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**Epidemiology and prevalence of oral candidiasis
in HIV patients from Chad**

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1. Introduction

1.1 HIV and AIDS: history and epidemic

The acquired immunodeficiency syndrome (AIDS) was recognized for the first time as a new disease in 1981, when the first clinical cases of AIDS were observed in a cluster of men who have sex with men and injecting drug users who presented with symptoms of *Pneumocystis carinii* infection, a rare opportunistic infection known to occur in people with a severely compromised immune system (Schliep and Yarrish 1999). Further symptoms were extensive mucosal candidiasis, cytomegalovirus infection and the development of rare malignancies as Kaposi's sarcoma (Gottlieb et al. 1981; Masur et al. 1981). In 1983, a retrovirus, which has later on been named the human immunodeficiency virus (HIV), was isolated and declared as the causative agent of the disease (Barre-Sinoussi et al. 1983; Gallo et al. 1984). Since its discovery, the disease has spread dramatically throughout the world and had infected over 38 million people by the year 2005 (UNAIDS 2006). Although the introduction of antiretroviral treatment in 1996 (Williams 1997) had led to a significant decrease of AIDS-related deaths (UNAIDS 2010), the HIV pandemic continues to be a big challenge in public health. In 2012, still 35.3 million people were living with HIV, 2.6 million new infections were occurring annually and still almost 2 million deaths per year were registered (UNAIDS 2013). Treatment still does not reach all affected people and even though a lot of prevention efforts have been done throughout the world, in some countries the epidemic continues to spread, due to obstacles as beliefs, value and education systems, ignorance, poverty, fear of discrimination, no access to a developed health care system and political instabilities within countries (UNAIDS 2010; UNAIDS 2013).

1.2 HIV transmission pathway and pathogenesis

“The HI virus is the causative agent of AIDS and AIDS is the end stage of a protracted pathogenic process in which the immune system of an infected person and its ability to control infections or malignant proliferative disorders are progressively destroyed” (Schüpbach 1999).

The HI virus is transmitted through blood transfusions (infected blood products) contaminated drug injecting needles or shared razor blades, perinatal transmission (birth and breastfeeding) and unprotected sexual intercourse (sperm and vaginal secretions)(CDC 1985; Curran 1985). Sexual intercourse is the primary mode of HIV infection worldwide, followed by mother to child transmission and in some regions injecting drug users (UNAIDS 2007). The HI virus causes progressive immune failure by infecting and killing vital cells of the immune system, such as macrophages, dendritic cells and in particular CD4 T-helper cells, which are responsible for the cell-mediated immune response. A decline of the CD4 T-helper cells below a critical level leads to a loss of adequate immune response and the body becomes progressively more susceptible to opportunistic infections (Ascher and Sheppard 1988; Levy 1993; Douek et al. 2009). Therefore, the measurement of CD4 T cell counts has become an important tool in the management and therapy of HIV-infected persons and is a criteria included in the CDC classification of HIV/AIDS (CDC 1992). AIDS is only the end stage of the progressive destruction of the human immune system, in which the host has become unable to defend itself against opportunistic infections (Schüpbach 1999).

1.3 HIV/AIDS epidemic in Sub-Saharan Africa

Nearly 95 % of the people infected with HIV/AIDS live in the developing world and of all people infected worldwide, 70 % live in Sub-Saharan Africa. 76% of all deaths related to AIDS occur in this region. The main affected group of individuals is those who are the most productive socially, reproductively and economically. Extinction of this group of people affects the country's economy and its further development. More than half the adults infected with HIV in Africa are women and there is also a high incidence of vertical transmission (UNAIDS 2013). The high incidence of infectious diseases (malaria, tuberculosis and others), poverty, malnutrition and limited access to health care worsens the situation of the HIV epidemic in Africa. HIV/AIDS remains one of the top leading causes of death in this region of the world (UNAIDS 2007). Main cause of AIDS death are the opportunistic infections (UNAIDS 2007). An opportunistic infection is defined as an infection which is caused by a microorganism normally non-pathogenic in healthy hosts, which acts as a pathogen under certain favorable circumstances such as a compromised immune system and causes

infection (Symmers 1965). One of the most common opportunistic infections in HIV/AIDS is oral candidiasis (OC) (Scully et al. 1991; Samaranayake et al. 2002).

1.4 The importance of oral lesions in people living in Sub-Saharan Africa

Early detection, monitoring and treatment of individuals infected with HIV, besides prevention and information campaigns, is an important task in the fight against the HIV epidemic and the fatal consequences that the progress of this disease may have on a country.

As resources and diagnostic tools in Sub-Saharan Africa are very scarce, the inspection of the oral cavity often remains the only (and an important) detection tool of a possible HIV infection, noting that HIV infection and AIDS are highly associated with several oral lesions (Wanzala et al. 1989). One of them, OC, is still reported to be strongly associated with the HIV infection in Africa and also remains the most common oral lesions associated with HIV around the world (Damtie et al. 2013; Kumar et al. 2013; Naidu et al. 2013). It may be more severe in African than in similar patients in Western Europe (Enwonwu 1994; Curtis et al. 2012; De Beaudrap et al. 2013) and significantly impair the nutrition and quality of life of the HIV-infected people, leading to malnutrition and rapid disease progression (Hodgson and Rachanis 2002). This emphasizes the importance of the early detection of these oral lesions to improve the morbidity associated by offering appropriate treatment and reduce the fatal consequences of the progress and continuous spread of the disease. With the advent of the antiretrovirals, in places where this treatment has been introduced, rates of OC and AIDS-related deaths have been reported to decrease (Nkuize et al. 2010; De Beaudrap et al. 2013; Meless et al. 2014), potentially signaling an improvement of the immune system under this regimen. But in many African countries the treatment coverage of patients in need of antiretrovirals remained for long insufficient; in many countries below 20%, which accounts for only one quarter of the people living with HIV in Sub-Saharan Africa that had access to antiretroviral treatment in the year 2006 (UNAIDS 2006). Therefore OC remains highly prevalent among HIV-infected individuals in Africa (Kamiru and Naidoo 2002; Damtie et al. 2013; Okoje et al. 2013) and an important tool in the diagnostic and management of the disease (Ranganathan et al. 2004), as in most cases it can be diagnosed by its clinical appearance alone (Thompson et al. 2010). It gives a hint to

HIV disease status, progression and eventual treatment failure (Greenspan 1997; Coogan et al. 2005).

1.5. Oral candidiasis

1.5.1 Oral candidiasis and HIV

OC, also known as “thrush” (Macher 1988), was included in the first descriptions of the acquired immune deficiency syndrome (AIDS) (Gottlieb et al. 1981; Klein et al. 1984; Fisher-Hoch and Hutwagner 1995), a state of disease caused by the HIV (Macher 1988). It is a common opportunistic infection in patients who suffer from this disease and one of the first clinical signs of a deteriorating immune system (Scully et al. 1991; Samaranayake et al. 2002). Therefore it has been included as a "symptomatic condition" highly associated with HIV infection in the CDC and WHO classification of HIV/AIDS (CDC 1992; WHO 2007); but it also affects patients with other immune disorders, patients undergoing chemotherapy against cancer (Baixench et al. 2008) or taking immunosuppressive drugs to protect transplanted organs, as well as sometimes healthy subjects (Gupta et al. 1994; Fisher-Hoch and Hutwagner 1995; Patterson 1999; Redding et al. 1999; Dongari-Bagtzoglou et al. 2009; Lopez-Pintor et al. 2013). Nevertheless it is more commonly seen in HIV-infected individuals and there seems to be a high association between the presence of the HIV and eventually the viral load, and appearance of OC (Schuman et al. 1998; Gottfredsson et al. 1999; Mercante et al. 2006; Fidel 2011; Cassone and Cauda 2012). OC is as well an indicator of severe immunosuppression (Klein et al. 1984; Schuman et al. 1998), correlating with CD4 T cell counts <200 CD4 T cells/ μ l (Mercante et al. 2006; Witzel et al. 2008). Infected individuals with the symptoms of OC progress more rapidly to AIDS and death than those without (Greenspan 1997). In a study of Lindan et al. (Lindan et al. 1992) in Kigali, Rwanda, the presence of a clinically detectable candidiasis in a known HIV-positive female was associated with a 40% risk of death within two years. Early recognition and treatment of these lesions are therefore very important in the management of HIV-infected patients.

1.5.2 Diagnosis of oral candidiasis

The diagnosis of OC has been based on the presumptive criteria set by the EC-Clearinghouse (ECC) classification in 1993, which has been and still is the most

widely used classification in clinical and epidemiological studies (EC-Clearinghouse 1993; Patton et al. 2013). According to this classification, there are two different clinical types of OC that are distinguished: the pseudomembranous candidiasis (PC) which is the most usual form (Leao et al. 2009), described as "white or yellow spots or plaques" which may have thick creamy, curd-like appearance, "that may be located in any part of the oral cavity and can be wiped off to reveal an erythematous surface which may bleed" (EC-Clearinghouse 1993; Thompson et al. 2010). The second type is the erythematous candidiasis (EC) described as "red areas located on the palate or the dorsum of the tongue". Both types belong to the category of "lesions highly associated with HIV" (EC-Clearinghouse 1993). But there are also various other different clinical presentation forms of OC known as: angular cheillitis (AC), median rhomboid glossitis (MRG), atrophic, nodular, plaque-like or hyperplastic candidiasis (Lalla et al. 2010; Tarcin 2011). All these forms may also be seen, but less frequent, although MRG and AC have been cited by Gazzard and Smith as well to be common in HIV-infected people (Gazzard and Smith 1990). Anyhow, these other forms are not included in the ECC classification. Several attempts have been made to classify these forms beginning with Lehner in 1964, who distinguished between acute and chronic forms and ending with a revised classification of Lehner by Axell et al. in 1997 (Axell et al. 1997; Parihar 2011). This last classification better considers the different clinical subdivision and unusual variants of OC.

It needs a trained and experienced medical personal to recognize and to distinguish between these different presentation forms. Sometimes these different forms may present as combinations (EC-Clearinghouse 1993) and some, like the atrophic form, occur in earlier stages and may frequently be missed or more difficult to diagnose (Gazzard and Smith 1990). OC may be quite often asymptomatic or accompanied by symptoms as cotton taste, dysgeusia or burning; it can furthermore spread to the esophagus (Tavitian et al. 1986; Gupta et al. 1994; Ally et al. 2001; Nishimura et al. 2013) and cause swallowing pain, or to other organs, which is associated with a high mortality rate (Gudlaugsson et al. 2003; Gautam et al. 2010). Systemic infection in HIV patients is rare but there are few case reports (Gautam et al. 2010; Anwar Khan 2012).

