within normal limits, ASCUS: atypical squamous cells of undetermined significance, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion)

The prevalence per 1000 smears was calculated for *Candida* and for squamous abnormalities (P2-3, P4, and P5-P9). Relative risks (RRs), linear regression, and confidence intervals were calculated using SPSS 10.0. For the prevalence per 1000 smears for koilocytosis (HPV) and dysbacteriosis (BV) in the four immigrant groups the data of the papers

by Boon *et al.*[28] and Verbruggen *et al.*[16] were used. *Candida* and squamous abnormalities are presented in a cross table (Table 4).

Results

Candida was detected in 5,796 smears (1.3%), whereas the remaining 439,875 smears were coded as free of *Candida*. In Table 2, the prevalence

Ethnicity	Candida		Koilocytosis (a)		Dysbacteriosis (b)	
	Prev	RR (95% CI)	Prev	RR (95% CI)	Prev	RR (95% CI)
Dutch (n=396270)	12.9	1.00 (reference)	1.67	1.00 (reference)	33.10	1.00 (reference)
Moroccan (n=3578)	11.7	0.91 (0.67-1.23)	1.40	0.84 (0.35-2.02)	36.33	1.10 (0.93-1.30)
Turkish (n=4301)	11.9	0.92 (0.70-1.21)	2.33	1.39 (0.75-2.60)	60.68	1.83*(1.63-2.07)
Surinamese (n=12701)	16.0	1.24*(1.08-1.42)	3.15	1.89*(1.37-2.60)	85.35	2.58*(2.43-2.74)
Other (n=28821)	13.5	1.05 (0.95-1.16)	2.50	1.50*(1.18-1.91)	51.52	1.56*(1.48-1.64)

Table 2 Data stratified by four groups of immigrants and Dutch women: prevalence per 1000 for *Candida*, koilocytosis and dysbacteriosis.
* Statistically significant
- a) numbers as per Boon *et al.* [28].
- b) numbers as per Verbruggen *et al.* [16].

per 1000 smears for *Candida* and the corresponding RRs for the four groups of Dutch immigrants are presented. The data for koilocytosis (O1) and dysbacteriosis (O3) are also included. The RR of having *Candida* was significantly higher for Surinamese women compared to the

other groups of women (1.24; CI 1.08-1.42). Furthermore, Surinamese women, together with women born in all other countries, featured significantly higher RRs for squamous lesion P4 (RR 1.47; CI 1.14-1.89 and RR 1.36; CI 1.13-1.62, respectively).

In Table 3, the data for *Candida* and squamous abnormalities are shown, as stratified by age groups. The prevalence of *Candida* decreases significantly with increasing age, as calculated by using linear regression analysis.

Age/years	*Candida*	P2-P3	P4	P5-9
	Prev.	Prev.	Prev.	Prev.
30 (n=58588)	17.4	15.2	6.1	7.1
35 (n=81050)	15.9	15.3	4.4	6.0
40 (n=79499)	15.8	14.8	3.8	4.4
45 (n=75568)	13.2	17.4	3.0	3.0
50 (n=71357)	10.4	18.6	2.4	2.1
55 (n=44830)	7.0	11.0	1.6	1.6
60 (n=34779)	5.0	8.7	1.2	1.2
All women (n=445671)	**13.0**	**15.1**	**3.4**	**3.9**

Table 3 Data stratified by age: prevalence per 1000 for *Candida* and for squamous abnormalities (P2-P3, P4, P5-P9).

In Table 4, the results of cross tabulation between *Candida* and squamous abnormalities are shown. There are no statistically significant differences between squamous abnormalities and presence of *Candida* (a Chi-square test performed produced an improbability chance of 7.5 %). Of the total

of 445,671 smears, 17 smears did not contain squamous cells, therefore the P-code could not be established.

Squamous abnormality	Candida		No Candida		Total	
	n	%	n	%	n	%
P1 (normal)	5644	97,38	430000	97.76	435644	97.75
P2-P3 (ASCUS)	109	1,88	6625	1.51	6734	1.51
P4 (LSIL)	24	0,41	1509	0.34	1533	0.34
P5-P9 (HSIL & carcinoma)	19	0,32	1724	0.39	1743	0.39
P1-P9	**5796**	**100**	**439858**	**100**	**445654**	**100**

Table 4 Cross tabulation: squamous abnormality and *Candida*.
(ASCUS: atypical squamous cells of undetermined significance, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion)

Discussion

Prevalence of *Candida* and (pre)neoplastic lesions was established in the Dutch cervical screening population, consisting of immigrants and native women. The relative risk of having *Candida* was highest for Surinamese women. Cotch *et al.*[9] also noted ethnic differences in vaginal colonization with *Candida*: black women were more likely to carry *Candida*, compared to Hispanic and white women. However, Eckert *et al.*[2] found no relationship between race and presence of *Candida* (white women versus all other) as did Wang and Lin in Taiwanese versus Mainlander and Aboriginal women.[21] In addition to a higher relative risk of having

vaginal *Candida*, Surinamese women were also more prone to have dysbacteriosis or koilocytosis (Table 2[16,28]). These results could reflect different behaviour encouraged by Surinamese culture. Surinamese female immigrants might display a higher use of oral contraceptives, together with a more active sexual lifestyle[30] compared to other groups of immigrants.

In our study, the overall prevalence of cytology-detected *Candida* in asymptomatic women was 1.3%. Wang and Lin[21] noted an overall prevalence of 3.4%, but the smears examined were taken from both symptomatic and asymptomatic women visiting venereal disease clinics. Although cytological examination undoubtedly underestimates the true prevalence of asymptomatic *Candida* compared to culture sampling[17,21], our screeners had no difficulty to recognize *Candida* in Papanicolaou-stained smears (figure 1: page 150).

The prevalence of *Candida* and the risk of developing cervical (pre)neoplastic changes declined with increasing age. This finding resembles results in other studies.[3,7,9,21,28] It is generally thought that high levels of reproductive hormones are an excellent source for growth of *Candida*, by providing a higher glycogen content in the vaginal environment. The fact that postmenopausal women appear more resistant to *Candida* colonization and that *Candida* rates during pregnancy are twice as high as in non pregnant women, illustrates this hormonal dependence.[3,7,9]

We established that presence of *C. vaginalis* is not associated with an increased risk for cervical (pre)neoplasia in the same smear. This result is concurrent with Wang and Lin[21] and Chakrabarti *et al.*[25], who also demonstrated no obvious associations between vaginal *Candida* and cytologic changes (CIN I, II, III). Whereas koilocytosis and HPV infection are associated with an increased risk of developing squamous abnormalities[11-14,31,32] and cytological changes are found more often in women with bacterial infections (such as Bacterial Vaginosis),[15,18,28] vaginal *Candida* is not a cofactor in the development of cervical cancer.

However, Guijon *et al.* noted that *Candida vaginalis* was present more often in women without CIN, compared to women with CIN.[24] In our study of 445,654 women, the prevalence of lesions P5-P9 in women with and without *Candida* was similar, although only 19 women with *Candida* had (pre)neoplastic changes. Because both the prevalence of vaginal *Candida* and the prevalence of squamous abnormalities are low, this number is small, but realistic. Therefore it is unlikely that there is a causal negative relationship between *Candida* and the development of cervical cancer.

In this study we show that presence of *Candida* is related to immigrant status. Presence of *Candida vaginalis* is not associated with an increased risk for squamous abnormalities in the same smear; therefore women carrying *Candida* are not at an increased risk of developing cervical cancer. Our results also show no protective effect of vaginal *Candida* in the development of (pre)neoplastic changes.

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Chapter 5

Candida and dysbacteriosis: a cytological, population-based study of 100,605 asymptomatic women concerning cervical carcinogenesis

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Abstract

OBJECTIVE: To examine whether presence of vaginal *Candida* or dysbacteriosis predisposes to an increased susceptibility for (pre)neoplasia in time.

METHODS: A retrospective, longitudinal, cohort study was performed, conducted from 100,605 women, who had two smears taken over a period of twelve years as part of the Dutch cervical screening program. From these women, a cohort of 1,439 women with *Candida* and a cohort of 5,302 women with dysbacteriosis were selected as two separate study groups. The control group consisted of women having completely normal cervical smears (n = 87,903). These groups were retrospectively followed in time. Odds ratios for squamous abnormalities in the follow-up smear of these three cohorts were established.

RESULTS: The dysbacteriotic cohort was significantly more likely to have low- and high-grade squamous intraepithelial lesions (LSIL and HSIL, including carcinoma) in the follow-up smear compared to the control group (ORs 1.85; CI 1.28-2.67 and 2.00; CI 1.31-3.05 respectively). In contrast, the *Candida* cohort had no significantly increased or decreased chance to have SIL. The equivocal diagnosis 'ASCUS' was rendered significantly more often in the follow-up smear of both study cohorts (OR *Candida* cohort 1.42; CI 1.03-1.95; OR dysbacteriotic cohort 1.44; CI 1.22-1.71).

CONCLUSION: In this study we show that presence of *Candida vaginalis* is not associated with an increased risk for SIL in time. In contrast, women with dysbacteriosis do have a significantly increased chance to develop (pre)neoplastic changes. These findings could be taken into account in further research concerning the predisposing factors for cervical carcinogenesis.

Introduction

Vaginal complaints account for large numbers of visits to general practices in The Netherlands. Approximately 50 per 1000 female patients per year visit their general practitioner (GP) with complaints of vaginal discharge, abnormal in amount, colour and/or smell.[1] Possible causes of the vulvovaginal discomfort include Bacterial Vaginosis and vulvovaginal candidiasis.[1,2] Roughly 75% of all sexually active women will experience one episode of vulvovaginal candidiasis (VVC) during their lives, but *Candida* can also be isolated from the genital tract of 20% of asymptomatic women during their childbearing years.[3,4] The mechanism whereby *Candida* organisms transform from commensal to pathogen is not yet established. It has been suggested that the normal vaginal flora, dominated by presence of lactobacilli, is protective against *Candida* vaginitis.[5] However, VVC occurs more frequently in women with a lactobacilli-predominated vaginal flora and is inversely correlated to a vaginal flora change with a mixed anaerobic vaginal flora.[6] In contrast, Bacterial Vaginosis is characterized by a lack of normally protective lactobacilli and an overgrowth of mainly anaerobic bacteria.[7,8] Since lactobacilli are part of the main defence mechanisms of the vagina, changes in the proportions of these *Lactobacillus* species are risk factors for receiving vaginal infections.[9-14] In addition, studies have noted that cervical cytological abnormalities occur more often in women with an abnormal vaginal flora than in those without this condition.[15-18] Therefore, it would be of interest to examine whether women carrying *Candida* are prone to acquire cervical cytological abnormalities in time compared to women known to have a disturbed, bacterial vaginal flora.

The Dutch national coding system for pathology findings in cervical cytology (KOPAC) is especially designed to store cytopathological findings including the presence or absence of *Candida* and dysbacteriosis.[19] Dysbacteriosis represents the microscopic diagnosis of a disturbed vaginal flora, in which lactobacilli are replaced by a mixture of anaerobic and aerobic bacteria, and is therefore closely related to the

clinical syndrome Bacterial Vaginosis.[17,20] In addition, squamous epithelial changes are coded from P2-3 (ASCUS or borderline) to P9 (clearly invasive squamous cell carcinoma) in the KOPAC system, allowing the recording of the occurrence of *Candida* and dysbacteriosis in each category.

The Leiden database contains the cytological findings of 100,605 women, who had two cervical smears taken over a period of twelve years, as part of the national screening program for cervical carcinoma. Our purpose was to perform a retrospective, longitudinal, population-based cohort study to assess a possible association between presence of vaginal *Candida*, dysbacteriosis, and the development of cervical cancer in time.

Materials and Methods

In the period between 1991 and 2003, the Leiden Cytology and Pathology Laboratory (LCPL) received almost 800,000 conventional smears. These smears originated from women who participated in the Dutch national screening program, since all women between the age of 30 and 60 in The Netherlands are invited once every 5 years to be tested for cervical cancer. All smears were coded according to the Dutch national coding system (KOPAC) for cervical cytology.[19] The O, in KOPAC, stands for inflammatory changes (Ontsteking) and this category is divided into nine different subgroups: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), no inflammatory changes (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific changes (O9). The P stands for plaveiselepitheel (squamous epithelium). Within this category P1 stands for normal or benign, P2-3 for ASCUS (borderline changes), P4 for mild dysplasia, P5 for moderate dysplasia, P6 for severe dysplasia, P7 for carcinoma in situ, P8 for microinvasive carcinoma and P9 for macroinvasive squamous cell carcinoma. All diagnoses of P5 or higher generate to a referral to the hospital for a colposcopic examination and if

required a biopsy. For the purpose of the study we grouped smears with P5 to P9 into a single category, called P5-P9. The relationship of the KOPAC P-codes with the Bethesda system is shown in Table 1. For each smear there is an O code

Codes of the KOPAC system	Description other systems	Bethesda system
P1	Normal	WNL
P2-P3	Borderline changes	ASCUS
P4	Mild dysplasia	LSIL
P5	Moderate dysplasia	HSIL
P6	Severe dysplasia	HSIL
P7	Carcinoma in situ	HSIL
P8	Micro invasive carcinoma	Carcinoma
P9	Squamous cell carcinoma	Carcinoma

Table 1 Relationship of the P-codes of the Dutch KOPAC and other classification systems.
(WNL: within normal limits, ASCUS: atypical squamous cells of undetermined significance, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion)

and a P code (P1, P2-3, P4, P5-P9), allowing for study of prevalence of both inflammation and (pre)neoplastic changes. The database contained 748,940 smears from 432,064 women. We selected all women whose first smear, taken as part of the national screening program, showed normal squamous epithelium (Bethesda classification WNL: within normal limits) and who had a subsequent smear made. Of this group of 100,605 asymptomatic women, women with a *Candida* diagnosis (n = 1,439) formed the first study cohort, whereas women with dysbacteriosis (n = 5,302) were selected as the second study cohort. The control group

consisted of women having completely normal smears (n = 87,903, see Table 2). All three cohorts were retrospectively followed in time. The mean follow-up time between the subsequent smears was 4.0 ($\pm$ 1.7) years. Odds ratios (OR) with 95% confidence intervals (CI) of the repeat smears were calculated separately for both the *Candida* cohort and the dysbacteriotic cohort (compared to the control cohort) by using SPSS 10.0.

O (ontsteking) = inflammation	O-codes of the smears of 100,605 women, screened for cervical carcinoma		Cohorts followed in time
	n	%	
O1 Koilocytosis	13	0.01	
O2 T. Vaginalis	334	0.33	
O3 Dysbacteriosis	5302	5.27	Dysbacteriotic cohort
O4 Candida	1439	1.43	*Candida* cohort
O5 G. Vaginalis	52	0.05	
O6 No inflammatory	87903	87.37	Control cohort
O7 Actinomyces	801	0.80	
O8 C. Trachomatis	171	0.17	
O9 Non-specific changes	4590	4.56	
Total	100,605	100	

Table 2 Pathogens detected in the cytologically normal cervical smear of 100,605 asymptomatic women.