1.5.3 Causative agent of oral candidiasis

The causative agent of OC is yeast fungi from the genus *Candida*. The yeast species *Candida* is a harmless commensal of the human which may colonize skin and mucous membranes like the gastrointestinal or urogenital tract. It is found in the oral flora of 15-60 % of healthy individuals (Sanchez-Vargas et al. 2005a; Yang et al. 2011). It causes infection when there is a disturbance in the host's specific (humoral) and non-specific (cellular) defense systems and virulence genes of the normally commensal *Candida* are activated (Cannon et al. 1995; Sturtevant 2000; Cassone and Cauda 2012). It may then cause superficial infection of the skin or mucous membranes or penetrate into deeper tissue layers, and disseminate in the blood system and organs causing candidemia (Pfaller and Diekema 2007).

More than 200 species of *Candida* are known today, but only few are pathogenic to humans. Out of these, *Candida albicans* (*C. albicans*) has been described to be the main pathogen of oral and systemic candidiasis and remains until date the main pathogen in this context (Thompson et al. 2010). However, other non-*C. albicans* yeast species (*spp.*) like *C. glabrata*, *Issatchenkia orientalis* (*I. orientalis*/*C. krusei*), *C. tropicalis* are increasingly being described to cause infection and they are becoming of increased importance as they show patterns of antifungal resistance to azoles, the antifungals still used as first line treatment (Krcmery and Barnes 2002; Snyderman 2003; Nadagir et al. 2008; Bassetti et al. 2009; Pappas et al. 2009). Especially patients with extensive exposure to antifungals or recurrent OC as it is in patients with advanced stages of AIDS are affected (Patel et al. 2006; Nadagir et al. 2008). A relatively new species closely related to *C. albicans* identified is *C. dubliniensis*. *C. dubliniensis* has been mainly recovered from the oral cavity of HIV-infected individuals and AIDS patients in association with OC (Sullivan et al. 1995; Coleman et al. 1997; Loreto et al. 2010). This species seems to be linked to HIV infection and may also be resistant to Fluconazole (Moran et al. 1997; Nadagir et al. 2008; Scheid et al. 2012). It is therefore important to be aware of the emerging new pathogens in this context. This rise in *Candida* species other than *C. albicans* and the continuous incidence of resistant species makes the management of the infection more and more difficult. Species determination and susceptibility testing should therefore be included in the management of the disease caused by these pathogens.

This is a problem in settings where further diagnostic facilities to determine and differentiate species and perform resistance analysis are not available.

1.6 Chad

1.6.1 The country's geography, climate and population

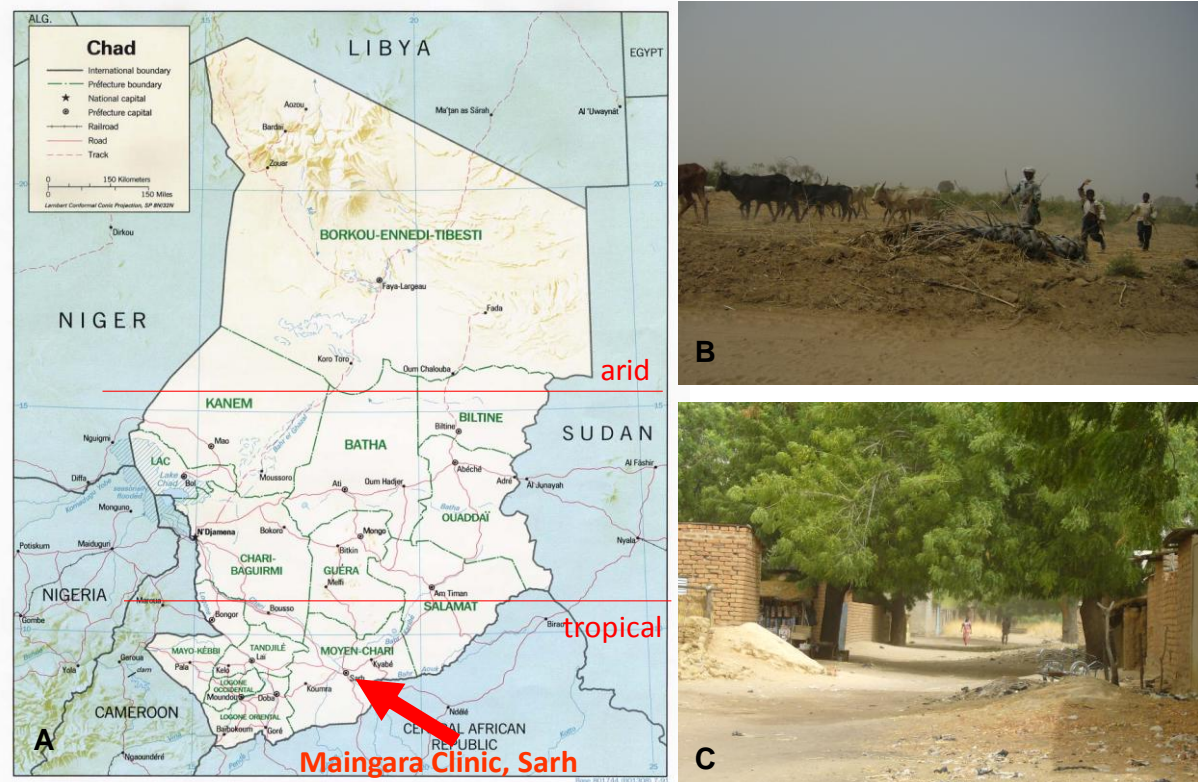


Figure 1: Map of Chad and landscape impressions. A. Map of Chad (from: <http://www.loc.gov/item/91681423/>, accessed 20.03.15). B. northern arid and C. southern tropical region. The clinic of Maingara, where the study took place was located in the southern tropical part of the country.

Chad is a land-locked country located in north central Africa measuring 1,284,000 square kilometers. In the language of the Buduma (an ethnic group who inhabits Lake Chad) it is called “big water” which refers to the Lake Chad, the second largest lake in West Africa and one of the most important wetlands on the African continent (Room 2008). It is enclosed by its neighbors: Libya in the north, Sudan in the east, Central African Republic in the south, Cameroon and Nigeria in the south-west and Niger in the north-west (Figure 1A). The population counts 11,193,452 inhabitants, of which most concentrate in the tropical south part of the country where the picture of wet savanna with an annual rainfall of 600-1200 mm/year dominates. The northern part is a Sahara-like region (dry savanna) with less than 200 mm of rainfall annually

(Figure 1B+C). The temperature varies between 13°C to 29°C in January and 25°C to 44°C in May in the northern part and between 15°C and 34°C in January and 23°C and 35°C in May in the southern part of the country (LCBC 2013).

Ndjamena is the capital with 808,000 inhabitants located at the west border of the country. The next biggest cities are Moundou, Sarh and Doba which are mainly concentrated in the south. Sarh is the third biggest city of Chad after Ndjamena and Moundou with around 100,000 inhabitants (<http://www.geoba.se/population.php?cc=TD>, accessed 19.06.14). The average population is very young with a median age of 16.9 years. 47% are under the age of 15 and only 2.9% are above 65. Till 2010, the life expectancy was 46 (WHO 2010).

80% of the Chadians live in rural areas from agriculture (although only 4% of the country's land is arable) and subsistence economy. It is one of the poorest countries in the world [Ranking 184 of 186 in the Human development Index (HDI)] (UNDP 2013). The geographical circumstances influence the differences in the socio-economic living styles. In the northern part of the country the population is largely nomadic. They have some livestock with mostly small ruminants and camels. In the southern part the population is more settled.

Chad has more than 200 different ethnic groups and more than 120 different languages and dialects. The biggest group is the Sara (27.7%) settled in the southern part of the country which speaks Sara. Further 26% of the population speaks Arabic, one of the official languages of Chad. The other official language is French. The illiteracy rate is high: 76% among women and 55% among men (UNESCO 2012).

The majority of the population is Muslim (approx. 55%) of which most live in the northern part of the country and speak Arabic. Further 35% are Christian and are more concentrated in the southern part of the country. The rest belongs to traditional African religions (animist, 7%) and others (CIA 2014).

1.6.2 History and politics

Chad became colonized in 1920 by the French and acquired its independence *in* 1960. Since, it has been marked by political instability with several civil wars and attempted coup d'états due to tensions between the Arab-Muslim north and Christian

south. The Darfur crisis in the neighboring Sudan which started in 2003 also affected the nation's instability with hundreds and thousands of refugees cumulating at the Chadian border. Despite all Mr. Idriss Deby who became president in 1990 managed to stay in power until today (Prunier 2008; BBC 2015).

1.6.3 Health care system

The health care system in Chad is marginally developed and insufficient to provide the necessary health care needed. Only 30% of the population has access to the health care system (WHO 2010). The physician density is 0.04 per 1,000 inhabitants which are concentrated in the urban areas and the existing hospitals are only rudimentarily equipped. In this context especially in the rural area, traditional healing methods are being favored. The situation is worsened by the political conflicts in Darfur, Sudan and Central African Republic which have led to a refugee's movement especially in the southern-east part of the country. Furthermore food crisis, missing sanitation and water sources affect the country's development and economy.

1.6.4 Chad and HIV

In Chad, the HIV prevalence was about 3.3% in 2007 and 2.7% in 2012 (UNAIDS 2007 ; CNLS 2012; UNAIDS 2013). The distribution is inhomogeneous within the country with a prevalence of 2.3 % in the rural and 7% in the urban area (UNGASS 2008). In the region of Sarh, the HIV prevalence of pregnant women was about 4% in 2012, and among sexual workers the prevalence was highest with 20% (CNLS 2012). The main transmission pathway remains the heterosexual unprotected intercourse (including paid sex) and the vertical transmission pathway from mother to child and breastfeeding. As in the overall region, women are more affected than men; in Chad 4% compared to 2.6% within the same age group. In Chad this is particularly due to polygamy (UNGASS 2008).

The HIV prevalence is not yet as high as in other African countries, but multiple factors promote the spreading of the disease. These include poverty, religious and cultural taboos, ignorance and lack of knowledge about the modes of transmission of the disease ongoing with high prevalence of unprotected sex, illiteracy of women, sex workers, high prevalence of sexually transmitted diseases, a rudimentary insufficient health care system, and persistence of the internal and external political conflicts with

the neighboring countries Sudan and Central African Republic which lead to a rising number of refugees at the south and south-eastern borders of Chad. In some regions it is reported that the prevalence may be as high as 10% (UNAIDS 2009; CNLS 2012).

A lot of efforts are being done to elucidate the population and projects for the prevention of the transmission of the disease, detection and a close follow-up of the infected individuals are being established (UNGASS 2008; CNLS 2012). The creation of the "Centre de Santé de Maingara" in Sarh, Chad in 2004 was part of one of those projects.

1.6.5 The clinic of Maingara in Sarh

Maingara is a city district of Sarh. The clinic (Figure 2), in which we found a collaboration partner to conduct our study, was founded with the support of the BELACD (Bureau d'Études et de Liaison des Actions Caritatives et de Développement) of Sarh, a non-governmental national aid organization, and could open its activities in 2004 to offer counseling, HIV testing, treatment and follow-up for HIV-infected individuals.



Figure 2: The clinic of Maingara

At the time of the study Dr. Lydia Kersch from Germany was the leading head of the center and the only doctor. The clinic team furthermore consisted of five trained

nurses, two social workers, three trained assistants for lab diagnostics and a secretary for patients' registration and file establishment. There was a section for HIV screening and counseling with social attendance, a day clinic equipped with an ultrasound and ECG machine, a lab with a microscope, photometer, and a CD4 T cell counting machine. Since August 2005 it had furthermore been supplemented with 18 beds to receive and treat severely ill cases.

Due to the educational and awareness training especially among the analphabetic youth, schools and young couples in the surrounding villages of the parish and the installment of institutions able to receive and to take care of affected people, the number of people presenting for a screening test rose constantly and a decline in HIV seropositivity of the tested persons could be observed from 2004 to 2006 (42% to 18.5%) (Dr. L. Kersch personal communication).

By the year 2006 the clinic was taking care of 1279 affected individuals (18% of the patients screened in Maingara) which were followed regularly at monthly intervals. 82% of the examined patients were in the reproductive age of 12 to 45 and 627 (49%) were under antiretroviral treatment at that time. Of these, 89.4% had been followed regularly, 11% went out of sight and 7 % went elsewhere.

By 2007, 71.3% of the patients were under antiretroviral treatment. Female patients were in the majority (60%) and also in the majority put under antiretroviral treatment. Still, the mortality rate in 2006 was high with 28.1% for the hospitalized patients and 15% for all the followed patients (Dr. L. Kersch personal communication).

Only a basic selection of drugs was available to treat and prevent the most common AIDS-related opportunistic diseases and diagnostic relied mainly on the clinician's subjective clinical impression. Antifungals were available in a very limited amount and therefore rarely prescribed.

1.6.6 HAART at the clinic of Maingara

The antiretroviral treatment available at the center was the highly active antiretroviral treatment (HAART) regimen. It is a combination of three antiretrovirals which was given according to the National Guidelines of Chad for antiretroviral therapy which refer to the WHO standards of 2006 (WHO 2006). The center was mainly financed by

the parish of Sarh and its international and private donors. Patients of the day clinic also had to provide a small contribution of about 3000 Francs CFA (approx. 4.60€) in quarterly payments. As the government did not yet provide antiretrovirals for free until the year 2007, patients had to contribute partially to the treatment costs (5000 Francs CFA/approx. 7.60€ per month). In case of inability to pay, the medication was given on credit when already started. To avoid HAART interruption, the patients were informed about the danger and consequences of an interruption always in the presence of a family member and had to oblige themselves in written form to take the medication as prescribed and take a responsible behavior towards others before the start of the treatment. Furthermore medical records were established and social workers looked up for the patients under HAART in their homes if they did not appear to their regular control. Nevertheless, 8.2% had an interruption of more than 45 days (Dr. L. Kersch personal communication).

1.7. Aim of the study

In Chad, no data on the prevalence of OC or oral colonization with *Candida* species in HIV-infected and healthy subjects were available. Neither there was known which kind of *Candida* species colonize the oral cavity of the Chadian population or cause infection in HIV-immuno-compromised patients and if they are susceptible to the existing antifungals. The clinical importance of OC in association with HIV in Chad was therefore unclear.

The first aim of the study was to determine the type of the existing yeasts colonizing the oral cavity of the Chadian healthy and HIV-infected population and test their sensitivity to five common antifungals in use (Amphotericin B, Nystatin, Fluconazole, Itraconazole and Caspofungin) with the goal to give a picture of the current situation and eventually improve the management of the oral fungal burden of HIV/AIDS patients in that specific country. Furthermore we wanted to define the prevalence of OC in the HIV-infected and healthy population, and analyze the influence and association of other factors like age, sex, HAART and antimicrobial therapy and CD4 T cell counts on that opportunistic disease.

2. Material and methods

2.1 Materials

2.1.1 Machines and instruments

Autoclave, steam sterilizer	<i>unknown</i>
AXIMA Assurance™ platform	Shimadzu Biotech, Duisburg, DE
Bruker MALDI Biotyper 2.0®	Daltonics, Bremen, DE
CD4 counter, cyFlow®	Partec, Münster, DE
Centrifuge, type 5417R	Eppendorf, Hamburg, DE
Dynex Revelation microplate reader	Dynex Technologies, Virginia, US
Incubator, type BB 6220 CU Heraeus®	Thermo Fisher Scientific, Langenselbold, DE
Microscope	Zeiss, Jena, DE
Multichannel pipet, Multipette®-Plus	Eppendorf, Hamburg, DE
pH-meter, HI 221	HANNA Instruments, Vöhringen, DE
Photometer, Mac Farland®	BioMérieux, Marcy, FR
Pipets "Reference", type 4810; 0,5-10 µl; 10-100 µl; 50-200 µl; 100-1000 µl	Eppendorf, Hamburg, DE
Pipet pump, Pipetus®-Akku	Hirschmann, Eberstadt, DE
Reading mirror, Microtiter®	Cooke Engineering Company, Virginia, US

Saramis MALDI Biotyper [®]	AnagnosTec, Potsdam, DE
Sterile bench	BDK Luft- und Reinraumtechnik GmbH, Sonnenbuehl-Genkingen, DE
Vortex REAX-top	Heidolph, Schwabach, DE
Weighing machine type BL 310	Sartorius, Göttingen, DE

2.1.2 Single-use material

Cryobank system	Mast Diagnostica, Reinfeld, DE
Eppendorf tubes 0,5 ml safe-lock	Eppendorf, Hamburg, DE
Eppendorf tubes 2,0 ml safe-lock	Eppendorf, Hamburg, DE
Flat bottom microdilution plates, 96 wells	Greiner, Kremsmünster, DE
Glas test tubes, round bottom	Roth, Karlsruhe, DE
Glas test tubes, flat bottom	Roth, Karlsruhe, DE
Graduated glas pipets 10 ml, 20 ml, 50 ml	Brand, Wertheim, DE
Graduated cylinder 1000 ml	Roth, Karlsruhe, DE
HIV testkits ImmunoComb [®] / Determine [®]	Orgenics, Yavne, IL
Inoculating loop white 1 µl	Sarstedt, Nürmbrecht, DE
Microscope slides and square cover glass	Knittel, Braunschweig, DE
Pipet tips, blue, 1000 µl	Sarstedt, Nümbrecht, DE

Pipet tips, yellow, 200 µl	Sarstedt, Nümbrecht, DE
Plastic test tubes 50 ml with cap	Sarstedt, Nümbrecht, DE
QIAamp DNA Mini Kit	Qiagen, Hilden, DE
Rice agar	Oxoid, Wesel, DE
Sterile agar gel transport swabs	Copan, Brescia, IT
Sterile Combitips 5 ml	Eppendorf, Hamburg, DE
Sterile cotton swabs	Copan, Brescia, IT
Sterile-Filter Corning® 0,22 µm (cellulose-acetate)	Corning GmbH, Wiesbaden, DE
Sterile Plastic Petri Dish 90mm Ø with cover	Greiner, Kremsmünster, DE
2.1.3 Addings and chemicals	
Agar (for STAIB-Agar)	Merck, Darmstadt, DE
Antibiotic Medium 3, AM3	Becton Dickinson GmbH, Heidelberg, DE
A-D(+)-Glucose-Monohydrate	Roth, Karlsruhe, DE
Creatinine (for STAIB-Agar)	Merck, Darmstadt, DE
Dimethyl Sulfoxide, DMSO, Hybri-Max®	Sigma-Aldrich, Steinheim, DE
Glucose	Roth, Karlsruhe, DE
<i>Guizotia abyssinica</i> seed (for STAIB-Agar)	Merck, Darmstadt, DE

KH₂PO₄, (for STAIB-Agar) Merck, Darmstadt, DE

Natrium chloride NaCl Merck, Darmstadt, DE

3-(N-morpholino) propansulfonic acid, MOPS Sigma-Aldrich, Steinheim, DE

Sabouraud powder medium Merck, Darmstadt, DE

RPMI -1640 media Sigma-Aldrich, Steinheim, DE

Antibiotics	Stock	Final concentration
Gentamicin (Merck, Darmstadt, DE)	16 mg/ml	16 µg/ml
Chloramphenicol (Sigma-Aldrich, Steinheim, DE)	16 mg/ml	16 µg/ml

Antimycotics

Amphotericin B Sigma, Taufkirchen, DE

Caspofungin MSD GmbH, Haar, DE

Fluconazole Discovery Fine Chemicals, Bournemouth, UK

Itraconazole Discovery Fine Chemicals, Bournemouth, UK

Nystatin Sigma, Taufkirchen, DE

2.1.4 Recipes for culture mediums

AM3	17.5 g/l AM3
	3 g/l glucose
RPMI	10.4 g/l RPMI
	34.53 g/l MOPS
	2 g/l glucose
Sabouraud agar	65 g/l Sabouraud
	1 ml Chloramphenicol (16mg/ml)
	1 ml Gentamicin (16mg/ml)
Staib-Agar:	5 % pulverized <i>Guizotia abyssinicia</i> seed
	0.1 % glucose
	0.1 % KH_2PO_4
	0.1 % creatinine
	1.5 % agar

2.1.5 Patients

Confirmed HIV-positive (HIV+) and -negative (HIV-) patients were recruited from the medical centre of Maingara in Sarh, Chad. As the majority of patients consulting at the clinic in Maingara were HIV-positive, a group of patients in a small dispensary 50 km away from Sarh was included to enlarge the control group.

2.2 Methods and study procedure

2.2.1 Ethics commission

Before realization, the research proposal was presented and approved by the ethics commission of the Georg-August-University of Göttingen (Application-N° 21/06/07) and the participating institutions in Chad (see appendix). All patients involved in the study were orally informed about the aim of the study and asked for their agreement before implementation according to the Helsinki Declaration (WMA 2013).