As described afore, the KOPAC system also contains a category to document presence of *Gardnerella vaginalis* (O5). The cervical smears in this category are dominated by clue cells and the adhering bacteria all have the same coccoid morphology.[19] In other words, in contrast to

dysbacteriosis, only one type of bacteria is encountered, but, similar to dysbacteriosis, lactobacilli are absent. However, due to a very small sample size (n = 52), women having only *Gardnerella vaginalis* in their cervical smears were not included in our analysis.

Results

In Table 3 the distribution of age in years among the *Candida* cohort, the dysbacteriotic cohort and the control group is shown. Note that, since cervical screening in the Netherlands targets women aged 30 to 60 years, all women were older than 30 and younger than 60 years of age. A chi-square test performed showed significant differences in age distribution between the three cohorts (P < 0.001, see Table 3).

Age in years	*Candida* cohort		Dysbacteriotic Cohort		Control cohort	
	n	%	n	%	n	%
30-40	706	49.1	2043	38.5	41405	47.1
40-50	560	38.9	2358	44.5	32386	36.8
50-60	173	12.0	901	17.0	14112	16.1
Total	1439	100	5302	100	87903	100

Table 3 Distribution of age.
Chi-square test *Candida* cohort (observed versus expected) = 17,858 (DF = 2, p<0.001)
Chi-square test dysbacteriotic cohort (observed versus expected) = 170,037 (DF = 2, p<0.001)

In Table 4 the frequency of the different P-outcomes from the second (or follow-up) smear of the *Candida* cohort, the dysbacteriotic cohort, and the control group are presented. In the second smear borderline changes (ASCUS) were established significantly more often in both study cohorts (OR Candida cohort: 1.42, CI 1.03-1.95; OR dysbacteriotic

cohort: 1.44, CI 1.22-1.71). In addition, the dysbacteriotic cohort was significantly more likely to have low-grade (LSIL) and high-grade squamous intraepithelial lesions (including carcinoma) in the second smear compared to the control group (ORs 1.85; CI 1.28-2.67 and 2.00; CI 1.31-3.05 respectively). In contrast, the *Candida* cohort had a lower chance to have LSIL (OR 0.42, CI 0.11-1.70), but a slightly higher chance (OR 1.22; CI 0.45-3.28) to develop high-grade squamous intraepithelial lesions (including carcinoma). However, these differences were not statistically significant.

Second smear (follow-up smear)	*Candida* cohort			Dysbacteriotic cohort			Control cohort
	n	OR	95%CI	n	OR	95%CI	n
P1 (normal)	1393	1.00	reference	5097	1.00	reference	85673
P2-P3 (ASCUS)	40	1.42*	1.03-1.95	149	1.44*	1.22-1.71	1737
P4 (LSIL)	2	0.42	0.11-1.70	32	1.85*	1.28-2.67	291
P5-P9 (HSIL and carcinoma)	4	1.22	0.45-3.28	24	2.00*	1.31-3.05	202
Total	**1439**			**5302**			**87903**

Table 4 Squamous abnormalities in the second (or follow-up) smear.
ASCUS = atypical squamous cells of undetermined significance
LSIL   = low-grade squamous intraepithelial lesion
HSIL   = high-grade squamous intraepithelial lesion
* Statistically significant

Discussion

In this study we investigated whether presence of vaginal *Candida* or dysbacteriosis, as determined in Papanicolaou stained cervical smears,

plays a role in cervical carcinogenesis. Nowadays, it is well known that infection with putative oncogenic human papillomaviruses contributes to the development of cervical cancer.[21-24] Although HPV infection is widely prevalent, only a few infected women will go on to develop cervical cancer, suggesting that other factors are involved in malignant progression. Cervical inflammation has been proposed as one of the cofactors in cervical carcinogenesis, since disturbance of the vaginal flora is known to increase the risk of acquisition of HPV infection.[25-28] Papanicolaou[29] and Mead *et al*[30] already confirmed that women with cervical carcinoma often had a dysbacteriotic flora, lacking the normally protective lactobacilli. In this study, we also noted that women with dysbacteriosis in their smears had a significantly but small higher risk of having (pre)neoplastic changes in time. This finding is partially supported by other studies that investigated women with dysbacteriosis.[15,17,25,31,32] However, two studies showed similar frequencies of dysbacteriosis among women with squamous intraepithelial lesions compared to those who did not have squamous intraepithelial lesions (SIL).[33,34] This discrepancy might be explained by the fact that the data in these studies were gathered simultaneously instead of in time. In addition, the presence of Bacterial Vaginosis was studied in women already suffering from cytological abnormalities.

We established that the equivocal diagnosis 'ASCUS' was rendered significantly more often in the second smear of both study groups. In the Bethesda system, ASCUS is defined as 'cellular abnormalities that are more marked than those attributable to reactive changes but that quantitatively or qualitatively fall short of a definitive diagnosis of squamous intraepithelial lesion'.[35] However, the histological correlate of this equivocal diagnosis is wide, ranging from a totally normal cervix mucosa to infiltrating carcinoma.[36] In addition, vaginal infections, such as vulvovaginal candidiasis, can mimic the cytomorphology of ASCUS by displaying nuclear enlargement and hyperchromasia in the vaginal epithelial cells.[37-39] The increased ASCUS scores noted in this study in the

cervical smears of women with *Candida* and dysbacteriosis can be due to cellular side-effects of the vaginal infection still present after four years. In this context, we stress the fact that due to the wide definition of ASCUS, it is not possible to make more concrete remarks about a possible causal relationship between *Candida* and ASCUS. Thus, randomized controlled trials are necessary to address this possible relationship.

In contrast, we established that presence of *Candida* is not associated with a significant increased risk for SIL in time. This result is confirmed by other studies, who also demonstrated no obvious associations between vaginal *Candida* and (pre)neoplasia.[18,40,41] Therefore, vaginal *Candida* is not a cofactor in the development of cervical cancer. This is consistent with the general hypothesis that the local cervicovaginal milieu plays a role in susceptibility to HPV infection, since women carrying *Candida* are likely to possess a healthy *Lactobacillus* predominated vaginal flora in contrast to women with dysbacteriosis.[21,22]

Finally, our results showed significant differences in age distribution between the *Candida* cohort, the dysbacteriotic cohort and the control group. This finding was expected, since the prevalence of dysbacteriosis gradually increases for women below 50 and then decreases to values observed among 30-year-old women,[17] whereas the prevalence of *Candida* declines with increasing age.[18]

In this study we show that presence of *Candida vaginalis* is not associated with an increased risk for SIL in time. In contrast, women with dysbacteriosis do have a significantly increased chance to develop (pre)neoplastic changes. These findings could be taken into account in further research concerning the predisposing factors for cervical carcinogenesis.

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Chapter 6

Candida and colonization with *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyses*: a cytological study

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Abstract

OBJECTIVE: To obtain insight into the consequences of a smear with *Candida* and the incidence of acquiring other vaginal infections as detected in repeat smears.

METHODS: A retrospective, longitudinal, cohort study was performed, conducted from 104,686 women, who had multiple smears taken over a period of twelve years as part of the Dutch cervical screening program. Women with *Candida* in their cervical smear were selected from this cohort and formed the first study group (n = 1,492), whereas 5,508 women with a dysbacteriotic flora (lacking lactobacilli) were selected as the second study cohort. The control cohort consisted of women who had a normal vaginal flora (n = 90,991). These three groups were retrospectively followed in time.

RESULTS: *Candida* occurred over 5 to 6 times more often in the first and second repeat smear of the *Candida* cohort compared to the control cohort. No other statistically significant differences between the *Candida* cohort and the control cohort were noted. However, in the dysbacteriotic women, *T. vaginalis*, *G. vaginalis* and *Actinomyces* were more frequently encountered in both repeat smears.

CONCLUSION: In contrast to dysbacteriosis, presence of vaginal *Candida* does not predispose to an increased susceptibility for *T. vaginalis*, *G. vaginalis* and *Actinomyces*. This subscribes the thought that women carrying *Candida* possess a healthy vaginal flora, dominated by presence of lactobacilli, which protects their vagina from other infective agents.

Introduction

Vulvovaginal candidiasis (VVC) is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract. It is a frequent diagnosis in the daily practice of gynaecology and accounts for large numbers of visits to general practices in The Netherlands.[1] However, *Candida* can also be isolated from the genital tract of approximately 20% of asymptomatic women during their childbearing years.[2,3] The mechanism whereby *Candida* organisms transform from commensal to pathogen is not yet established. It has been suggested that the normal vaginal flora, dominated by presence of lactobacilli, is protective against *Candida* vaginitis.[4] Since changes in the proportions of normally protective *Lactobacillus* species are risk factors for receiving vaginal infections,[5-9] it would be of interest to examine whether women carrying *Candida* are prone to acquire other vaginal infections in time.

In a cervical smear, pathogens such as *Candida*, *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyces* can be detected. This implies that large cytological databases can be exploited to study the possible relationship between *Candida* and colonization of other pathogens. A study of asymptomatic women with vaginal *Candida* is possible in the Leiden database, containing the cytological findings of 104,686 women, who had more than two cervical smears taken over a period of twelve years, as part of the national screening program for cervical carcinoma. In the Dutch screening programs, women do not choose to be investigated but are invited by a central organization to visit their physicians for a cervical smear. Hence, all infective agents that are detected in a smear are subclinical.

Our purpose was to perform a retrospective, longitudinal, population-based cohort study to obtain insight into the consequences of a smear with *Candida* and the incidence of acquiring other vaginal infections as detected in repeat smears.

Materials and Methods

In the period between 1991 and 2003, the Leiden Cytology and Pathology Laboratory (LCPL) received almost 800,000 conventional smears. These smears originated from women who participated in the Dutch national screening program, since all women between the age of 30 and 60 in The Netherlands are invited once every 5 years to be tested for cervical cancer. All smears were coded according to the Dutch national coding system (KOPAC) for cervical cytology.[10] The O, in KOPAC, stands for inflammatory changes (Ontsteking) and this category is divided into nine different subgroups: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), no inflammatory changes (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific changes (O9).

The database contained 748,940 smears from 432,064 women. We selected all women whose first smear was taken as part of the national screening program and who had at least one subsequent smear made. Of this group of 104,686 asymptomatic women, women with a *Candida* diagnosis (n = 1,492) were selected as the first study group. To be able to compare women carrying *Candida* in time with women known to have a disturbed vaginal flora, women with dysbacteriosis (n = 5,508) were included as a second study cohort. The control group consisted of women having a normal vaginal flora (n = 90,991). All three cohorts were retrospectively followed in time. This longitudinal study was performed in two steps, whereby the first and the second repeat smears of the three cohort groups were analysed (respectively n = 1,492, n = 783: *Candida* cohort, n = 5,508, n = 2,979: dysbacteriotic cohort, and n = 90,991, n = 43,050: control cohort, see Table 1). Odds ratios (OR) with 95% confidence intervals (CI) of the repeat smears were calculated for the *Candida* cohort, the dysbacteriotic cohort, and the control cohort by using SPSS 10.0.

O (ontsteking) = inflammation	O-codes of the smears of 104,686 women, screened for cervical carcinoma		Cohorts followed in time
	n	%	
O1 Koilocytosis	394	0.38	
O2 T. Vaginalis	362	0.35	
O3 Dysbacteriosis	5508	5.26	Dysbacteriotic cohort
O4 Candida	1492	1.43	*Candida* cohort
O5 G. Vaginalis	62	0.06	
O6 No inflammation	90991	86.92	Control cohort
O7 Actinomyces	826	0.79	
O8 C. Trachomatis	229	0.22	
O9 Non-specific changes	4822	4.61	
Total	**104,686**	**100**	

Table 1 Pathogens detected in the cervical smear of 104,686 asymptomatic women.

Age in years	*Candida* cohort		Dysbacteriotic cohort		Control cohort	
	n	%	n	%	n	%
30-40	423	28.4	1188	21.6	25183	27.7
40-50	703	47.1	2466	44.8	39673	43.6
50-60	366	24.5	1854	33.6	26135	28.7
Total	**1492**	**100**	**5508**	**100**	**90991**	**100**

Table 2 Distribution of age.

Results

In Table 2 the distribution of age in years among the *Candida* cohort, the dysbacteriotic cohort and the control cohort is shown. Since cervical screening in the Netherlands targets women aged 30 to 60 years, all women were older than 30 and younger than 60 years of age. The mean follow-up time between the subsequent smears was 3.9 ($\pm$ 1.7) and 3.6 ($\pm$ 1.6) years respectively.

First follow-up smear	*Candida* cohort			Dysbacteriotic cohort			Control cohort
	n	OR	95%CI	n	OR	95%CI	n
O1 Koilocytosis	4	1.36	0.50-3.66	21	2.19*	1.39-3.44	190
O2 T. Vaginalis	4	2.03	0.75-5.50	25	3.90*	2.54-5.99	127
O3 Dysbacteriosis	42	1.15	0.84-1.56	921	7.74*	7.13-8.40	2357
O4 Candida	79	6.81*	5.37-8.65	39	1.03	0.75-1.43	747
O5 G. Vaginalis	2	2.43	0.59-9.98	14	5.23*	2.90-9.44	53
O6 No inflammation	1254	1.00	reference	4078	1.00	reference	80761
O7 Actinomyces	8	1.40	0.69-2.82	59	2.95*	2.24-3.89	396
O8 C. Trachomatis	0			8	1.65	0.80-3.40	96
O9 Non-specific changes	99	1.02	0.83-1.25	343	1.08	0.97-1.21	6264
Total	**1492**			**5508**			**90991**

Table 3 Pathogens detected in the first follow-up smear.

* Statistically significant

In Table 3 (first follow-up smear) and 4 (second follow-up smear), the frequency of the different O-outcomes from the subsequent smears of the *Candida* cohort, the dysbacteriotic cohort and the control group are presented. In the first follow-up smear, *Candida* occurred over 6 times

more often among the *Candida* cohort compared to the control cohort (OR 6.81; CI 5.37-8.65). This trend persisted in the second smear (OR 5.26; CI 3.52-7.86). However, *Trichomonas vaginalis*, *Gardnerella* and *Actinomyces* did not appear significantly more often in the first and second follow-up smear of the *Candida* cohort compared to the controls. In contrast, in the dysbacteriotic cohort, *T. vaginalis*, *Gardnerella vaginalis*, and *Actinomyces* were more often detected in the first and the second follow-up smear compared to the control cohort.