2.2.2 Patient acquisition

All patients who presented for consultation at the clinic of Maingara in Sarh during the study period were orally informed (for the majority could not read or write) about the aim of the study (see appendix). Only those who gave their informed oral consent were included into the study, irrespectively of their age or sex. The majority was HIV-infected and came to their regular monthly health control, or had just been tested HIV-positive. To establish a control group, a group of patients had been seen in the “dispensary” (small health center) of Bemouli, in a rural area 50 km away from Sarh, a small health care point providing basic health attendance by a nurse for a normal population. Confirmed HIV-negative patients who presented at the clinic of Maingara were as well included.

2.2.3 History taking and clinical inspection/examination

After informing the patient about the study and getting his/her agreement, informations on the medical history, age, sex, HIV status, current opportunistic infections and medications, HAART, and the last CD4 T cell count were noted or taken from medical records. A brief clinical examination was done, the oral cavity inspected at day light additionally with a small torch and the observations noted. From patients presenting again during the study period, a consecutive sample and examination was taken to evaluate disease progression and effect of antimycotics or HAART if given.

2.2.4 HIV testing

The HIV status of the patients was determined by a rapid test (Determine[®] HIV-1/2), and if positive it was confirmed through another rapid test kit (ImmunoComb[®] II HIV 1&2 Bispot). If both were positive, the patient was considered HIV-positive.

2.2.5 HAART

The antiretroviral therapy available in Chad was TRIOMUNE 30[®] for patients with a body weight under 60 kg and TRIOMUNE 40[®] for patients above 60 kg. TRIOMUNE is a combination of two nucleosid reverse transcriptase Inhibitors (NRTIs) Stavudine (30 or 40 mg), Lamivudine (150 mg) and one non-nucleosid reverse transcriptase inhibitor (NNRTI) Nevirapine (200 mg). The daily treatment regimen consisted of one tablet twice a day (WHOPAR 2011; NAM 2014) Patients received treatment according to the National Guidelines of Chad for antiretroviral therapy which had been based on the WHO Standards (WHO 2006). It was indicated when the patient had a CD4 T cell count <200 CD4 T cells/ μ l or was in a WHO clinical stage III or IV. Patients with a WHO clinical stage II could also get the therapy when CD4 T cells were between 200 and 350 CD4 T cells/ μ l (Table 1). In case of intolerability of Nevirapine or tuberculosis treatment with Rifampicin, patients received a combination with Efavirenz (NNTRI) and in very few cases of suspected therapy failure or Kaposi sarcoma, the protease inhibitor (PI) Indinavir was given.

Table 1: Indication for HAART according to the National Guidelines of Chad

	WHO clinical stage I	WHO clinical stage II	WHO clinical stage III	WHO clinical stage IV
CD4 T cells				
≥ 350	no HAART	no HAART	HAART possible	HAART
200-350	no HAART	HAART possible	HAART	HAART
≤ 200	HAART	HAART	HAART	HAART

2.2.6 Swabs

The oral cavity of the patients was sampled by taking swabs with a sterile cotton swab from visible oral lesions or when no visible symptoms, going over tongue, palate and side cheek pockets. Each swab was directly inoculated onto 1/8th of a Sabouraud (SAB) agar plate (Figure 3). Due to the lack of an incubator the plates were cultured over night at room temperature (approximately between 26°C and 28 °C at night time and 30-36°C at day time) in the time from April to June. The plates were controlled for the growth of yeasts after 24 and 48 hours. If positive, the number of colony-forming units (CFU) was counted. A culture counting from 1 to 15 CFU was considered as “low fungal burden” (LFB) and a counting from above 15 CFU or confluent growth on a 1/8th surface of the plate as “high fungal burden” (HFB). Samples of several colonies from each morphologically distinct appearance were stored on slant agar at 4°C until they were transferred to Germany, where they were recultivated and separated for further analysis. The samples were collected over a period of time of 3 months, between April and June 2007.

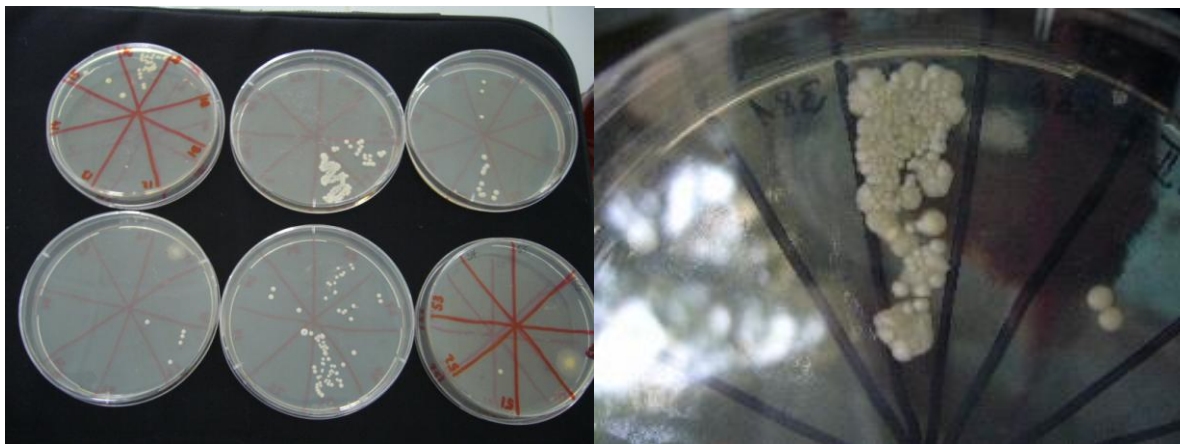


Figure 3: Growth of yeast on the agar plates from the direct smear of the patients. On each plate there was space for eight swab specimens. CFU were counted by hand. In the right picture confluent growth with mixed species.

2.2.7 Differentiation (Germany)

All collected samples were recultivated in Germany on Sabouraud agar. Swabs which presented apparent mixed cultures by colony morphology were separated and purified before differentiation. After verification and identification of yeasts under the microscope, two different methods were applied: the standard phenotypic methods

with cultivation on different culture mediums, like rice and Staib agar, API differentiation kits, and in case of difficult identification polymerase chain reaction (PCR). Additionally, all samples were identified with two commercially available MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time Of Flight) identification systems: Bruker MALDI Biotyper 2.0 and AnagnosTec Saramis. Before use, all samples were cultivated over night at 37°C and in case of slow-growing organisms over two to three days.

2.2.7.1 Microbiological and biochemical differentiation

2.2.7.1.1 Microscopy

All isolated species were first identified as yeasts under the microscope by staining with methylene blue. Specimens with several colonies with distinct morphology were separated and purified before further differentiation process (Figure 4).



A. Microscopic image of yeasts **B. Mixed culture (on SAB agar)** **C. Purified culture (on SAB agar)**

Figure 4: Images of yeasts and yeast culture. **A.** Typical ellipsoid shape of living yeasts stained with methylene blue under the microscope (X1000). **B.** Growth of two morphologically distinct yeasts (*C. albicans* (opaque) and *I. orientalis* (dull) on SAB agar. **C.** Purified yeast culture (*P. fabianii*) with visible colonies.

2.2.7.1.2 Rice agar, Staib agar and API

All species identified as yeasts were cultivated on rice-tween agar covered with a glass slide (semi-anaerobic conditions) at 26.5 °C for 48 h and checked for the growth of chlamyospores and pseudomycelium under the microscope (Figure 5). The isolates with visible growth of chlamyospores were again cultivated in the same way on Staib agar to differentiate between *C. albicans* and *C. dubliniensis* (Staib and Morschhauser 1999; Loreto et al. 2010). All the other isolates were differentiated with the API system (API 32 C and API 20 C AUX) and in case of difficult identification a PCR had been performed.

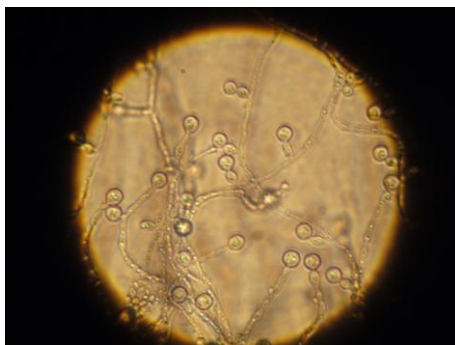


Figure 5: Chlamydospore formation by *C. albicans* on rice-tween agar. Induced pseudomycelium and chlamydospores formation by *C. albicans* on rice-tween agar.

2.2.7.1.3 PCR

For the samples that could not be identified by the biochemical standard methods, a PCR was performed to amplify the ITS2 rDNA region followed by sequencing of the PCR product (Chen et al. 2000). Fungal DNA from a single large colony was isolated with the QIAamp DNA Mini Kit. The ITS2 rDNA region was amplified and the product sequenced (SeqLab, Göttingen, Germany) and identified in the CBS yeast sequence database (<http://www.cbs.knaw.nl/>, accessed 20.05.14).

2.2.7.2 Differentiation with the MALDI-TOF

For the confirmation of the species identification, two MALDI-TOF systems were available for an evaluation period and the strains were included into that study (Bader et al. 2011).

2.2.7.2.1 Bruker MALDI Biotyper 2.0 system

For yeast identification (Bader et al. 2011) with the MALDI BioTyper 2.0 system (Figure 6), cells of approximately five colonies from Sabouraud agar plates were suspended in 300 µl water and inactivated by addition of 900 µl 96 % ethanol. The cells were spun down and the pellet air dried at room temperature, resuspended in 50 µl 70 % formic acid and extracted by addition of an equal volume of acetonitrile and thorough mixing. Cellular debris were removed by centrifugation (17,000×g for 2 min), 1 µl of the clear supernatant was spotted onto a polished steel carrier (Figure 6), allowed to dry, overlaid with 1 µl of HCCA matrix (saturated solution of α -cyano-4-hydroxycinnamic acid in 50 % acetonitrile, 2.5 % trifluoroacetic acid, Bruker Daltonics) and allowed to dry again. The matrix could be stored for a maximum of two weeks at room temperature in a dark container.

Measurement was done with the MALDI BioTyper 2.0 (library version 3.0) and FlexControl software on a Microflex LT20 mass spectrometer (20 Hz nitrogen laser), using a bacterial test standard (Bruker Daltonics) as a molecular weight standard. Spectra were detected in positive linear mode, mass range 2 – 20 kDa. Intensity of the laser was controlled by the FlexControl software driven in automatic mode, at the settings recommended by the manufacturer. Only species identifications with scores >2.000 were accepted, but proposed identifications at the genus level only were rejected.