Second follow-up smear	*Candida* cohort			Dysbacteriotic cohort			Control cohort
	n	OR	95%CI	n	OR	95%CI	n
O1 Koilocytosis	2	1.21	0.30-4.91	10	1.71	0.89-3.29	93
O2 T. Vaginalis	0			8	2.71*	1.28-5.73	47
O3 Dysbacteriosis	15	1.03	0.62-1.73	291	5.68*	4.93-6.53	815
O4 Candida	27	5.26*	3.52-7.86	23	1.27	0.83-1.95	288
O5 G. Vaginalis	0			4	6.36*	1.99-20.3	10
O6 No inflammation	690	1.00	reference	2436	1.00	reference	38725
O7 Actinomyces	6	1.99	0.88-4.51	36	3.39*	2.36-4.86	169
O8 C. Trachomatis	0			3	2.65	0.78-9.00	18
O9 Non-specific changes	43	0.84	0.61-1.14	168	0.93	0.79-1.09	2885
Total	**783**			**2979**			**43050**

Table 4 Pathogens detected in the second follow-up smear.
* Statistically significant

Discussion

The mechanism whereby *Candida* organisms transform from commensal to pathogen is not yet established. It has been suggested that the normal vaginal flora, dominated by presence of lactobacilli, is protective against

*C*andida vaginitis.[4] This hypothesis is strengthened by the fact that in vitro studies have demonstrated that *Candida albicans* is inhibited by culture supernatants of *Lactobacillus acidophilus.*[11,12] In addition, vaginal candidiasis often develops after systemic use of broad spectrum antibiotics.[13] However, clinical studies show that women with yeast vaginitis have the same frequency and concentration of *Lactobacillus* species as women without recurrent infections.[14,15] Besides, vulvovaginal candidiasis occurs more frequently in women with a lactobacilli-predominated vaginal flora, as compared to those with a flora change with a mixture of anaerobic and facultative anaerobic bacteria.[16] Moreover, the use of oral or vaginal forms of lactobacilli does not prevent post-antibiotic vulvovaginitis.[17] Hence, the role of lactobacilli in preventing *Candida* to transform from commensal to pathogen remains unclear. Since changes in the proportions of normally protective *Lactobacillus* species are risk factors for receiving other vaginal infections[5-9] we examined whether asymptomatic women carrying *Candida* have an increased risk to acquire other vaginal infections in time.

We established that women, who initially carried *Candida,* were prone to have a subsequent smear with *Candida.* Since the Dutch cervical cancer screening program invites women by a central organization to visit their physicians for a cervical smear, the *Candida* detected in these women is subclinical. It should be noted however, that different levels of tolerance for vaginal complaints could lead to a small minority of relatively "asymptomatic" women*,* who are in fact suffering from candidiasis. Therefore, our results imply that *Candida* commensalism and/or vulvovaginal candidiasis can last many years and/or happen intermittently. This finding is endorsed by the fact that approximately 75% of all women will develop VVC of which about half will suffer a second VVC event, while *Candida* can also be isolated from the genital tract of approximately 20% of asymptomatic women in their childbearing years.[2,3]

Furthermore, we noted that women carrying *Candida* are not predisposed to acquire other vaginal pathogens such as *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyces* in time, because no statistically significant differences between the *Candida* cohort and the control cohort were established. However, women suffering from dysbacteriosis, characterized by a sharp decrease of the normally protective lactobacilli and an overgrowth of mainly anaerobic bacteria, are at risk of being colonized with *T. vaginalis*, *G. vaginalis* and *Actinomyces*. This result is probably due to lack of the normally protective *Lactobacillus* species, since other studies confirmed that a decrease in the amount of H2O2-producing lactobacilli is a risk factor for receiving vaginal infections, such as Bacterial Vaginosis,[5,6] *Chlamydia trachomatis*[6,7] and *Trichomonas vaginalis*.[8] Interestingly enough, women with dysbacteriosis were not at risk of having a subsequent smear with *Candida* and vice versa. This leads us to the idea that *Candida* can only survive in the vagina when a healthy *Lactobacillus* predominated flora is present. By competing for nutrients, interfering in the adherence to vaginal epithelial cells[18] and the use of bacteriocin,[19] this *Lactobacillus* flora might sustain *Candida* commensalism, thereby possibly protecting these asymptomatic women against the development of C*andida* vaginitis. Another hypothesis involves the thought that *Candida* can not survive in a vaginal flora dominated by an overgrowth of anaerobic bacteria. This thought has been subscribed by other studies, which confirmed a negative correlation between presence of Bacterial Vaginosis and vaginal candidiasis.[20,21] In addition, Rodrigues *et al.* noted that bacterial amines, such as putrescine and cadaverine produced by anaerobes (e.g. *G. vaginalis*), inhibit germ tube formation by *C. albicans*.[22] Further studies are needed to evaluate and determine the (clinical) impact of *Lactobacillus* species and anaerobes on the presence of vaginal *Candida*.

We established that presence of asymptomatic vaginal *Candida* does not predispose to an increased susceptibility for *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyces*. This subscribes the thought that

women carrying *Candida* possess a healthy vaginal flora, dominated by presence of lactobacilli, which protects their vagina from other infective agents and might even prevent *Candida* species to transform from commensal to pathogen.

Acknowledgement

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Chapter 7

Symptomatic candidiasis: using self sampled vaginal smears to establish the presence of *Candida*, lactobacilli, and *Gardnerella vaginalis*

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Abstract

OBJECTIVE: To establish the association between the bacterial flora and *Candida* overgrowth in symptomatic women.

METHODS: In a prospective cohort study, 10 symptomatic women with recurrent vulvovaginal candidiasis (RVVC) were taught how to prepare vaginal smears of their own vaginal fluids on day 7, day 14, day 21 and day 28. These air dried smears were sent to the laboratory with self reporting forms of vaginal symptoms on the four sampling days. The 40 smears were stained with the PAS-method and examined by three different cytopathologists for presence of *Candida*. Thereafter, the smears were restained with Giemsa-stain to determine presence of lactobacilli, *Gardnerella vaginalis* ('clue cells') and neutrophils. Sensitivity, specificity and positive predictive values of the vaginal symptomology and the microscopic analysis of the smears were calculated by using SPSS 10.0.

RESULTS: All three cytopathologists unequivocally established *Candida* blastospores and (pseudo)hyphae in 27 out of the 40 PAS-stained vaginal smears, whereas in the remaining 13 smears *Candida* was not found. All 10 patients had *Candida* in their smears during the second half of their menstrual cycle. Neutrophils were seen in 16 of the 27 smears positive for *Candida*. Lactobacilli were present in all smears of the RVVC patients, but *Gardnerella vaginalis* was not seen. The sensitivity of complaints for the presence of *Candida* was 89%, but the specificity only 31% and the positive predictive value 57%.

CONCLUSION: Self sampled smears are a reliable means to establish presence of *Candida* in symptomatic patients with candidiasis. *Candida* is associated with a lactobacillus-predominated vaginal flora, but with the absence of *Gardnerella vaginalis*. We also found that the sensitivity of vaginal complaints was high, but specificity low.

Introduction

Vulvovaginal candidiasis (VVC) is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract. It is a frequent diagnosis in the daily practice of gynaecology. VVC also accounts for large numbers of visits to Dutch general practitioners.[1] Five percent of all women who experienced an initial episode of VVC will develop recurrent vulvovaginal candidiasis (RVVC) defined as 4 or more episodes of symptomatic vaginitis per year.[2] Altered defense mechanisms of the vaginal microclimate and reactions to the hormonal status are thought to play a major role in the development of a recurrent episode of candidiasis.[2-7] In general, efforts to prevent recurrence by suspending antibiotics, hormone therapy, tight fitting clothes, thong panties, panty liners and sometimes even sexual intercourse have not been effective.[3,8,9] Symptoms such as vaginal pruritus and abnormal discharge can also be due to other agents or to non-infectious causes and the diagnosis of recurrent candidiasis should always be based upon vaginal examination and microscopy.[10] However, use of a speculum may be uncomfortable for the patient and requires an office visit. Therefore, women with RVVC might benefit from taking vaginal smears themselves. Self collected urine samples have already been used successfully in screening for presence of *Chlamydia trachomatis*.[11] In addition, Tabrizi *et al.*[12] and Novikova *et al.*[13] used self collected vaginal swabs to check for *Candida* vaginitis to the satisfaction of both patients and physicians. Periodic Acid Schiff (PAS) staining of vaginal smears is a histochemical method that stains the (pseudo)hyphae and vegetative yeast forms of *Candida*. In addition, the same smears can be used to differentiate the bacterial flora. The present study was undertaken to exploit self sampled vaginal smears of women with recurrent vulvovaginal candidiasis to show the presence or absence of *Candida*, lactobacilli and *Gardnerella* in stained slides. In addition, the sensitivity, specificity, positive and negative predictive values of symptoms such as pruritus, abnormal discharge and soreness were analyzed statistically.

Materials and Methods

We conducted a prospective cohort study in 10 women with RVVC. Participants were recruited between 2004 and 2005 from the Leiden Cytology and Pathology Laboratory (LCPL), which is specialized in the evaluation of women with chronic vulvovaginal symptoms. Female patients aged >18 years who had a history of >4 episodes of VVC in the previous year (including current episodes) were enrolled in the study. At least 1 of the previous VVC episodes had to have been diagnosed by a general practitioner. The 10 patients had similar social and economic status, were healthy, and suffered from a primary idiopathic form of RVVC for no apparent reason. All patients had regular menstrual cycles (approximately 28 to 32 days) and did not use oral contraceptives during the study period. The women were aged between 30 and 46. The women included in the study were taught to conduct vaginal smears of their own vaginal secretions on day 7, day 14, day 21 and day 28 of their menstrual cycle, whereby the first day of their menstrual period was assumed to be day 1. The patients were also asked to report presence of vaginal pruritus, any discharge and soreness on the four sampling days. The four air dried smears were sent to the LCPL together with a diary of the vaginal status. The smears were stained with the PAS-method before microscopic evaluation took place. In the PAS-method, which depends on oxidation of polysaccharides to aldehydes and their staining with Schiff's reagent, the cell walls of yeasts (blastospores and (pseudo)hyphae) appear bright red. In addition, squamous cells containing glycogen stain red. Three different cytopathologists examined the PAS-stained smears independently at 100x magnification. They noted presence of *Candida* (pseudo)hyphae and spores. Up-and-down focusing was used when thick clusters of vaginal epithelial cells were present in order to distinguish the spores and hyphae between the red-stained squamous cells. The pathologists used standardized forms to systematically record their microscopic findings. Then, the smears were restained with Giemsa-stain and screened for presence of lactobacilli,

polymorphonuclear neutrophils and *Gardnerella vaginalis* forming 'clue cells'.

Sensitivity, specificity, positive and negative predictive values of the vaginal complaints and the microscopic analysis of the self administered smears were calculated by using SPSS 10.0.

The study protocol received approval from the Institutional Ethical and Scientific Review Committee.

Results

The quality of the air dried smears was excellent, with abundant well-spread single cells and occasional thick cell clusters. The presence or absence of blastospores and (pseudo)hyphae was established by the results of all three cytopathologists, independently. The three cytopathologists found *Candida* in 27 out of the 40 PAS-stained vaginal smears without disagreement and the remaining 13 smears were judged free of *Candida*. By PAS-staining, yeast spores and (pseudo)hyphae were unequivocally detectable by their bright red cell walls (see figures 1 and 2: page 151). In some smears, *Candida* was only seen in one portion of the smear, emphasizing screening the full smear carefully and completely. Presence of lactobacilli was assessed semi-quantitatively (1+, 2+ and 3+). Lactobacilli were present in all Giemsa-stained smears (figures 3 and 4: page 152). Notably, *Gardnerella vaginalis* 'clue cells' were not seen in any of the smears during microscopic evaluation.

All 10 RVVC patients had *Candida* in their smears on day 21 of their menstrual cycle (see Table 1), although signs of inflammation (polymorphonuclear neutrophils) were absent in three patients. The relative number of lactobacilli noted during screening was not associated with presence or absence of *Candida* (see Table 2).

Patients	Presence of *Candida* spores and (pseudo)hyphae			
	Day 7	Day 14	Day 21	Day 28
A	No	No	Yes	Yes
B	Yes	Yes	Yes	No
C	Yes	Yes	Yes	Yes
D	No	Yes	Yes	No
E	No	Yes	Yes	No
F	No	Yes	Yes	No
G	No	Yes	Yes	No
H	Yes	Yes	Yes	Yes
I	Yes	Yes	Yes	Yes
J	No	No	Yes	Yes

Table 1 Presence of Candida spores and (pseudo)hyphae as diagnosed equally by three different cytopathologist in PAS-stained vaginal smears.

Patients		*Candida* on microscopy of the smear		Total
		Yes	No	
Presence of lactobacilli	1+	3	3	**6**
	2+	16	8	**24**
	3+	8	2	**10**
Total		**27**	**13**	**40**

Table 2 Lactobacilli on microscopy of self sampled vaginal smears.

The sensitivity, specificity, and the positive and negative predictive value of any vaginal complaints are presented in Table 3. Specific complaints

Patients		*Candida* on microscopy of the smear		Total
		Yes	No	
Any complaints	Yes	24	9	**33**
	No	3	4	**7**
Total		**27**	**13**	**40**

Table 3 The sensitivity, specificity, positive and negative predictive values of any complaints (abnormal discharge, pruritus and/or soreness) for *Candida* on microscopy of the smear.

Sensitivity = 89%

Specificity = 31%

Positive predictive value = 73%

Negative predictive value = 57%

Patients		*Candida* on microscopy of the smear		Total
		Yes	No	
Signs of inflammation	Yes	16	4	**20**
	No	11	9	**20**
Total		**27**	**13**	**40**

Table 4 The sensitivity, specificity, positive and negative predictive values of signs of inflammation on microscopy of self-sampled vaginal smears for *Candida* on microscopy of the smear.

Sensitivity = 59%

Specificity = 69%

Positive predictive value = 80%

Negative predictive value = 45%

such as pruritus, abnormal discharge or soreness showed rather high sensitivity, but lower specificity rates (pruritus: sensitivity, specificity, positive and negative predictive values respectively 74%, 46%, 74%, and 46%, abnormal discharge: respectively 74%, 54%, 77%, and 50% and

soreness: respectively 52%, 54%, 70%, and 35%). The sensitivity, specificity, positive and negative predictive values of presence of polymorphonuclear neutrophils in the smears is given in Table 4.

Discussion

The present study was undertaken to examine feasibility of using self sampled vaginal smears of patients with RVVC, to establish presence of *Candida* cytologically and to grade the concomitant bacterial flora. In addition, the sensitivity, specificity and positive predictive value of symptoms in women with recurrent candidiasis was established.

The age of the patients in this study was in concordance with the general concept that vulvovaginal candidiasis is a hormone-dependent disease where the incidence increases after menarche and peaks among women between 30 to 40 years of age.