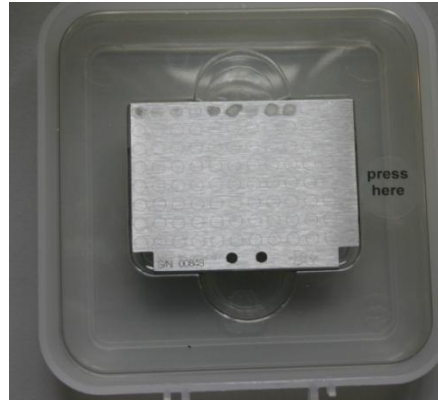
2.2.7.2.2 AnagnosTec Saramis system

For yeast identification (Bader et al. 2011) with the Saramis system (“Spectral Archive and Microbial Identification System”), cells from a single colony on a Sabouraud agar plate were directly applied onto the steel carrier, dried for a short time (~2 min) and lysed by suspension in 0.5 µl 25 % formic acid. The sample was allowed to air dry at room temperature, overlaid with 1 µl HCCA matrix (saturated solution of α -cyano-4-hydroxycinnamic acid in acetonitrile:ethanol:water 1:1:1 acidified with 3% v/v trifluoroacetic acid) (AnagnosTec) and again allowed to air dry.

Measurement was done on an AXIMA Assurance™ platform in positive linear mode, mass range 2 – 20 kDa, using *E. coli* strain CCUG 10979 as a molecular weight standard. Intensity of the 50 Hz nitrogen laser was under control of the acquisition software, at the settings recommended by the manufacturer. Only hits within the Superspectra database (Saramis™ Premium, version 3.3.1) with scores >80 % were accepted, but identifications proposed from the single spectra database were excluded.



Bruker MALDI Biotyper 2.0 system



Polished steel carrier

Figure 6: MALDI Biotyper 2.0 system

2.2.8 Antifungal susceptibility testing

Antifungal susceptibility testing of the isolates obtained in this study was done according to the NCCLS reference method for broth dilution antifungal susceptibility testing of yeast; Approved Standard, M27-A2 (Guidelines of the Clinical and Laboratory Standard Institute, CLSI) (NCCLS 2002). The antifungals tested were Fluconazole, Itraconazole, Nystatin, Amphotericin B and Caspofungin.

2.2.8.1 Broth mediums and preparation of the microdilution plates

The broth microdilution test was performed in sterile microdilution plates with 96 flat bottom wells (Greiner). The broth mediums used were RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) to a pH of 7.0 at room temperature (21.3°C) and AM3 (Antibiotic Medium 3). The RPMI 1640 medium was being used for the testing of Caspofungin and the azoles and the AM3 medium for the testing of Amphotericin B and Nystatin. Amphotericin B, Nystatin and Itraconazole were dissolved in dimethyl sulfoxide (DMSO), Fluconazole in methanol and Caspofungin in water. The final drug concentration for the microdilution plates was prepared and

adapted for each antifungal agent according to the MIC (minimal inhibiting concentration) ranges described in the NCCLS protocol (0.063 - 1.5 µg/ml for Amphotericin B, 0.016 - 24 µg/ml for Nystatin, 0.016 - 16 µg/ml for Caspofungin and Itraconazole, and 0.250 - 256 µg/ml for Fluconazole). 100 µl of medium with the highest drug concentration was dispensed into the wells of column 1, column 11 contained the lowest concentration and column 12 served as the positive growth control, containing 100 µl of a sterile drug-free medium. The trays were stored at -70°C and thawed one hour before use.

2.2.8.2 Inoculum stock suspension:

The inoculum used was prepared from colonies cultured for 24 h under aerobic conditions at 37°C, which were suspended in sterile saline (NaCl). The turbidity was adjusted with a spectrophotometer to a value of 0.5 McFarland.

2.2.8.3 Working suspension and dispersion onto the plates:

The final working suspension was prepared by diluting 1:100 in physiological NaCl followed by a 1:20 dilution in RPMI 1640 medium for Caspofungin and the azoles and in AM3 medium for Nystatin and Amphotericin B. The inoculum had 5×10^2 to $2,5 \times 10^3$ cells per ml.

The wells were dispensed with 100 µl of the prepared inoculum suspensions with a multichannel pipette, starting with the positive control well going downwards in the row. For the quality control of the plates, the strains recommended by CLSI (*C. parapsilosis* (ATCC 22019) and *I. orientalis* (ATCC 6258) were included in the testing procedure.

2.2.8.4 Incubation and reading of the results

The plates were incubated in a humid box at 36-37°C and analyzed after 48 hours for the presence or absence of visible growth with the help of a reading mirror. The growth of each well was compared to the growth control well and noted down as visible growth, low visible growth, no growth or contamination. Furthermore, the cells in each well were resuspended with a pipette and the optical density at 60 nm (OD 600) measured with a microplate spectrophotometer. The results were then compared with the results of the visual reading to evaluate and control the plausibility of the measured results. The minimal inhibitory concentration (MIC) for Amphotericin

B and Nystatin was defined as the lowest concentration in which at least 90 % of growth of the sample was inhibited, defined as MIC₉₀, for Caspofungin and the azoles, as the lowest concentration in which at least 50 % of growth was inhibited (MIC₅₀).

To minimize deviation, each organism was tested 2 to 4 times and the average MIC was calculated. An isolate was considered resistant if the average MIC was greater than their respective CLSI clinical breakpoints ($\geq 64 \mu\text{g/ml}$ for Fluconazole, $\geq 1 \mu\text{g/ml}$ for Amphotericin B and Itraconazole). For Nystatin and Caspofungin no clinical breakpoints have yet been defined.

2.2.9 Storage

All isolates were stored in a Cryobank (Mast Diagnostica[®]) - System at -70 °C.

2.2.10 Statistics

Statistical significance was calculated using Student's T-test for quantitative variables, Chi-Square test for percentages and for small samples sizes the Fisher's exact test, where p-values <0.05 were considered as significant.

3. Results

3.1 Patient cohort

3.1.1 Distribution according to age and gender

A total number of 589 patients were seen during the study period. The age distribution was between two and 70 years, whereas the majority of the patients was between 19 and 35 years old (n=263). The average age was 34 in Maingara and 28 in Bemouli. Children below 12 years of age were represented in Maingara with 1.2% (n=5) and 2.0% (n=3) in Bemouli. From the five children in Maingara, two were HIV-positive and three HIV-negative. Seven patients were above the age of 60: three from Maingara, out of which one patient was HIV-positive. For a small fraction of patients the exact age remained unknown. In all age groups, female patients were dominant. In total, the distribution consisted of 73.5% (n=433) female and 26.5% (n=156) male patients (Figure 7).

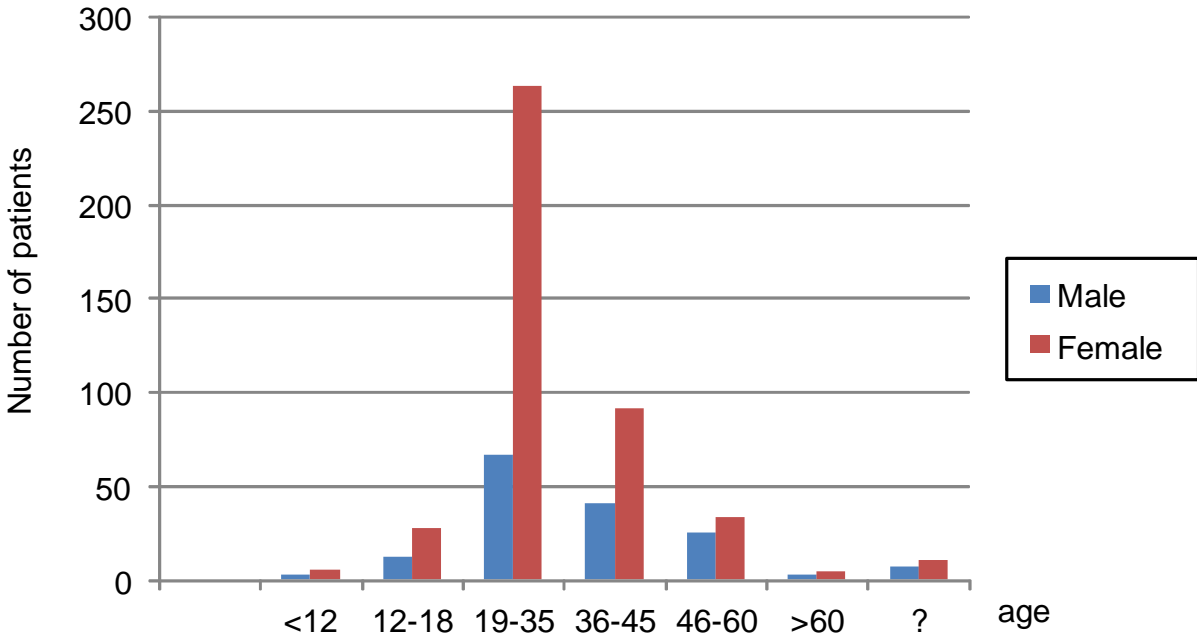


Figure 7: Distribution of the patients according to their age and gender in the general cohort. Patients aged 19-35 (mean 34) and the female gender was most represented.

3.1.2 Distribution of the patients according to their HIV status

From the total number of 589 patients consulted, 441 were from the clinic in Maingara and 148 from the medical dispensary (health center) in the village of Bemouli. From the cohort consulted in Maingara, 384 (87.1%) patients were HIV-positive. The patients for the control group of HIV-negative patients were recruited from Maingara (27.8%; n=57) and Bemouli (72.2%; n=148).

For the majority, patients recruited from Bemouli remained untested. HIV testing had not been performed among these patients, due to ethical considerations, as the medical dispensary was a small normal health care point providing basic health attendance by a nurse for a normal population. HIV prevalence in rural areas of Chad was very low and affected individuals or severe ill cases used to directly go to the specialized centers or hospitals in the urban area (CNLS 2012). The cohort of patients from Bemouli was therefore presumed to be HIV-negative. In the eleven cases of the patients where yeast growth was observed, HIV testing was performed, after their informed consent. All were confirmed to be HIV-negative, supporting the assumption that this clientele could be used as an additional HIV-negative control group. Furthermore clinical oral impression was without suspicious peculiarities. Patients from Bemouli were therefore considered HIV-negative for the purpose of this study and as there were no relevant significant differences between the HIV-negative patients from Maingara and Bemouli, these patients were combined into one group.

3.1.3 Gender distribution in the different subgroups

As in the overall cohort, in the HIV-positive and HIV-negative group female patients were most represented with 75 % (n=287) in the HIV-positive and 71% (n=146) in the HIV-negative group (Figure 8).

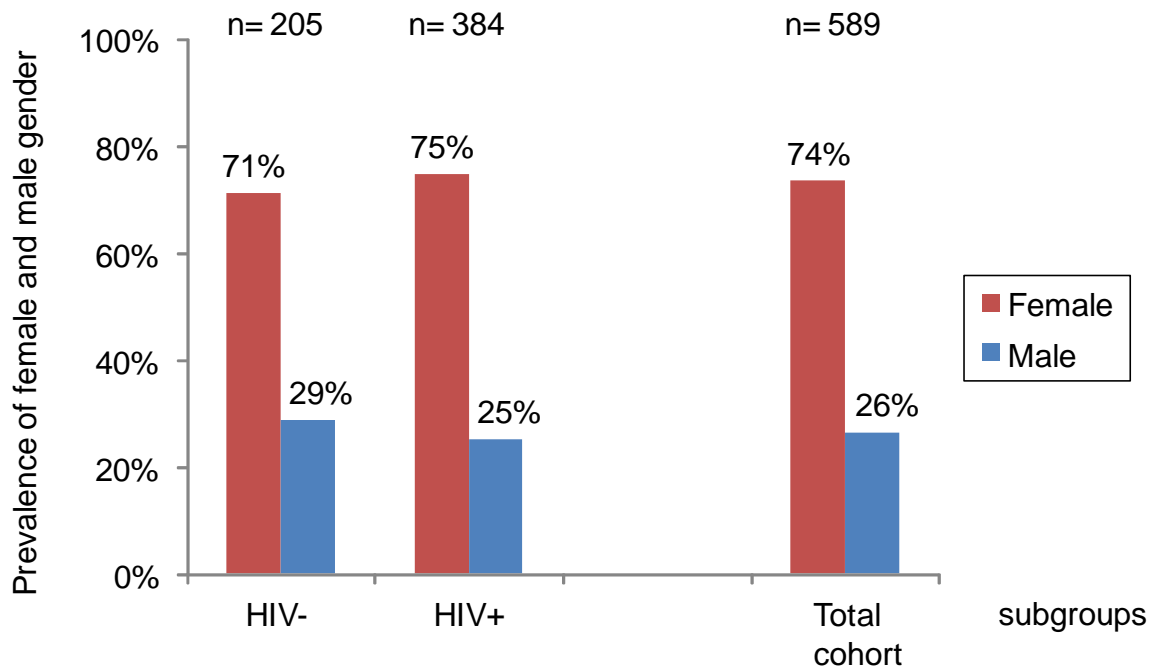


Figure 8: Distribution of the gender in the different subgroups. HIV-positive patients were recruited only from the clinic in Maingara, HIV-negative individuals mainly from Bemouli. Female patients were significantly more represented than male patients in all subgroups. HIV+= HIV-positive; HIV-= HIV-negative; n= number of patients.

3.2 Prevalence of fungal colonization

In a first step, we analyzed to what degree the different patient subgroups (HIV-positive with and without HAART, HIV-negative control) were colonized with oral yeasts. For this analysis only samplings from the first visit of each patient were analyzed and categorized. Patients who had received antifungal or antibacterial treatment within the last three weeks before and at time of the first sampling were fully excluded from this evaluation and patients having received less than 25 days of HAART were excluded from the HAART+ group. (In a separate chapter (3.11) patients with antibacterial but without antifungal treatment will be included again for the analysis of the influence antibacterial treatment on oral colonization). After implementing all exclusion criteria, a total of 534 patients were included for further analysis: 343 HIV-positive, 56 HIV-negative from Maingara and 135 HIV-negative from Bemouli. 52.5% (n=180) of the HIV-positive patients were under HAART. The age and gender distribution in the HIV-positive subgroups were similar to the HIV-positive overall cohort.

A total of 130 swabs from the 534 patients included in the study were yeast-positive (=24.4%) at the patients' first visit. There was no significant difference in oral yeast colonization between the different subgroups: HIV-positive patients only had a slightly higher prevalence of oral yeast colonization (25.4%) than HIV-negative patients (22.5%). There was also no significant difference between the HIV-negative patients from Maingara and Bemouli (data not shown).

HIV-positive patients were therefore further divided into two subgroups: "with HAART" (HAART+) for those who had received HAART for at least 25 days and "without HAART" (HAART-) for the patients, who were without HAART at the time of and prior to examination. Here we found a significant difference between HAART treated and HAART non-treated patients: HIV+/HAART+ patients had a significantly ($p=0.003$) lower colonization rate (18.9%; $n=34$) than HIV+/HAART- patients (32.7%; $n=53$), but not ($p=0.111$) as compared to the HIV-negative control (22.5%; $n=31$) (Figure 9).

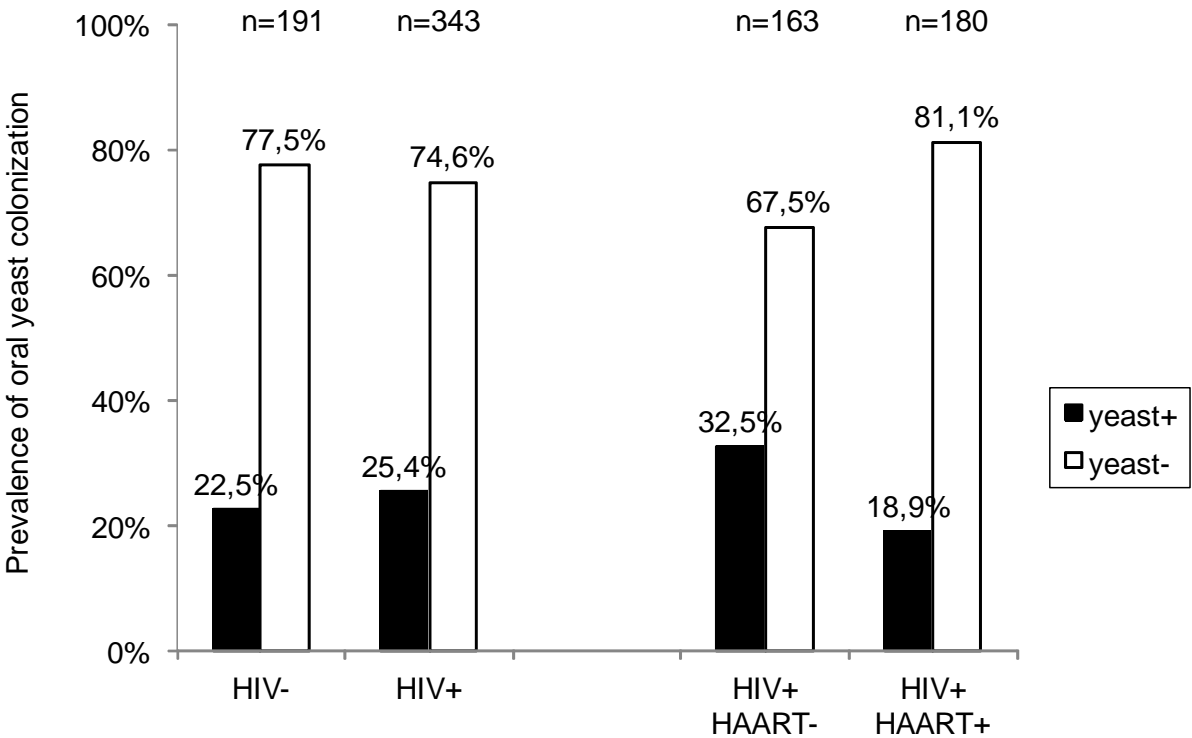


Figure 9: Prevalence of yeast growth in the oral cavity in the different subgroups. HIV+/HAART+ patients had a significant lower colonization rate than HIV+/HAART- patients, but similar to the control group. n= number of patients; yeast+= yeast-positive; yeast-= yeast-negative.

3.3 Degree of oral fungal burden in HIV-negative and -positive patients

A semi-quantitative analysis of the degree of fungal burden based on CFU counts on the agar plate of the positive oral swabs between the study group and the control group revealed no significant difference ($p=0.7$). The rate of high fungal burden (HFB; $CFU>16$ on a $1/8^{\text{th}}$ agar plate) was 32.2% for the HIV-positive and 35% for the HIV-negative patients (Figure 10). In contrast, within the HIV-positive subgroups, patients receiving HAART had a significantly ($p=0.02$) lower prevalence of HFB (18%, $n=6$) than those without HAART (42%; $n=22$), but not significantly lower than the controls ($p=0.09$) (Figure 10).