In this study we defined *Candida* infection by presence of blastospores and (pseudo)hyphae in stained smears. Zdolsek *et al.* noted that the sensitivity of a positive wet smear in establishing candidiasis was higher than by a positive culture alone.[14] We find it very effective to use self sampled smears to determine presence of *Candida*. First, the presence or absence of blastospores and (pseudo)hyphae was equally established by all three cytopathologists, independent of each other. The cytopathologists had no difficulty to identify the brightly red stained spores and the elegant (pseudo)hyphae protruding from epithelial cells. This finding is in agreement with results from the study by Novikova *et al.*, who noted that recurrent episodes of *Candida* were easily detectable in self sampled methylene blue stained vaginal smears.[13] Second, presence of lactobacilli and neutrophils could also be determined without difficulty in the Giemsa-stained smears. Third, staining makes it possible to preserve the smears and to use them for teaching purposes or quality control. Fourth, the patients in this study were very enthusiastic about self sampling, since we could confirm that they suffered from candidiasis without having to examine their vagina. In some cases we photographed

the fungi and sent these images to the patient, which was most appreciated. Obviously, the study size was small, but the use of self sampling and standardized staining sets to simplify the analysis of vaginal discharge will be further evaluated.

All our patients had *Candida* on day 21 of their menstrual cycle. Spacek *et al.* confirmed that most patients (56%) with RVVC experienced discomfort in the second half of their cycle.[15] This finding might be related to estrogen levels that are elevated during the last half of the menstrual cycle. Estrogen seems to increase the glycogen content in the vaginal epithelial cells, thereby providing a carbohydrate source necessary for multiplication of the yeasts and facilitating their adherence to epithelial cells.[16]

In addition, symptoms such as pruritus, abnormal discharge, and soreness have a rather high sensitivity but lower specificity. This finding is consistent with results published by Mårdh *et al.* and Zdolsek *et al.*[14,17] Since vaginal pruritus and abnormal discharge can also be due to other agents or to non-infectious causes, such as allergic and hypersensitivity vulvitis, the diagnosis 'Candida infection' should thus always be confirmed by culture or (wet) smear.[10]

Lactobacilli were present in all smears of the patients, whereas *Gardnerella vaginalis* 'clue cells' were not seen in any of the smears. Studies by Sobel *et al.* and Hawes *et al.* confirm that women with yeast vaginitis have the same frequency and concentration of *Lactobacillus* species as women without recurrent infections.[18,19] Moreover, Zdolsek *et al.* noted that vulvovaginal candidiasis occurs more frequently in women with a lactobacilli-predominated vaginal flora, as compared to those with a flora change with a mixture of anaerobic and facultative anaerobic bacteria.[20] In forty smears, we expected to find at least 12 smears with 'clue cells', but none were found. This may be related to amines produced by *Gardnerella vaginalis* having a negative influence on the growth of *Candida*.[21] The recurrence of *Candida* infection might not be related to a deficiency of the normally protective vaginal bacterial flora.

Finally, polymorphonuclear neutrophils were present in 16 out of the 27 smears with *Candida*. This may be related to the observation made by Fidel *et al*., that VVC correlates with a vaginal infiltration of polymorphonuclear neutrophils, whereas protection against *Candida* infection appears non-inflammatory.[4,9] These results could indicate that some of the symptoms of women with (R)VVC may be due to an aggressive innate response by neutrophils, instead of being caused by a shift in the healthy vaginal flora. Larger randomized controlled trials are necessary to address this question.

This study raises some fascinating questions about the microbial regulation of the vaginal biosphere. Are bacterial species independent of conditions favoring yeasts or are they somehow interconnected by quorum sensing?[22] Further studies may be directed towards the interaction between the various members of the vaginal flora. This study should open molecular methodology for determining the interaction of these microbes.

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Chapter 8

General discussion and conclusions

General discussion and conclusions

This thesis addresses asymptomatic and symptomatic presence of vulvovaginal *Candida*. We have examined how Dutch general practitioners diagnose and treat *Candida* vaginitis (chapter 2 and 3). We also studied prevalence and differences in ethnicity of vulvovaginal *Candida* in the western region of The Netherlands (chapter 4). Furthermore, we addressed the question of whether women with *Candida* are more likely to develop cervical (pre)neoplasia (chapter 4 and 5). The question of whether women carrying *Candida* are predisposed to acquire (other) pathogens in time was considered in chapter 6. Finally, we surveyed the bacterial flora of women with recurrent symptomatic candidiasis using self sampled vaginal smears (chapter 7).

Prevalence of vulvovaginal Candida

The Dutch general practitioners guideline states that about 50 per 1000 female patients per year visit their general practitioner with vaginal complaints, with as much as 25 to 35% of the cases due to vaginal candidiasis.[1] In chapter 2, 380 general practitioners (GPs) in the western region of The Netherlands were asked to estimate the yearly number of office visits due to or concerning vulvovaginal candidiasis. The Dutch GPs reported an average of up to 105 cases of vaginal candidiasis annually, with most general practices attending a population of approximately 1000-1500 women. One of the reasons for this discrepancy might be found in the fact that many physicians in this study diagnosed vulvovaginal candidiasis based on symptoms and clinical findings only, rather than by more objective diagnostic procedures, which may lead to an overestimation of *Candida* vaginitis. Another reason may be due to confusion from the International Classification of Primary Care (ICPC) coding system, which GPs in The Netherlands are required to use to document all consultations.[2] The ICPC-category X72 (vulvovaginal candidiasis) was used by the Dutch GPs in chapter 2 to calculate the number of cases of vulvovaginal candidiasis, but the GPs

might not only have used this category to report candidiasis, but also to document other kinds of vaginitis. However, it has been estimated that roughly 75% of all sexually active women will experience one episode of vulvovaginal candidiasis (VVC) during their lives.[3-8] Most studies estimate a VVC prevalence of 5 to 15 percent, depending on the population studied. In addition, *Candida* can also be isolated from the genital tract of approximately 20% of asymptomatic healthy women during their childbearing years.[3,4,8,9] Wang and Lin[10] noted an overall prevalence of *Candida* of 3.4%, but the cervical smears examined were taken from both symptomatic and asymptomatic women visiting venereal disease clinics, perhaps biasing the data. In chapter 4, the baseline prevalence of *Candida*, as established in cervical smears of more than 400,000 asymptomatic women, was 13 per 1000. Although cytological examination might underestimate the true prevalence of asymptomatic *Candida* compared to cultivation on selected media,[10,11] it does show the level of prevalence in the general population.

Age distribution of vaginal Candida

Vulvovaginal candidiasis is usually seen in women of childbearing age. The incidence of VVC increases dramatically in the second decade of life, peaks in the third and fourth decade and declines in females older than 40 years.[4,8,10,12] Postmenopausal women appear more resistant to *Candida* colonization, although the incidence of VVC rises among women using hormone replacement therapy.[4,8,12,13] In chapter 4, we confirm that the prevalence of asymptomatic *Candida* declines with increasing age. This reduction in prevalence of *Candida* might be due to decreased levels of reproductive hormones. High levels of reproductive hormones are generally thought to provide a better source for growth of *Candida* by inducing higher glycogen contents in the vaginal epithelial cells.[14] In addition, estrogens have a direct effect on the growth of *Candida* and its adherence to the vaginal epithelium.[15] A hormonal dependence is emphasized by several studies showing an increased

vaginal colonization with species of *Candida* following high-dose oestrogen oral contraceptives.[16,17] In addition, the vagina shows an increased susceptibility to infection by *Candida* during pregnancy, resulting in both a higher prevalence of vaginal colonization and a higher rate of symptomatic vaginitis.[18]

Ethnic differences in presence of vaginal Candida in asymptomatic women
Differences in the prevalence of vaginal *Candida* among ethnic groups have been studied previously, but with conflicting results.[3,10,12] In chapter 4, we found that asymptomatic Surinamese women were more likely to have *Candida* compared to other groups of immigrants and indigenous Dutch women. Patient delay (due to different levels of tolerance for vaginal discharge) and health care system avoidance might lead to more "asymptomatic" carriers of *Candida* among these women. However, Surinamese women are also more likely to have koilocytosis[19] and dysbacteriosis,[20] suggesting cultural differences in sexual behaviour. Other risk factors for carrying *Candida* involve pregnancy, use of high-oestrogen oral contraceptives, use of antibiotics, uncontrolled diabetes mellitus, and a college or higher education.[4,5,10] Variations in cultural behaviour might have influenced the presence of these predisposing factors, thereby contributing to the ethnic differences in the baseline prevalence of asymptomatic *Candida* established in chapter 4.

Accuracy of the clinician's diagnosis of symptomatic vulvovaginal candidiasis (VVC)
Women with candidiasis can present with varying degrees of vulvar pruritus and vaginal discharge. Occasionally, vaginal soreness, irritation, vulvar burning, and external dysuria are reported.[4] Vulvovaginal examination of these symptomatic women often reveals epithelial erythema and swelling of the labia and vulva, together with adherent whitish liquid or 'granular' discharge.[6] However, due to lack of specificity of these signs and symptoms, all patients with symptomatic vaginitis should be diagnosed by microscopic examination of vaginal

secretions.[21,22] A 10% potassium hydroxide (KOH) slide mounting medium may be used to reveal (pseudo)hyphae and/or blastospores, which may reach sensitivity of up to 50 to 60% for yeasts.[1,21-24] If microscopy is negative for presence of *Candida* but VVC is suspected on the basis of symptoms and signs, a vaginal culture should be performed[1,3,21-25] especially when dealing with patients with recurrent vaginal complaints.[26-28] Alternative diagnostic tools in diagnosing VVC include PCR and (Papanicolaou) stained vaginal smears.[25,29-32]

In chapter 2, we examined how GPs in The Netherlands diagnose and treat *Candida* vaginitis by sending questionnaires to 1160 Dutch general practitioners. In this study, the Dutch GPs reported large numbers of patients suffering from VVC annually, although they scarcely perform vaginal examination, microscopy and culture-sampling. This finding implies that women in The Netherlands are frequently diagnosed with vulvovaginal candidiasis and treated with antimycotics solely based on symptoms and inspection only, whereas the Dutch general practitioners guideline suggests performing microscopy and/or culture when confronted with vaginal complaints.[1] In chapter 3, we compared the microscopic diagnoses made by Dutch GPs with those made by the pathologist, with the latter being a 'gold standard', to find out if the Dutch GPs were capable of detecting (pseudo)hyphae and blastospores when they did perform microscopy. In only about half (54) of the 104 vulvovaginal candidiasis cases the clinician agreed with the pathologist that the smear was positive for *Candida*. The GPs frequently confused hairs with (pseudo)hyphae and had great difficulty identifying the small fungal blastospores, suggesting that the results were probably mainly due to lack of experience in microscopy. Wiesenfeld *et al.*[33] confirmed that a majority of primary care clinicians do not commonly perform microscopy. In addition, Mårdh *et al.*[21] and Schaaf *et al.*[22] established that the predictive value of vaginal complaints alone such as itching, burning and white, curdy discharge is low. Other studies have shown that without the benefit of microscopy or culture as many as half of the women

routinely diagnosed with vulvovaginal candidiasis may suffer from other underlying conditions.[3,23,34,35] Not using microscopy or culture could therefore lead to an overestimated prevalence and incidence of 'vulvovaginal candidiasis'. Since vaginal complaints account for more outpatient visits than any other reason that women seek health care in The Netherlands, lack of experience in microscopy could thus lead to inappropriate treatment in numerous cases.[33,36,37] Therefore, office-based techniques to diagnose vaginitis should be better taught in medical and postgraduate education. The use of standardized staining sets to simplify the analysis of vaginal discharge and to preserve the slides for training purposes should be provided in the interest of the public's health.

Presence of asymptomatic vaginal Candida and preneoplasia
Cervical carcinoma is the second most common cancer among women worldwide.[38] Infection with putative oncogenic human papillomaviruses is thought to contribute to the development of cervical cancer.[39-42] Although HPV infection is widely prevalent, only a few infected women will eventually develop cervical cancer. This suggests that other factors are involved in malignant transformation of cervical epithelium. Inflammation of the cervix has also been proposed as one of the cofactors in cervical carcinogenesis, since disturbance of the vaginal flora is known to statistically increase the risk of acquisition of HPV infection and cervical cytological abnormalities occur more often in women with an abnormal vaginal flora than in those without this condition.[20,42-49] In chapter 4, we examined the relationship between presence of *Candida* and squamous (pre)neoplasia, by using data sets of systematically coded vaginal smears. We established that presence of *Candida* is not associated with cervical (pre)neoplasia in the same smear. This result is concurrent with findings in other studies, that show no obvious associations between vaginal *Candida* and cervical cytological abnormalities.[10,50] Thus, presence of vaginal *Candida* does not seem to be a cofactor in the development of cervical cancer, in contrast to koilocytosis, HPV

infection, and bacterial infections (such as Bacterial Vaginosis) which are associated with an increased risk of developing squamous abnormalities.[20,39-41,44,45,51-53] However, since we performed a cross-sectional study of asymptomatic women it may be impossible to infer a causal relationship between *Candida* and squamous abnormalities. Therefore, we conducted a retrospective, longitudinal, cohort study of 100,605 women who had two smears taken between 1991 and 2003 in chapter 5, whereby the first smear of these women showed normal squamous epithelium. From this group of 100,605 asymptomatic women, women with a *Candida* diagnosis (n = 1,439) were selected as the first study cohort. To be able to compare women carrying *Candida* in time with women known to have a disturbed vaginal flora, women with dysbacteriosis (n = 5,302) were selected as a second study group. The control cohort consisted of women having completely normal smears (n = 87,903). These groups were retrospectively followed in time and odds ratios for squamous abnormalities in the follow-up smear of the three cohorts were established. In this chapter, we confirm that women with dysbacteriosis, characterized by a lack of normally protective lactobacilli and an overgrowth of mainly anaerobic bacteria,[54] are at risk of developing cervical (pre)neoplasia. However, presence of *Candida* was not associated with cervical carcinogenesis. Therefore, vaginal *Candida* does not seem to be a cofactor in the development of cervical cancer. This is consistent with the general hypothesis that the local cervicovaginal milieu plays a role in susceptibility to HPV infection, since women carrying *Candida* are likely to possess a healthy *Lactobacillus* predominated vaginal flora in contrast to women with dysbacteriosis.[44,45]

Candida and susceptibility for other pathogens in asymptomatic women

The mechanism whereby *Candida* organisms transform from commensal to pathogen is not yet established. It has been suggested that the normal vaginal flora, dominated by presence of lactobacilli, is protective against C*andida* vaginitis,[55] although VVC occurs more often in women with a lactobacilli-predominated vaginal flora and is inversely correlated to a mixed anaerobic vaginal flora.[56-58] Since lactobacilli are part of the main defence mechanisms of the vagina, changes in the proportions of these *Lactobacillus* species are risk factors for receiving vaginal infections.[59-64] Therefore, we examined whether women carrying *Candida* are prone to acquire other vaginal pathogens in time compared to women known to have a disturbed vaginal flora (dysbacteriosis). In chapter 6, we conducted a retrospective, longitudinal cohort study from 104,686 women with multiple smears taken between 1991 and 2003, and selected women with a *Candida* diagnosis (n = 1,492) as a first study cohort and women with dysbacteriosis (n = 5,508) as a second study cohort. The control cohort consisted of women having a completely normal vaginal flora (n = 90,991). These three cohorts were retrospectively followed in time and the incidence of vaginal pathogens as detected in repeat smears was noted. We established that women, who initially carried *Candida*, are prone to have a subsequent smear with *Candida*. In contrast, women carrying *Candida* are not predisposed to receive other vaginal pathogens such as *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyces* in time. However, women with dysbacteriosis, lacking the normally protective lactobacilli,[54] are at risk of becoming colonized with *T. vaginalis*, *G. vaginalis* and *Actinomyces*. In contrast, women with dysbacteriosis are not prone to having a subsequent smear with *Candida*. This finding leads us to two hypotheses: first, *Candida* can only survive in the vagina when a healthy *Lactobacillus* pre-dominated flora is present. By competing for nutrients, interfering in the adherence to vaginal epithelial cells[65] and the use of bacteriocin,[66] this *Lactobacillus* flora might sustain *Candida* commensalism. Second, *Candida* can not survive in a vaginal flora

dominated by an overgrowth of anaerobic bacteria. This thought has been subscribed by other studies, which confirmed a negative correlation between presence of Bacterial Vaginosis and vaginal candidiasis.[67,68] One study even noted that bacterial amines, such as putrescine and cadaverine produced by anaerobes (e.g. *G. vaginalis*), inhibit germ tube formation by *C. albicans*.[69] However, further studies are needed to evaluate and determine the (clinical) impact of *Lactobacillus* species and anaerobes on the presence of vaginal *Candida*.