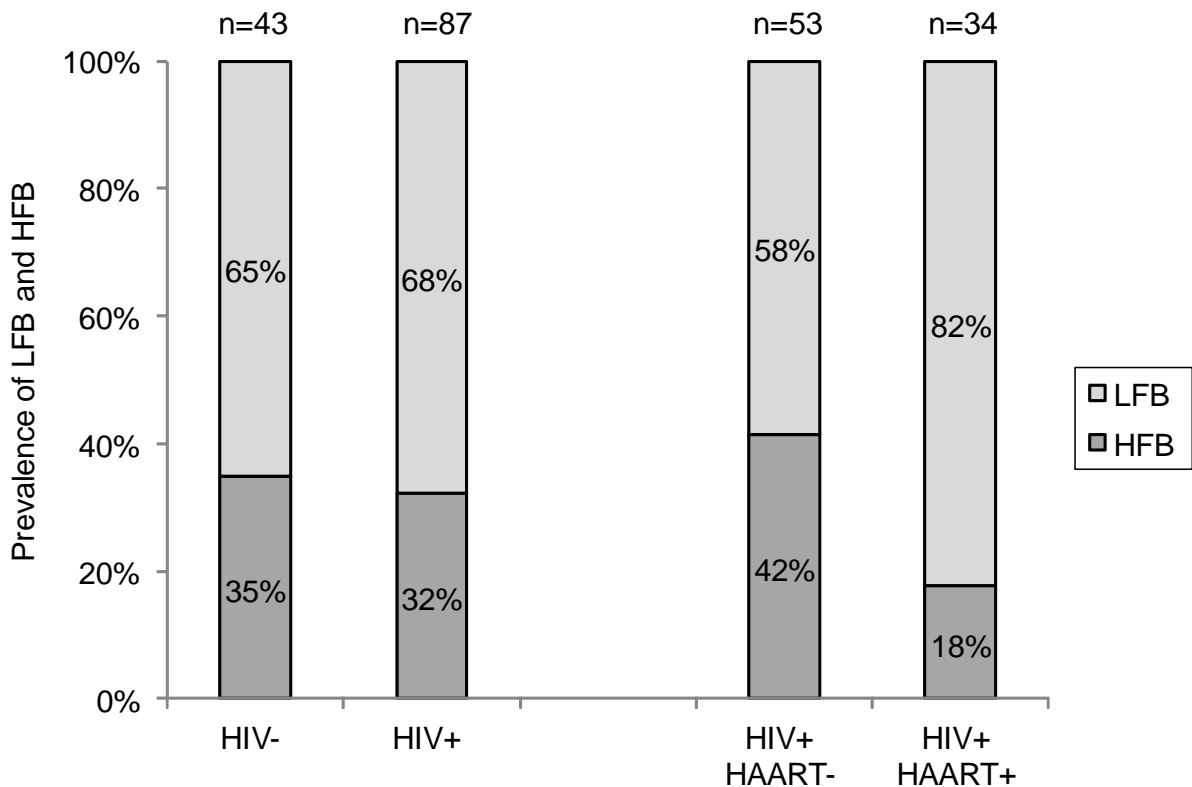
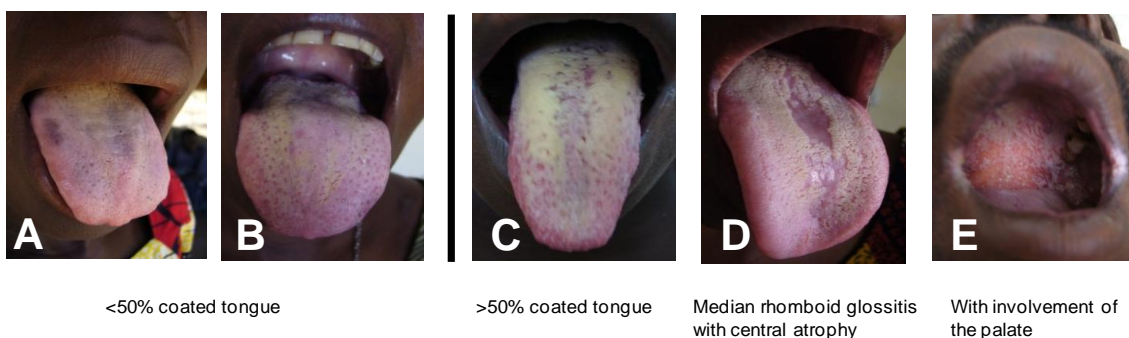


Figure 10: Prevalence of low and high fungal burden in the positive oral swab. There was a significant difference in oral fungal burden between HIV+/HAART+ and HIV+/HAART- patient. HIV+/HAART+ patients had a significantly ($p=0.02$) lower rate of high fungal burden (18%) than HIV+/HAART- patients (42%). n = number of patients, LFB= low fungal burden= colonization with 1-15 CFU/ $1/8^{\text{th}}$ of an agar plate, HFB= high fungal burden= colonization with >15 CFU/ $1/8^{\text{th}}$ of an agar plate.

3.4 Classification of oral symptoms and diagnosis of oral candidiasis

Diagnosis of OC was more challenging than expected, as the typical clinical presentation of OC ("whitish plaque which can be whipped off easily revealing erythematous area beneath") (EC-Clearinghouse 1993) was rare. Subsequently, we therefore based our diagnostic and classification criteria on those proposed by Lehner (Lehner 1964; Parihar 2011) who classified OC based on clinical, mycological, histological, serological and therapeutic criteria. As additional tests such as exfoliative cytology or tissue biopsy for confirmation or rejection of OC were not available on site and antimycotics were rare, we classified our patients only according to the clinical observations and the result of the culture from the oral swab. Based on the symptoms observed and additionally inspired by the scoring index for oral mucositis proposed by McGuire et al. (2002) patients were first subdivided into the three following subgroups:

- **noS** = **asymptomatic**: no visible alterations in the oral cavity
- **mS** = **mild symptoms**: whitish or yellowish coated tongue with <50% affected area (Figure 11A+B).
- **sS** = **severe symptoms**: one of the following symptoms or the combination of them: thick whitish or yellowish coated tongue with >50% affected area and/or atrophy and/or erythema and/or other mucosal sites affected like palate or side cheek pockets (Figure 11C-E).



A+B: mild symptoms

C-E: severe symptoms

Figure 11: Examples of classified symptomatic patients. Patients classified with "mild symptoms" only had a tongue coating affecting <50% of the tongue (A+B); patients with "severe symptoms" a thick white or yellowish coating affecting >50% of the tongue area (C), and/or atrophy (D), and/or additionally other mucosal sites affected (E).

Together with the results from mycological culture (yeast negative vs. yeast positive), four different patient groups were defined (Table 2):

- **Yeast-** : all patients with absence of yeast (irrespective of symptoms)
= non-carriers
- **YnoS** : Yeast positive patients with no symptoms
= asymptomatic yeast carriers
- **YmS** : Yeast positive patients with mild symptoms
= yeast carriers with mild symptoms
- **YsS** : Yeast positive patients with severe symptoms
= yeast carriers with severe symptoms

Only patients from group YsS will further be discussed and classified as patients with oral candidiasis (Table 2).

Table 2: Classification of the patients including clinical presentation and oral swab culture

Swab culture	Symptoms	Classification	
Yeast-	Asymptomatic or symptomatic	Yeast-/no, mild or severe symptoms	uncolonized
Yeast+	Asymptomatic	Yeast+/no symptoms (YnoS)	colonized
	Symptomatic	Yeast+/mild symptoms (YmS)	colonized?
		Yeast+/severe symptoms (YsS)	Oral candidiasis (OC)

According to the symptoms observed and the result of the oral swab, four different clinical symptom groups were defined.

3.5 Prevalence of symptoms and oral yeast colonization in the different symptomatic groups

3.5.1 Prevalence of symptoms

HIV-positive patients were significantly more often symptomatic than the controls ($p=0.03$). The respective prevalence of symptoms in HIV-positive patients was 40% and 30% in HIV-negative patients. There was no significant difference in the prevalence of symptoms between HIV+/HAART- (44%) and HIV+/HAART+ (36%) patients ($p=0.13$), but a significant difference in the prevalence of severe symptoms (HIV+/HAART-: 26% and HIV+/HAART+: 12%; $p\leq 0.01$). HIV+/HAART- patients also had a significantly higher prevalence of severe symptoms (26%) than the controls (15%) ($p\leq 0.01$) (Figure 12).

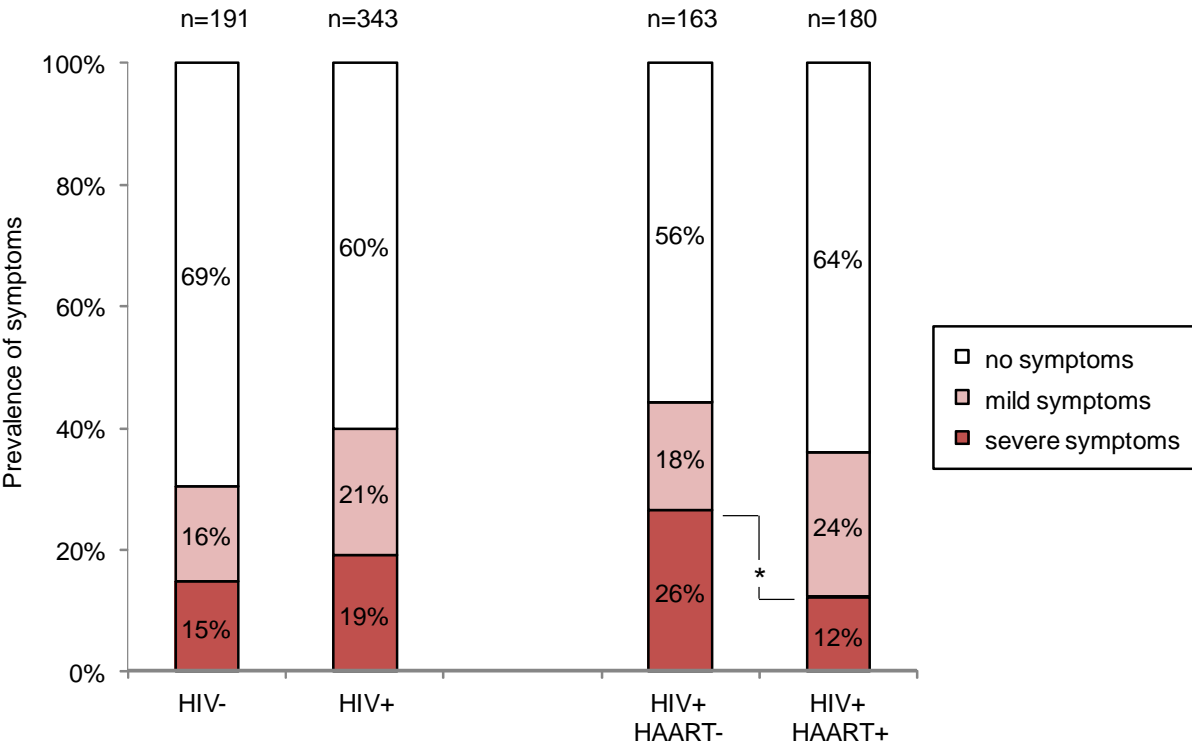


Figure 12: Prevalence of symptomatic patients in the different subgroups. HIV+/HAART- patients were significantly more often symptomatic than the controls ($p\leq 0.01$) and had significantly more severe symptoms than HIV+/HAART+ patients ($*= p\leq 0.01$) and HIV-negative patients ($p\leq 0.01$). n= number of patients.

3.5.2 Prevalence of yeasts in the oral cavity of asymptomatic patients

Asymptomatic HIV-negative and HIV-positive patients were in up to 80% of the cases yeast-free and there was no significant difference ($p=0.09$) in the prevalence of oral yeast carriage in the HIV-positive subgroups (Figure 13).