Recurrent symptomatic vulvovaginal candidiasis

Five percent of all women who experienced an initial episode of VVC will develop recurrent vulvovaginal candidiasis (RVVC) defined as 4 or more episodes of symptomatic vaginitis per year.[70] Since symptoms such as vaginal pruritus and abnormal discharge can also be due to other agents or to non-infectious causes, the diagnosis of recurrent candidiasis should always be based upon vaginal examination and microscopy.[71] However, use of a speculum may be uncomfortable for the patient and requires an office visit. Therefore, women with (recurrent) candidiasis might benefit from taking vaginal smears themselves. In chapter 7, we examined self sampled vaginal smears of women with (recurrent) symptomatic vulvovaginal candidiasis to show the presence or absence of *Candida*, lactobacilli and *Gardnerella* in stained slides. We defined candidiasis by presence of blastospores and (pseudo)hyphae in stained smears, consistent with Zdolsek *et al*.[23] It proved to be very effective to use self sampled smears to determine presence of *Candida*, since presence or absence of blastospores and (pseudo)hyphae was equally established by all three pathologists, independent of each other. This finding is in agreement with results from the study by Novikova *et al.*, who noted that recurrent episodes of *Candida* were easily detectable in self sampled methylene blue stained vaginal smears.[72] In addition, presence of lactobacilli and neutrophils could also be determined without difficulty. Another advantage of staining self-sampled slides is that staining makes

it possible to preserve the smears and to use them for teaching purposes or quality control. The largest benefit however was shown by the patients in this study: they were very enthusiastic about self-sampling, since we could show that they suffered from candidiasis without having to examine their vagina. While our initial study size was small, the use of self-sampling and standardized staining sets to simplify the analysis of vaginal discharge will be further evaluated.

We noted that all patients suffered from vaginal candidiasis on day 21 of their menstrual cycle, as did Spacek *et al.*[73] This finding might be explained by the fact that estrogen levels are elevated during the last half of the menstrual cycle, thereby providing a glycogen source necessary for multiplication of the yeast and facilitating adherence to epithelial cells.[14,15,74]

In addition, we established that presence of symptoms such as pruritus, abnormal discharge, and soreness has a rather high sensitivity but low specificity. This finding is consistent with results published by Mårdh *et al.* and Zdolsek *et al.*[21,23] Since vaginal pruritus and abnormal discharge can also be due to other infectious agents or to non-infectious causes, the diagnosis 'RVVC' should thus always be confirmed by culture or wet-smear.[71]

Lactobacilli were present in all smears of our patients, whereas adherent *Gardnerella vaginalis* (clue cells) was not seen in any of the smears. Studies by Sobel *et al.* and Hawes *et al.* confirm that women with yeast vaginitis have the same frequency and concentration of *Lactobacillus* species as women without recurrent infections.[56,57] Moreover, Zdolsek *et al.* noted that vulvovaginal candidiasis occurs more frequently in women with a lactobacilli-predominated vaginal flora, as compared to those with a flora distribution of a mixture of anaerobic and facultative anaerobic bacteria.[58] Therefore, we suggest that recurrence of VVC might not be caused by a deficiency of the normally protective vaginal bacterial flora.

In addition, we noted presence of polymorphonuclear neutrophils in 16 out of the 27 smears with *Candida*. Fidel *et al.* also found that VVC

correlates with a vaginal infiltration of polymorphonuclear neutrophils, whereas protection against *Candida* infection appeared non-inflammatory.[75,76] These results could indicate that the symptoms of women with (R)VVC are caused by an aggressive innate response of the body (or genital organs), possibly due to chemicals produced by the fungi, instead of being caused by a shift in the healthy vaginal flora. However, larger randomized controlled trials are needed to address this hypothesis.

Asymptomatic versus symptomatic women with Candida
Women in The Netherlands between the ages of 30 and 60 years are invited once every 5 years to be tested for cervical cancer, a program that has continued since its inception in 1996.[81] In this thesis we established that a number of these asymptomatic women screened for cervical cancer also have *Candida* in their smears. This confirms other studies reporting that *Candida* can be isolated from the genital tract of asymptomatic women during their fertile years.[3,4,8,9] The natural history of asymptomatic colonization with *Candida* is unknown, but it is supposed that *Candida* gains access to the vaginal lumen from the adjacent perianal area.[4] Other mechanisms whereby *Candida* may reach the vagina might include the fingers, male genitalia, and inert objects such as contraceptive diaphragms.[4] However, presence of vaginal *Candida* alone is not a sufficient criterion for diagnosing vulvovaginal candidiasis, since the clinical entity is based on symptomatic vaginitis (e.g. pruritus, discharge) and the finding of *Candida* (pseudo)hyphae and/or spores in a wet mount or culture of the vaginal discharge.[21-23] The exact mechanism by which *Candida* organisms become problematic or symptomatic is not yet fully established. Presence of risk factors, such as changes in the host vaginal environment and virulence factors expressed by strains of *C. albicans* have all been suggested to contribute to the transition from commensalism to pathogenicity. In this thesis we have not addressed the question of why some women carry *Candida*

without developing symptomatic vaginitis, while others suffer from VVC. We did, however, establish differences in ethnicity among asymptomatic women carrying *Candida,* implying that differences in cultural behaviour may affect the vaginal flora and might influence the presence of asymptomatic *Candida.* We also suggest that recurrence of VVC might not be due to an alteration in the normally protective vaginal bacterial flora, but perhaps to an abnormal response of the body (to metabolic products released by the fungi). Larger randomized controlled studies may clarify this hypothesis.

General conclusions

1. The overall (asymptomatic) baseline prevalence of *Candida* in the western region of The Netherlands is 13 per 1000 women, decreasing with increasing age.

2. The RR of having *Candida* is significantly higher for asymptomatic Surinamese women compared to other groups of immigrants and indigenous Dutch women.

3. Dutch GPs diagnose (recurrent) vulvovaginal candidiasis based on empiricism rather than diagnostic procedures. In addition, it is difficult for Dutch GPs to recognize blastospores and (pseudo)hyphae in stained smears of symptomatic women.

4. Presence of vaginal *Candida* in asymptomatic women is not associated with an increased risk for (pre)neoplastic cells in the same smear and in follow-up smears.

5. Presence of vaginal *Candida* does not predispose to an increased susceptibility for *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyces* overgrowth.

6. Recurrent vulvovaginal candidiasis is associated with presence of lactobacilli and polymorphonuclear neutrophils in the vaginal smear, but with the absence of *Gardnerella vaginalis.*

7. Cytology (using self sampled vaginal smears) provides an excellent diagnostic tool in establishing and monitoring (recurrent) vulvovaginal candidiasis.

Future perspectives

We have established that Dutch GPs report large numbers of patients suffering from VVC annually, but without performing adequate diagnostic procedures such as vaginal examination, microscopy and culture on selective media to confirm their diagnosis. In addition, we noted that when Dutch GPs do perform microscopy, they often cannot recognise (pseudo)hyphae and blastospores. Without performing the necessary diagnostic procedures there may be an overestimated prevalence and incidence of 'vulvovaginal candidiasis', since the predictive value of subjective vaginal complaints such as itching, burning and white, curdy discharge is low. We believe that office-based techniques to diagnose vaginitis should therefore be better taught in medical and postgraduate education. We also found that conventional cytology is an excellent means to diagnose vulvovaginal candidiasis, since it has the advantage of providing a permanent record of the patient's vaginal flora, yields comparable results when performed by different cytopathologists, and is a reproducible value. The use of standardized stained slide reference sets to simplify the analysis of vaginal discharge and to preserve the material for training purposes should be instituted in workshops and outreach programs for clinicians.

We also discovered that women lacking the normally protective H_2O_2-producing lactobacilli are more likely to develop (other) infections and cervical (pre)neoplasia with time, whereas women carrying *Candida* are not. This finding is consistent with the general hypothesis that the local cervicovaginal milieu may play a role in susceptibility to (HPV) infection, or that women carrying *Candida* are likely to possess a healthy *Lactobacillus* predominated vaginal flora in contrast to women with dysbacteriosis. In addition, we established that women with

dysbacteriosis are unlikely to have a subsequent smear with *Candida* over time and vice versa. Furthermore, *Gardnerella vaginalis* associated clue cells were not seen in any of the examined patients with recurrent candidiasis. These findings suggest that *Candida* can not survive in a vaginal flora dominated by an overgrowth of anaerobic bacteria. However, further studies are needed to examine whether Bacterial Vaginosis and vulvovaginal candidiasis are indeed more or less exclusive of each other. PCR of 16S RNAs might provide an excellent tool to map the vaginal flora and to determine the (clinical) impact of presence of vaginal *Candida* and of anaerobes on the vaginal flora. Future research should also be directed towards the protective role of lactobacilli and the mechanism whereby *Candida* transforms from commensal to pathogen.

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Vulvovaginal *Candida*:

a study of (a)symptomatic women

APPENDIX

Summary

The research described in this thesis concerns presence of asymptomatic vaginal *Candida* and vulvovaginal candidiasis. Vulvovaginal candidiasis (VVC) is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract. It is a frequent diagnosis in the daily practice of gynaecology and accounts for large numbers of visits to general practices in The Netherlands. Acute vulvar pruritus and vaginal discharge are the usual presenting complaints, but neither symptom is specific to VVC. Vulvovaginal examination frequently reveals epithelial erythema and swelling of the labia and vulva, together with adherent whitish discharge. Due to lack of specificity of signs and symptoms all patients with symptomatic vaginitis should be diagnosed on the basis of microscopic examination of their vaginal secretions. However, *Candida* (pseudo)hyphae and/or blastospores are also often discovered in cervical smears of asymptomatic women during their childbearing years.

Nationwide cytological screening for cervical cancer and its precursor lesions has been introduced in The Netherlands in 1988. Since 1996 all women between the ages of 30 and 60 receive a letter of invitation once every five years to have a cervical smear taken, usually made by their own general practitioner. These smears are coded according to the Dutch national coding system for cervical cytology (KOPAC). KOPAC is an acronym: the letters K (kompositie = composition), O (ontstekingsverschijnselen = inflammation), P (plaveiselepitheel = squamous epithelium), A (andere afwijkingen endometrium = other abnormalities endometrium) and C (cylinderepitheel endocervix – endocervical cylindrical epithelium) are used to indicate the composition and morphology of the smears. The O-category consists of nine different subgroups: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis (O3), *Candida* (O4), *Gardnerella vaginalis* (O5), no inflammatory changes (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) and non-specific changes (O9). The P-category is also subdivided into 9 categories: P1 stands for normal or benign squamous epithelium, P2-3

for ASCUS (borderline changes), P4 for mild dysplasia, P5 for moderate dysplasia, P6 for severe dysplasia, P7 for carcinoma in situ, P8 for microinvasive carcinoma and P9 for macroinvasive squamous cell carcinoma. All diagnoses of P5 or higher generate to a referral to the hospital for a colposcopic examination and if required a biopsy. Parts of the research described in this thesis involve cervical smears that were taken as part of the national screening program in the western region of The Netherlands and sent to the Leiden Cytology and Pathology Laboratory (LCPL) for KOPAC-analysis. The Leiden database was used to study possible relationships between presence of asymptomatic *Candida*, immigrant status, (pre)neoplasia, and the incidence of acquiring other vaginal infections in time, by the cytological identification of *Candida* (O4) and other elements of the vaginal flora in smears.

An introduction to and the rationale for the studies in this thesis are provided in chapter 1.

In chapter 2 we examined how general practitioners (GPs) in The Netherlands diagnose and treat vaginal candidiasis, by sending questionnaires to 1160 Dutch GPs. On average, the GPs reported 105.6 consultations concerning vaginal candidiasis per practice per year. Only 61 (16.1%) Dutch GPs always or often performs microscopy when diagnosing candidiasis, while 143 (37.6%) GPs never use a microscope to confirm their diagnosis. Furthermore, only 30 (7.9%) GPs take *Candida* cultures regularly, whereas 154 GPs (40.5%) never take a vaginal swab to diagnose acute candidiasis. Treatment of choice is mostly miconazol (50%) or clotrimazol (24%). This leads to the conclusion that GPs often diagnose 'vulvovaginal candidiasis' in their practices, but often do not perform the laboratory examinations required to confirm their putative diagnosis, which could lead to wrong diagnoses and maltreatment with antimycotics, without cure of the patients' vaginal complaints.

Chapter 3 describes the accuracy of microscopic diagnoses of vaginal discharge made by Dutch general practitioners (GPs). The GPs performed microscopy of 324 stained vaginal smears of patients with symptomatic vaginitis and noted presence or absence of (pseudo)hyphae and blastospores. These smears were then sent to the pathologist for confirmation of the microscopic diagnosis of the GPs, whereby the cytological diagnosis of the pathologist was considered the 'gold' standard. In 104 of the 342 cases *Candida* was established by the pathologist. Sensitivity and specificity of the microscopic diagnoses of the clinicians were 52% and 89% respectively. The most frequent reason for a false positive diagnosis was presence of hairs, whereas the most frequent reason for a false negative diagnosis was understaining of the smear. This study shows that even in stained smears it is difficult for GPs to recognize blastospores and/or (pseudo)hyphae.

In chapter 4 we examined the relationship between *Candida vaginalis* and (pre)neoplasia, and the prevalence of *Candida* and (pre)neoplasia related to age and ethnicity. Data were collected from almost 500,000 asymptomatic women invited for mass screening between 1995 and 2002. Prevalence and relative risks were established for *Candida* and squamous abnormalities in Dutch women and four groups of immigrants. The prevalence of *Candida* was significantly higher in the cohort of 30 year old women and lower in the cohorts of 45, 50, 55 and 60 year old women. In addition, the relative risk (RR) of having *Candida* was higher for Surinamese women (1.24; CI 1.08-1.42). No statistically significant relationship between (pre)neoplasia and *Candida* was observed. Presence of *Candida* is therefore not associated with an increased risk for squamous abnormalities in the same smear.

The purpose of chapter 5 was to examine whether presence of vaginal *Candida* or dysbacteriosis predisposes to an increased susceptibility for (pre)neoplasia in time. In women with dysbacteriosis, an overgrowth of

coccoid bacteria and an almost complete absence of *Lactobacillus* forms are seen in cervical smears, whereas presence of *Candida* is often accompanied by a normal *Lactobacillus* flora. A retrospective, longitudinal, cohort study was performed, conducted from more than 100,000 women, who had two smears taken over a period of twelve years as part of the Dutch cervical screening program, whereby the first smear of these women showed normal squamous epithelium. From these women, a cohort of 1,439 women with *Candida* and a cohort of 5,302 women with dysbacteriosis were selected as two separate study groups. The control group consisted of women who had completely normal cervical smears (n = 87,903). These groups were retrospectively followed in time, with an interval of 4.0 (+ 1.7) years between the first and second smear. The dysbacteriotic cohort was significantly more likely to have low- and high-grade squamous intraepithelial lesions (LSIL and HSIL) including carcinoma in the follow-up smear compared to the control group (ORs 1.85; CI 1.28-2.67 and 2.00; CI 1.31-3.05 respectively). In contrast, the *Candida* cohort had no significantly increased or decreased chance to have SIL. The equivocal diagnosis 'ASCUS' was rendered significantly more often in the follow-up smear of both study cohorts (OR *Candida* cohort 1.42; CI 1.03-1.95; OR dysbacteriotic cohort 1.44; CI 1.22-1.71). In this study we show that presence of *Candida vaginalis* is not associated with an increased risk for SIL in time. In contrast, women with dysbacteriosis do have a significantly increased chance to develop (pre)neoplastic changes. These findings could be taken into account in further research concerning the predisposing factors for cervical carcinogenesis.

Chapter 6 was used to obtain insight into the consequences of a smear with *Candida* and the incidence of acquiring other vaginal infections as detected in repeat smears by performing a retrospective, longitudinal, cohort study. From more than 100,000 women, who had multiple smears taken over a period of twelve years as part of the Dutch cervical

screening program, women with *Candida* in their cervical smear were selected as the first study cohort (n = 1,492). In addition, 5,508 women with a dysbacteriotic flora (lacking lactobacilli) were selected and formed the second study cohort. The control cohort consisted of women who had a normal vaginal flora (n = 90,991). These three cohorts were retrospectively followed in time. *Candida* occurred over 5 to 6 times more often in the first and second repeat smear of the *Candida* cohort compared to the control cohort. No other statistically significant differences between the *Candida* cohort and the control cohort were noted. However, in the dysbacteriotic women, *T. vaginalis*, *G. vaginalis* and *Actinomyces* were more frequently encountered in both repeat smears. This subscribes the thought that women carrying *Candida* possess a healthy vaginal flora, dominated by presence of lactobacilli, which protects their vagina from other infective agents.

In chapter 7 we evaluated the association between the bacterial flora and *Candida* overgrowth in symptomatic women. In a prospective cohort study, 10 women with recurrent vulvovaginal candidiasis (RVVC) were taught how to prepare vaginal smears of their own vaginal fluids on day 7, day 14, day 21 and day 28. These air dried smears were sent to the laboratory with self reporting forms of vaginal symptoms on the four sampling days. The 40 smears were stained with the PAS-method and examined by three different cytopathologists for presence of *Candida*. Thereafter, the smears were restained with Giemsa-stain to determine presence of lactobacilli, *Gardnerella vaginalis* ('clue cells') and neutrophils. All three cytopathologists unequivocally established *Candida* blastospores and (pseudo)hyphae in 27 out of the 40 PAS-stained vaginal smears, whereas in the remaining 13 smears *Candida* was not found, indicating that self sampled smears form a reliable means to establish presence of *Candida* in symptomatic patients with candidiasis. All 10 patients had *Candida* in their smears during the second half of their menstrual cycle. Neutrophils were seen in 16 of the 27 smears positive for *Candida*.

Lactobacilli were present in all smears of the RVVC patients, but *Gardnerella vaginalis* was not seen. The sensitivity of complaints for the presence of *Candida* was 89%, but the specificity only 31% and the positive predictive value 57%.

Finally, the findings of the studies described in this thesis are discussed in chapter 8. Data in this thesis do not lead to one general conclusion, but do show the beneficial effect of cytology in determining the presence of vulvuvaginal *Candida* in symptomatic and asymptomatic women. Evaluation of cytology of (stained) smears yields comparable results when performed by different cytopathologists. In addition, besides (pseudo)hyphae and/or blastospores, presence of lactobacilli and neutrophils can also be determined without difficulty. Another advantage of using (stained) smears to establish presence of *Candida* is that staining makes it possible to preserve the smears and to use them for teaching purposes or quality control.

In this thesis, we noted that Dutch GPs diagnose (recurrent) vulvovaginal candidiasis on the basis of gut feelings rather than adequate diagnostic procedures. In addition, we found that it is difficult for Dutch GPs to recognize blastospores and (pseudo)hyphae in stained smears of symptomatic women. Furthermore, presence of vaginal *Candida* in asymptomatic women is not associated with an increased risk for (pre)neoplastic cells in the same smear and in follow-up smears and does not predispose to an increased susceptibility for *Trichomonas vaginalis*, *Gardnerella vaginalis* and *Actinomyces*. Finally, we noted that recurrent vulvovaginal candidiasis is associated with presence of lactobacilli and polymorphonuclear neutrophils in the vaginal smear, but with absence of *Gardnerella*. Future research should focus on the effect of *Candida* and dysbacteriosis on the vaginal ecosystem and the relationship between the vaginal flora and the development of cervical (pre)neoplasia.

Samenvatting

Dit proefschrift gaat over (symptomatische) vulvovaginale candidiasis en over de asymptomatische aanwezigheid van *Candida* in de vagina. Vulvovaginale candidiasis (VVC) wordt veroorzaakt door een abnormale groei van gisten in de mucosa van de vagina en vulva en wordt in de volksmond 'vaginale schimmelinfectie' genoemd. Het is een diagnose die geregeld gesteld wordt in de Nederlandse huisartsenpraktijk en die vaak gepaard gaat met hinderlijke jeukklachten en toegenomen witte, brokkelige vaginale afscheiding. Bij lichamelijk onderzoek is meestal een rode, wat gezwollen vagina (en vulva) waarneembaar, samen met plakkerige, witte afscheiding. Omdat deze symptomen en tekenen weinig specifiek zijn voor een schimmelinfectie, moet de diagnose VVC altijd gebaseerd zijn op (microscopisch) onderzoek van de vaginale afscheiding. Microscopie van een direct preparaat, gemaakt door een druppel vaginale afscheiding te mengen met een druppel 10% kaliumhydroxide, vertoont (pseudo)hyphae en blastosporen als sprake is van een schimmelinfectie. Echter, *Candida* (pseudo)hyphae en/of sporen zijn behalve tijdens microscopisch onderzoek van de afscheiding van vrouwen met een schimmelinfectie ook met enige regelmaat terug te vinden in baarmoederhalsuitstrijkjes van asymptomatisch vrouwen. Landelijke cytologische screening voor baarmoederhalskanker en voorloperlaesies vindt in Nederland plaats sinds 1988. Sinds 1996 ontvangen alle vrouwen in Nederland tussen dertig en zestig jaar een schriftelijke uitnodiging om een uitstrijkje te laten maken door hun eigen huisarts. Deze uitstrijkjes worden gescreend en gecodeerd volgens het Nederlandse coderingssysteem voor cervixcytologie (KOPAC). KOPAC is een acroniem, waarbij de vijf letters staan voor: K (kompositie van het uitstrijkje), O (ontstekingsverschijnselen), P (plaveiselepitheel), A (andere afwijkingen endometrium) en C (cylinderepitheel endocervix). Deze letters worden gebruikt om de compositie en morfologie van de uitstrijkjes te beschrijven. De O-categorie bestaat uit negen verschillende subgroepen: koilocytosis (O1), *Trichomonas vaginalis* (O2), dysbacteriosis

(O3), *Candida* (O4), *Gardnerella vaginalis* (O5), geen onsteking (O6), *Actinomyces* (O7), *Chlamydia trachomatis* (O8) en aspecifieke ontsteking (O9). De P-categorie wordt ook onderverdeeld in 9 subklasses: P1 staat voor normaal of benigne plaveisel epitheel, P2 voor abnormale plaveiselcellen, P3 voor atypische squameuze metaplasie, P4 voor milde dysplasie, P5 voor matige dysplasie, P6 voor ernstige dysplasie, P7 voor carcinoma in situ, P8 voor microinvasief carcinoom en P9 voor macroinvasief plaveiselcelcarcinoom. Alle diagnoses vanaf P5 of hoger worden doorverwezen naar het ziekenhuis voor colposcopisch onderzoek en zo nodig een biopsie.

Delen van de studies beschreven in dit proefschrift gaan over de uitstrijkjes, die werden afgenomen in het westen van Nederland als onderdeel van het bevolkingsonderzoek en verstuurd naar Leiden voor KOPAC-analyse. De Leiden database is gebruikt om mogelijke relaties tussen aanwezigheid van asymptomatische *Candida*, etniciteit, leeftijd, (pre)neoplasie en de kans op het oplopen van andere vaginale infecties in de tijd te onderzoeken.

In hoofdstuk 1 wordt een algemene inleiding op het onderzoek beschreven en is uiteengezet wat de motieven zijn om de studies uit te voeren.

In hoofdstuk 2 wordt onderzocht hoe Nederlandse huisartsen de diagnose 'vaginale schimmelinfectie' stellen, door enquêtes te versturen naar 1160 huisartsen in de regio Leiden. Gemiddeld genomen vermelden de aangeschreven huisartsen ongeveer 106 consulten betreffende schimmelinfecties per praktijk per jaar. Slechts 61 (16.1%) van de ondervraagde Nederlandse huisartsen verrichten altijd microscopie van de vaginale afscheiding om de diagnose VVC te kunnen stellen, terwijl 143 (37.6%) huisartsen aangeven, dat zij nooit een microscoop hanteren om de door hun gestelde diagnose te bevestigen. Bovendien nemen slechts 30 (7.9%) huisartsen met enige regelmaat (cervix) kweken af,

terwijl 154 (40.5%) huisartsen nooit kweken afnemen om VVC aan te tonen. Hieruit concluderen wij dat huisartsen regelmatig 'vulvovaginal candidiasis' diagnosticeren in hun praktijk op basis van hun klinische blik, in plaats van gebruik te maken van microscopie of kweek om hun diagnose te bevestigen niet verrichten. Uit onderzoek is echter gebleken dat subjectieve klachten en tekenen, zoals vaginale jeuk en toegenomen afscheiding, ook voorkomen bij allerlei andere vormen van (niet) infectieuze vaginitis. Dit zou kunnen betekenen dat huisartsen regelmatig de verkeerde diagnose stellen en dientengevolge de verkeerde behandelingen starten, waardoor de patiënte niet van haar klachten afkomt.

Hoofdstuk 3 beschrijft een onderzoek naar de betrouwbaarheid van diagnostiek van schimmelinfecties. In gekleurde preparaten van afscheiding van vrouwen met een schimmelinfectie zijn *Candida* (pseudo)hyphen en/of blastosporen beter zichtbaar dan in het directe preparaat. De analyse is gebaseerd op 342 casussen, waarbij de microscopie van de gekleurde uitstrijkjes werd uitgevoerd door Nederlandse huisartsen. Deze uitstrijkjes werden vervolgens naar de patholoog gestuurd voor bevestiging dan wel ontkenning van de microscopische waarnemingen/diagnoses van de huisartsen. De sensitiviteit en specificiteit van de microscopische diagnoses van de huisartsen waren respectievelijk 52% and 89%. Deze studie laat zien dat het voor huisartsen zelfs in gekleurde uitstrijkjes moeilijk is om blastospores en/of (pseudo)hyphae te herkennen.

In hoofdstuk 4 wordt verslag gedaan van de analyse van een databestand van ongeveer 500.000 uitstrijkjes, waarbij wordt gekeken naar het verband tussen aanwezigheid van *Candida* en (pre)neoplasie, gekoppeld aan leeftijd en etniciteit. Uit het onderzoek blijkt dat de kans op het hebben van een uitstrijkje met *Candida* afneemt met de leeftijd. Bovendien hebben Surinaamse vrouwen een verhoogd relatief risico op

het hebben van *Candida* in een uitstrijkje (RR 1.24; CI 1.08-1.42). Na uitgebreid onderzoek blijkt er geen relatie te zijn tussen de aanwezigheid van *Candida* en het hebben van plaveiselcelafwijkingen in hetzelfde uitstrijkje.

In hoofdstuk 5 wordt een retrospectieve studie beschreven, gebaseerd op de gegevens van ruim 100.00 vrouwen, die twee uitstrijkjes hebben laten maken in het kader van het bevolkingsonderzoek over een periode van twaalf jaar. Doel van de studie was om te onderzoeken of de aanwezigheid van vaginale *Candida* dan wel dysbacteriose in de tijd leidt tot een verhoogde kans op de ontwikkeling van voorloperstadia van baarmoederhalskanker. Bij vrouwen met dysbacteriose kan een overgroei van coccoide bacteriën en een afwezigheid van beschermende lactobacillen worden waargenomen in de baarmoederhalsuitstrijkjes, terwijl *Candida* over het algemeen gepaard gaat met aanwezigheid van beschermende lactobacillen. Een groep vrouwen met *Candida* (n = 1,439) en een groep vrouwen met dysbacteriose (n = 5,302) werd geselecteerd en vergeleken met een compleet gezonde controle cohort (n = 87,903). De uitkomsten laten zien dat de groep vrouwen met dysbacteriose in de eerste uitstrijk significant meer kans hadden op het ontstaan van plaveiselcelafwijkingen in de follow-up uitstrijk vergeleken met de vrouwen van de controle groep, terwijl de groep vrouwen met *Candida* geen verhoogde kans had op het ontstaan van plaveiselcelafwijkingen in het vervolg uitstrijkje.

In hoofdstuk 6 wordt gekeken naar de consequenties van een uitstrijkje met *Candida* en het oplopen van andere infecties in de tijd, door middel van een retrospectief onderzoek. Van meer dan 100.000 vrouwen, die meer dan 1 uitstrijkje hadden laten maken in het kader van het bevolkingsonderzoek over een periode van twaalf jaar, werden vrouwen met *Candida* (n = 1,492) in hun eerste uitstrijkje geselecteerd als het eerste cohort. Vrouwen met dysbacteriose (afwezigheid van

lactobacillen) in het eerste uitstrijkje (n = 5,508) werden geselecteerd als het tweede cohort. Het controle cohort bestond uit vrouwen met een compleet normale vaginale flora (n = 90,991). De uitkomsten tonen dat *Candida* 5 tot 6 keer vaker voorkomt in de vervolguitstrijkjes van de vrouwen met *Candida* ten opzichte van het controle cohort. Geen andere statistisch significante verschillen werden waargenomen tussen de *Candida* vrouwen en het controle cohort. Echter, *T. vaginalis*, *G. vaginalis* en *Actinomyces* werden significant vaker waargenomen in de vervolg uitstrijkjes van de vrouwen met dysbacteriose. Dit onderschrijft de gedachte dat vrouwen die *Candida* bij zich dragen een gezonde vaginale flora hebben, gedomineerd door aanwezigheid van lactobacilli, die hun vagina beschermt tegen andere infecties.

In hoofdstuk 7 evalueren we de bacteriële flora en *Candida* overgroei in symptomatische vrouwen. In een prospectieve studie wordt 10 patiënten met herhaalde vulvovaginale candidiasis (RVVC) uitgelegd hoe ze uitstrijkjes moeten maken van hun eigen afscheiding op dag 7, dag 14, dag 21 en dag 28 van hun menstruele cyclus. Deze aan de lucht gedroogde vaginale uitstrijkjes worden naar het laboratorium gezonden met bijgaand een formulier waarop patiënten hun klachten tijdens de cyclus konden noteren. De veertig uitstrijkjes werden gekleurd volgens de PAS-methode en beoordeeld door drie verschillende cytopathologen op de aanwezigheid van *Candida*. Daarna werden de uitstrijkjes opnieuw gekleurd, ditmaal volgens de Giemsa-kleuring om de aanwezigheid van lactobacilli, *Gardnerella vaginalis* ('clue cellen') en neutrofiele granulocyten te kunnen noteren. Alle drie cytopathologen constateerden onafhankelijk van elkaar *Candida* blastospores en (pseudo)hyphae in 27 van de 40 PAS-gekleurde vaginale uitstrijkjes, terwijl de overgebleven 13 uitstrijkjes volgens alle drie pathologen geen *Candida* bevatten. Dit toont aan dat zelf afgenomen uitstrijkjes goed kunnen worden gebruikt om de aanwezigheid van *Candida* aan te tonen. Verder hadden alle 10 patienten *Candida* in het uitstrijkje dat was afgenomen op de 21e cyclusdag.

Daarnaast werden neutrofiele granulocyten waargenomen in 16 van de 27 uitstrijkjes positief voor *Candida*. Lactobacilli waren aanwezig in alle uitstrijkjes van de RVVC patienten, maar *Gardnerella vaginalis* werd niet eenmaal gezien. De sensitiviteit van klachten voor de aanwezigheid van *Candida* was 89%, maar de specificiteit was slechts 31% en de positief voorspellende waarde 57%.

In hoofdstuk 8 wordt een overzicht gegeven van alle onderzoeksresultaten en worden enkele suggesties gedaan voor vervolgonderzoek. De resultaten in dit proefschrift leiden niet tot één uiteindelijke conclusie, maar laten het voordelige effect zien van cytologie op het vaststellen van de aanwezigheid van vulvovaginale *Candida* in symptomatische en asymptomatische vrouwen. Evaluatie van cytologie van (gekleurde) uitstrijkjes levert eenduidige en reproduceerbare resultaten op, wanneer het wordt uitgevoerd door verschillende cytopathologen. Bovendien kan naast de aanwezigheid van (pseudo)hyphae en/of blastosporen, ook de aanwezigheid van lactobacilli en neutrofiele granulocyten zonder problemen worden vastgesteld. Een ander voordeel van het gebruik van (gekleurde) uitstrijkjes om de aanwezigheid van *Candida* vast te stellen, is dat kleuring het mogelijk maakt om de uitstrijkjes te bewaren en te gebruiken voor onderwijsdoeleinden of kwaliteitscontrole.

In dit proefschrift hebben we verder opgemerkt dat Nederlandse huisartsen (herhaalde) vulvovaginale candidiasis diagnosticeren op basis van hun klinische blik in plaats van adequate diagnostische procedures. Bovendien hebben we bemerkt dat het voor Nederlandse huisartsen lastig is om blastosporen en (pseudo)hyphae te herkennen in (gekleurde) uitstrijkjes van symptomatische vrouwen. Daarnaast is de aanwezigheid van vaginale *Candida* in asymptomatische vrouwen niet geassocieerd met een verhoogd risico op plaveiselcelafwijkingen in hetzelfde uitstrijkje en in vervolg uitstrijkjes. Ook geeft aanwezigheid van vaginale *Candida* geen verhoogd risico op een uitstrijkje met *Trichomonas vaginalis*, *Gardnerella*

vaginalis of *Actinomyces* in de toekomst. Als laatste noteren we dat herhaalde vulvovaginale candidiasis geassocieerd is met de aanwezigheid van lactobacilli en neutrofiele granulocyten in de afscheiding, maar met afwezigheid van *Gardnerella vaginalis*. Toekomstig onderzoek zou zich moeten richten op het effect van *Candida* en dysbacteriose op het vaginale ecosysteem en de relatie tussen de vaginale flora en het ontstaan van plaveiselcelafwijkingen.

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List of publications

Engberts MK, Korporaal H, Vinkers M, van Belkum A, van Binsbergen JJ, Helmerhorst ThJM, van der Meijden WI. Vulvovaginal candidiasis: diagnostic and therapeutic approaches used by Dutch general practitioners. Submitted for publication

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Engberts MK, Verbruggen BSM, van Haaften M, Boon ME, Heintz APM. *Candida* and dysbacteriosis: a cytological, population-based study of 100,605 asymptomatic women concerning cervical carcinogenesis. Submitted for publication

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Engberts MK, Verbruggen BSM, van Haaften M, Boon ME, Heintz APM. Symptomatic candidiasis: using self sampled vaginal smears to establish the presence of *Candida*, lactobacilli, and *Gardnerella vaginalis*. Submitted for publication

Curriculum Vitae

Maria Karin (Marian) Engberts werd op 18 juli 1978 geboren te Gasselte. In 1996 behaalde zij haar diploma aan het Gymnasium Celeanum te Zwolle. In september 1996 begon zij aan de studie Farmacie aan de Rijksuniversiteit Groningen, maar gelukkig werd zij in mei 1997 ingeloot voor de studie Geneeskunde in Groningen. Tijdens haar studie Geneeskunde was zij onder meer actief binnen de medische faculteitsvereniging Panacea, waar zij in 1999 de rol van secretaris vervulde binnen het bestuur. Daarnaast verbleef zij in 2001 een half jaar in Nieuw-Zeeland, alwaar zij haar wetenschappelijke stage verrichtte in het Starship Children's hospital te Auckland. Zij behaalde in 2003 haar artsexamen na het afronden van haar keuze co-schap obstetrie/gynaecologie in het Diakonessenhuis te Utrecht. Na het afsluitende artsexamen verhuisde zij naar Utrecht en begon in december 2003 als AGNIO obstetrie/gynaecologie, wederom in het Diakonessenhuis (opleider: dr. M. van Haaften). In mei 2004 werd zij fertiliteitsarts en startte zij met haar promotieonderzoek onder leiding van professor dr. A.P.M. Heintz. Het onderzoek werd in nauwe samenwerking uitgevoerd met het Leids Cytologisch en Pathologisch Laboratorium te Leiden (begeleider: mw dr. M.E. Boon, directeur). Marian begint binnenkort aan haar opleiding tot gynaecoloog binnen het cluster van het Utrechts Medisch Centrum te Utrecht.
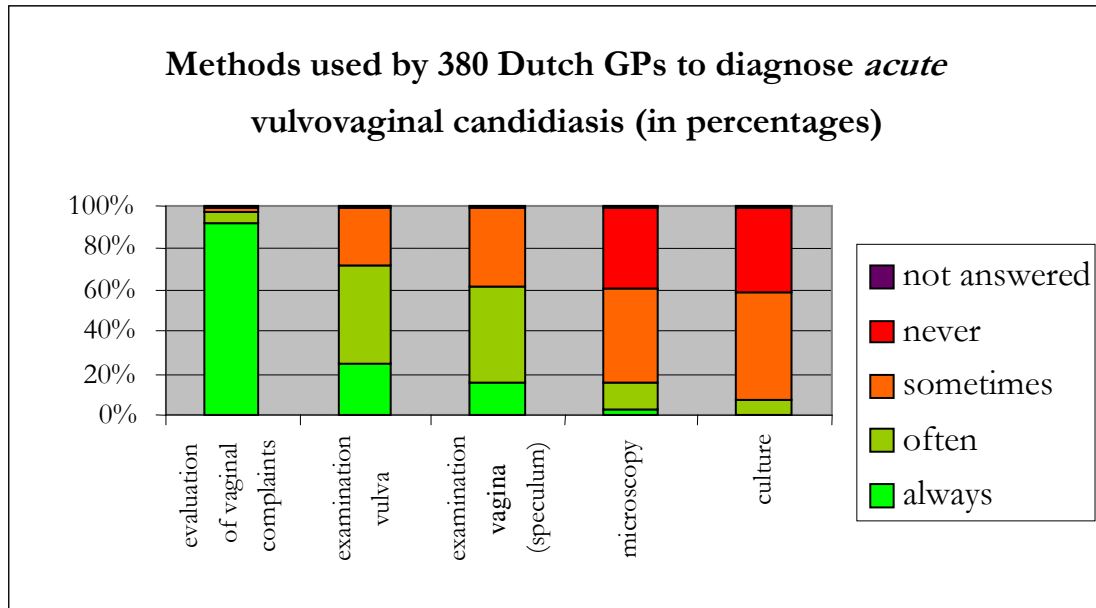
Figure 1, Chapter 2: Methods used by 380 Dutch GPs to diagnose *acute* vulvovaginal candidiasis.
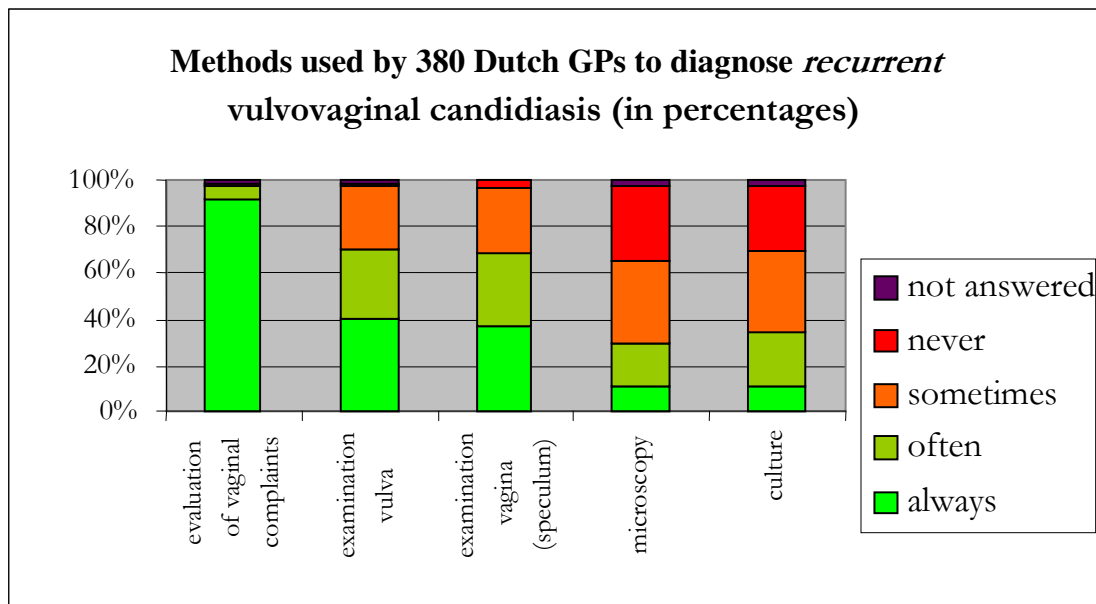
Figure 2, Chapter 2: Methods used by 380 Dutch GPs to diagnose *recurrent* vulvovaginal candidiasis.
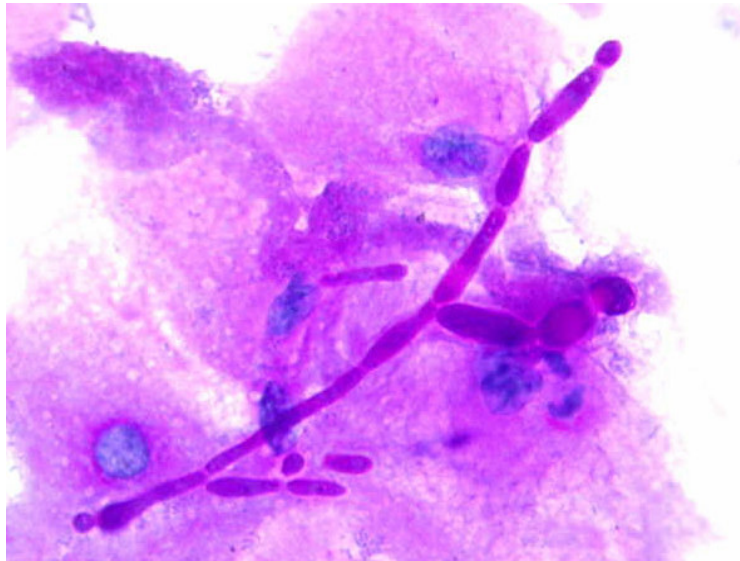
Figure 1, Chapter 3: Presence of *Candida* (pseudo)hyphae and a few blastospores (red) as established by the pathologist in a smear restained by the PAS-method (original magnification: 1000 times).
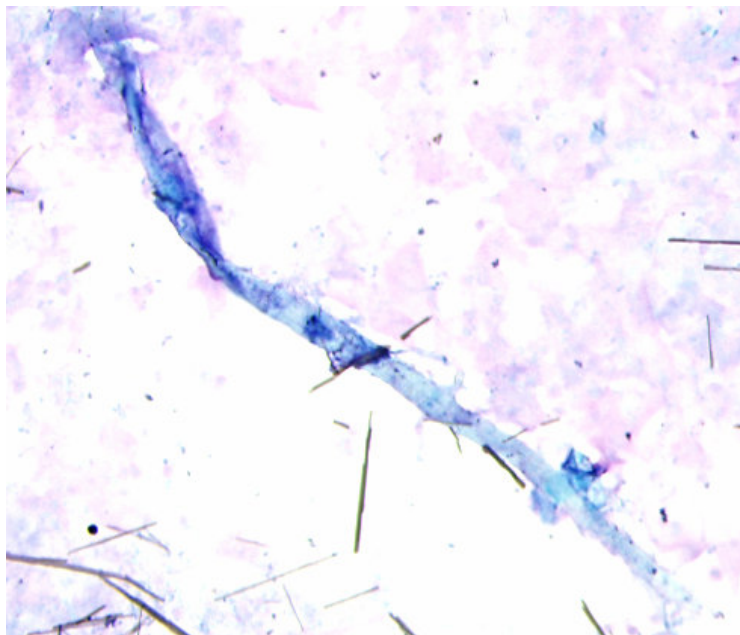

Figure 2, Chapter 3: Presence of a hair (blue) in a Bioclin-stained vaginal smear. The GP mistook this hair for (pseudo)hyphae. Note that this hair is over one hundred times larger than a fungus. Moreover, there is a twist in the structure, indicating that we are dealing with a curled hair, probably from the genital area. Furthermore, crystals (black) are present due to aging (>1 year) of the Bioclin staining solution (original magnification: 200 times).
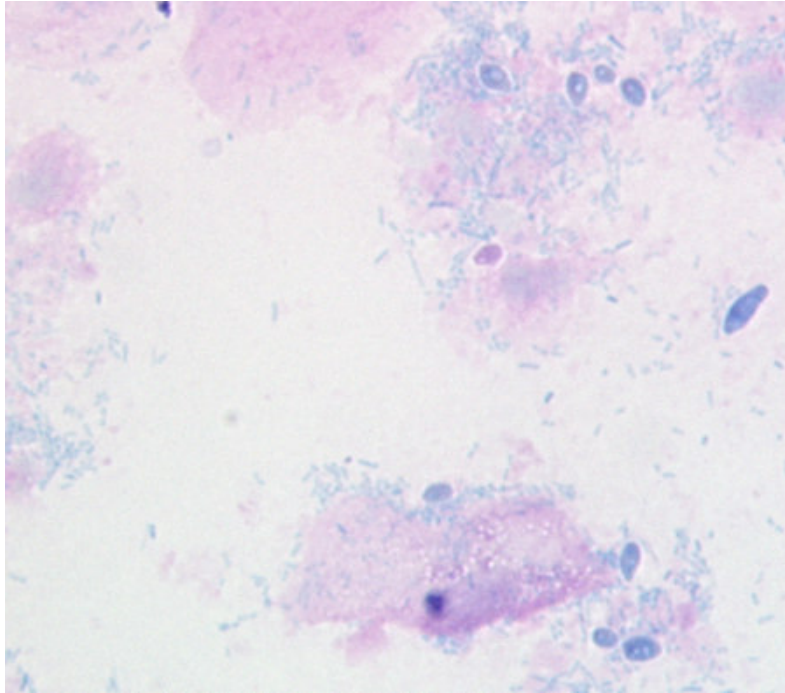
Figure 3, Chapter 3 (Case): Presence of small blastospores and lactobacilli (blue) in a Bioclin-stained vaginal smear. The GP failed to notice these blastospores, although they were present in large numbers in one part of the slide (original magnification: 1000 times).
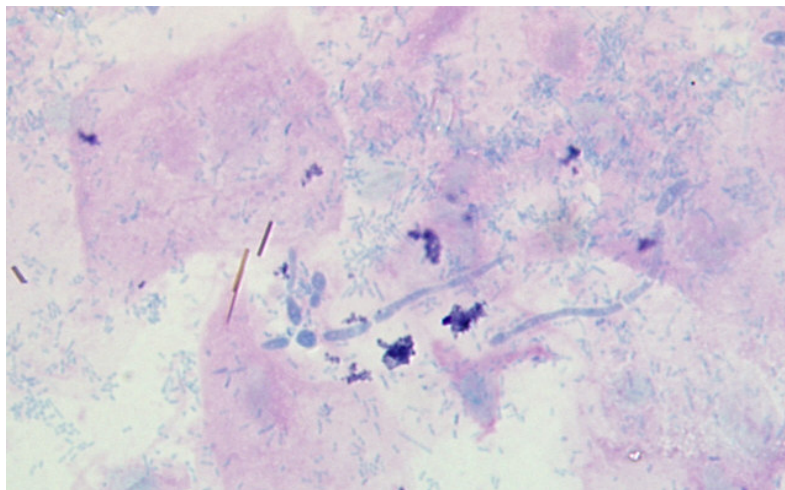
Figure 4, Chapter 3 (Case): Presence of only two (pseudo) hyphae (blue) in a Bioclin-stained vaginal smear, which the GP failed to notice (original magnification: 1000 times).
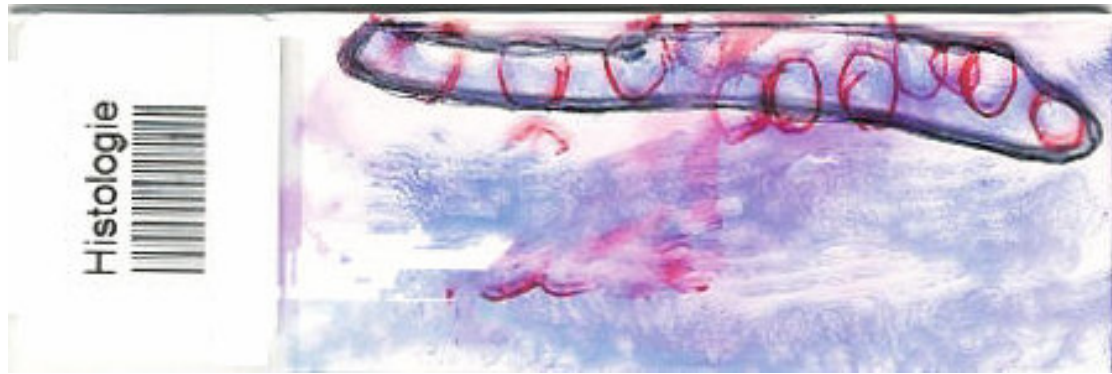
Figure 5, Chapter 3 (Case): The Bioclin-stained vaginal smear. The blastospores and short (pseudo)hyphae were found exclusively in the marked area of the smear.
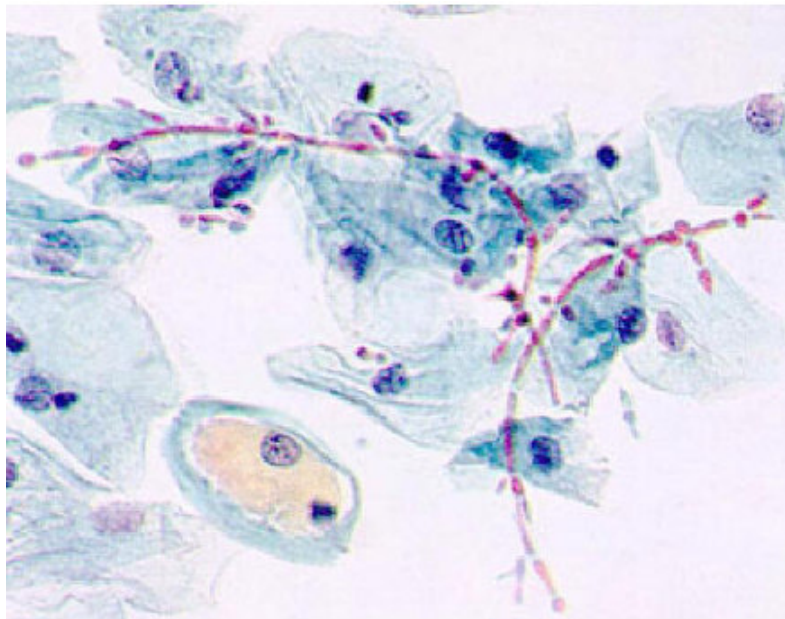
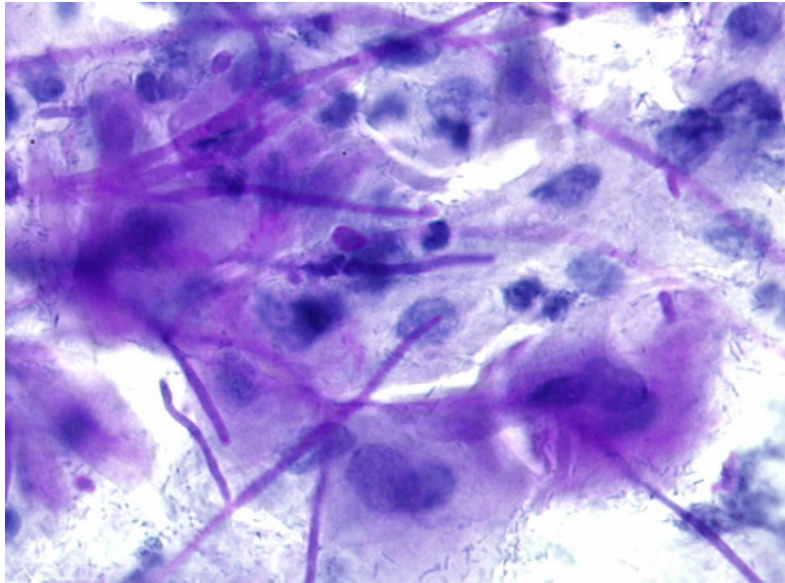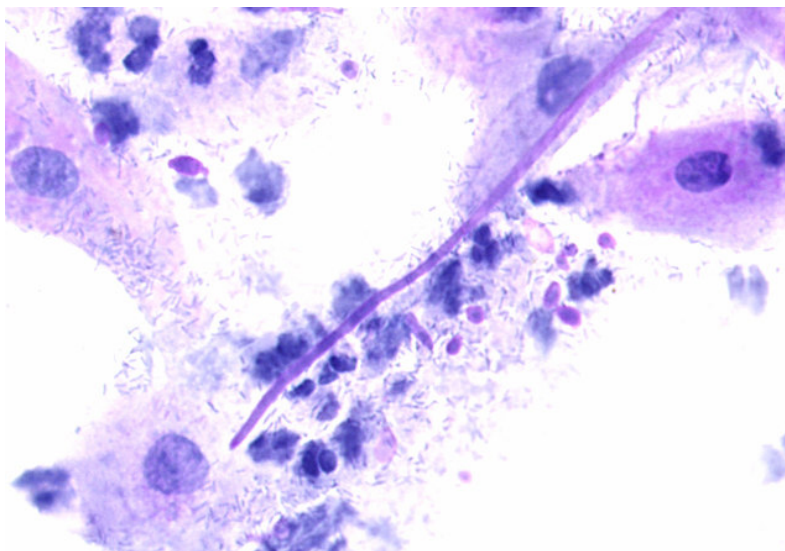**Figure 1, Chapter 4**: *Candida vaginalis* in a Papanicolaou-stained smear.

Figure 1, Chapter 7: Presence of red (pseudo)hyphae and two spores in the self sampled, PAS-stained vaginal smear of patient H on day 21, as unequivocally established by three different cytopathologists (magnification: 100 times).

Figure 2, Chapter 7: Presence of red (pseudo)hyphae and spores in the self sampled, PAS-stained vaginal smear of patient D on day 14, as unequivocally established by three different cytopathologists. Notably, lactobacilli (blue, 2+) and polymorphonuclear neutrophils (blue) are also visible (magnification: 100 times).
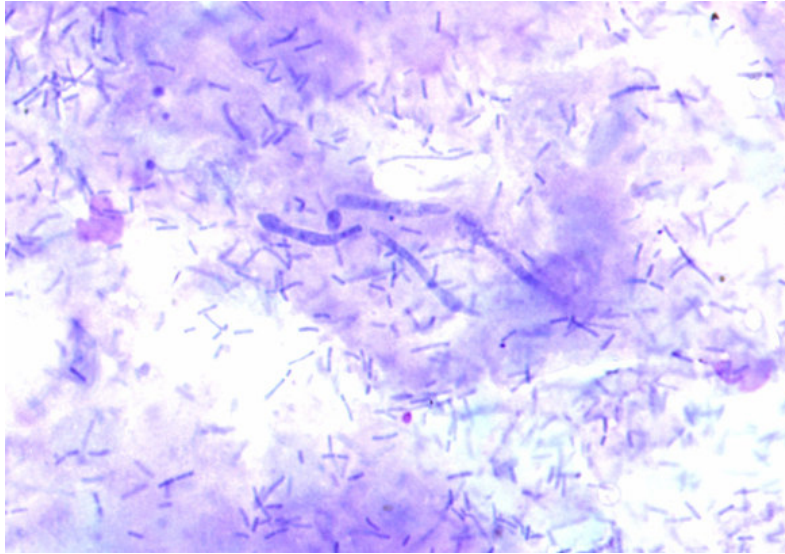
Figure 3, Chapter 7: Presence of blue (pseudo)hyphae and spores in the self sampled, Giemsa-stained vaginal smear of patient E on day 21, as unequivocally established by three different cytopathologists. As can be seen, lactobacilli (blue, 3+) are easily detectable in the Giemsa-stained smear (magnification: 100 times).
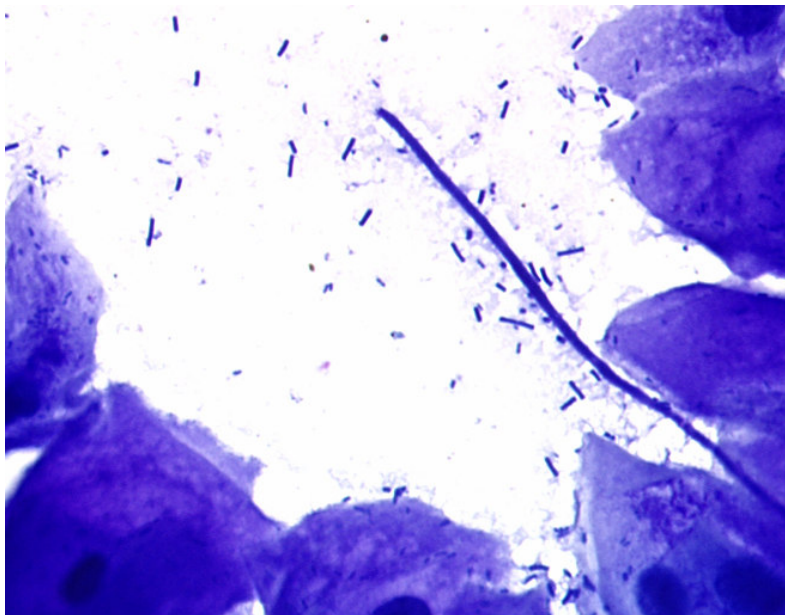

Figure 4, Chapter 7: Presence of blue (pseudo)hyphae in the self sampled, Giemsa-stained vaginal smear of patient G on day 14, as noted by three different cytopathologists. For photographic purposes, a blue filter was used to enhance visibility. Again, lactobacilli (blue, 1+) were easily notable in the Giemsa-stained smear (magnification: 100 times).