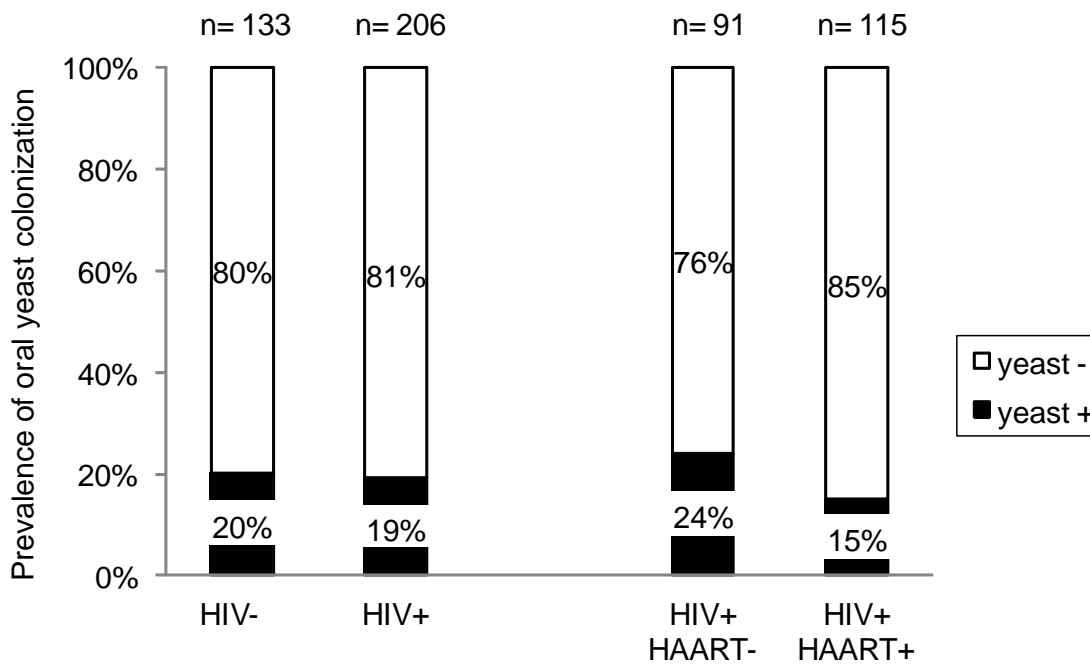


Figure 13: Prevalence of yeast in the oral cavity of asymptomatic patients. Asymptomatic HIV-negative and HIV-positive patients were mainly (in up to approximately 80% of the cases) free of yeast colonization. n= number of patients

3.5.3. Prevalence of yeasts in the oral cavity of symptomatic patients

In HIV-positive patients the severity level of symptoms significantly correlated with the presence or absence of yeasts in the oral cavity ($p \leq 0.01$ for HIV+/HAART- and $p=0.05$ for HIV+/HAART+ patients). The oral cavity of patients with mild symptoms was in 83% (HAART-) and 81% (HAART+) of the cases yeast-free and up to 60% (HAART-) and 41% (HAART+) of the patients with severe symptoms where yeast-positive. In HIV-negative patients that correlation was not obvious (Figure 14).

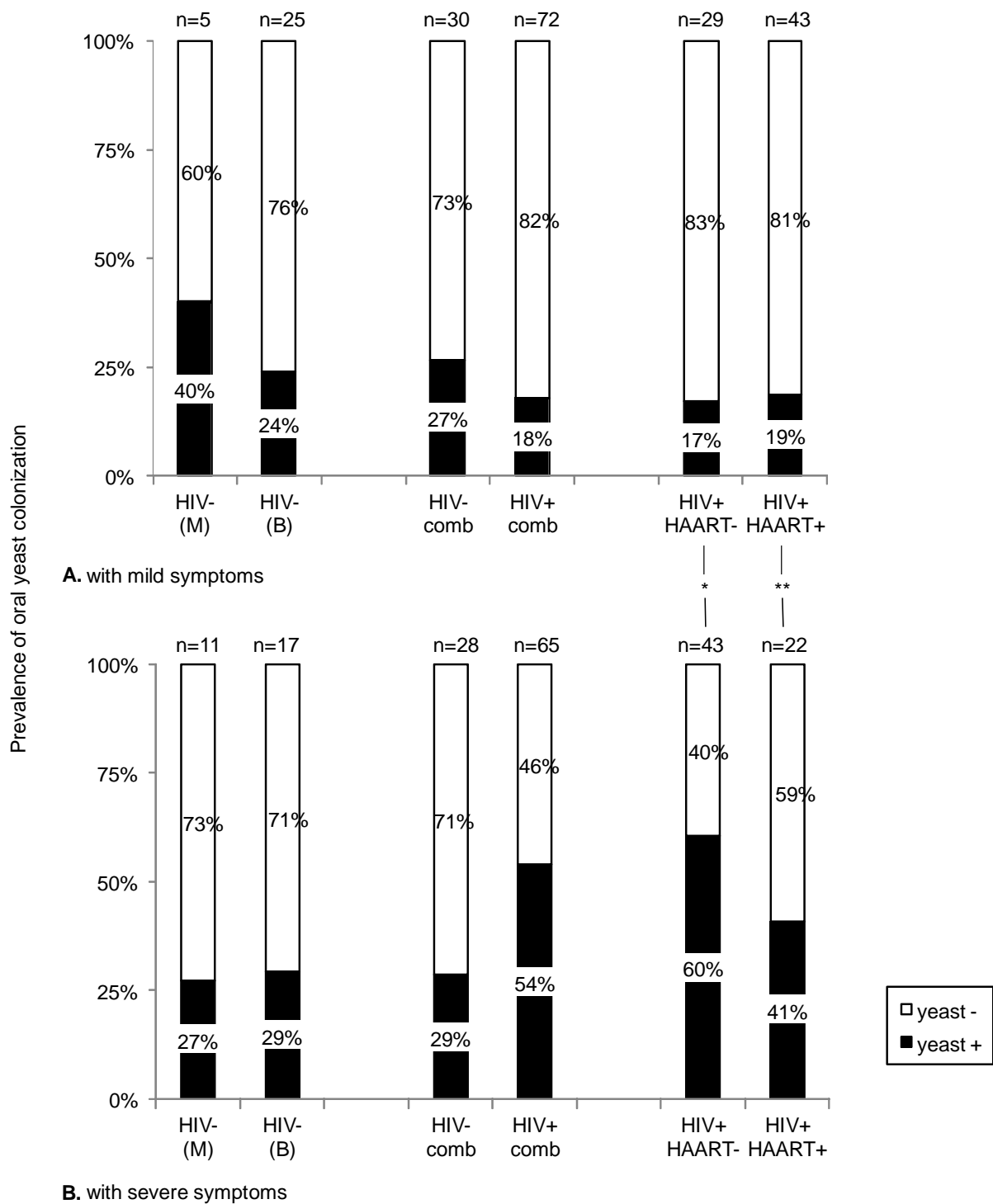


Figure 14: Prevalence of yeast in the oral cavity in symptomatic patients. Mild symptoms in HIV-positive patients were more associated with the absence of yeasts in the oral cavity and severe symptoms significantly correlated with the presence of oral yeast colonization in HIV+/HAART- (*= $p \leq 0.01$) and HIV+/HAART+ patients (**= $p = 0.05$). **A.** patients with mild symptoms, **B.** patients with severe symptoms; yeast-= yeast-negative; yeast+= yeast-positive; n= number of patients.

3.6 Association between oral fungal burden and symptoms in the different subgroups

In all subgroups, asymptomatic yeast carriers (YnoS) mainly had a low fungal burden. In HIV-negative patients there was a higher prevalence of high fungal burden in patients with mild to severe symptoms (YmS and YsS). HIV+/HAART+ symptomatic patients had the lowest prevalence of high fungal burden and only in HIV+/HAART- patients high oral fungal burden was significantly associated with severe symptoms ($p \leq 0.01$) (Figure 15).

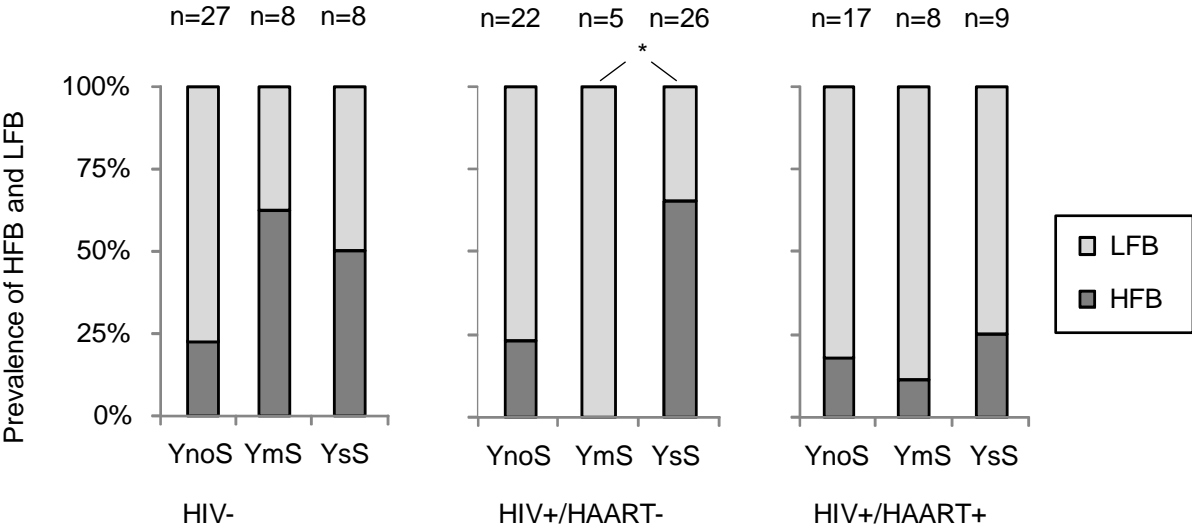


Figure 15: Prevalence of high oral fungal burden according to symptoms in the different subgroups. A high oral fungal burden was significantly associated with severe symptoms in HIV+/HAART- patients (*= $p \leq 0.01$). In HIV+/HAART+ patients this association was not obvious. YnoS= yeast positive/no symptoms; YmS= yeast positive/mild symptoms; YsS= yeast positive/severe symptoms; LFB= low fungal burden; HFB= high fungal burden.

3.7 Prevalence of oral candidiasis in the different subgroups

For the patients classified with mild symptoms, the only symptoms noted were a mildly coated tongue (<50% of the area). Swab cultures showed a low prevalence of yeast colonization (Figure 14A) and colonized patients from that group (YmS) only had a low fungal burden (Figure 15). It is therefore highly probable, that these patients were only colonized with *Candida*, so that a classification into any form of OC was not undertaken in this group, but rather classified as "colonized patients".

Only yeast-positive patients with severe symptoms (YsS) were classified as patients with oral candidiasis. The prevalence of OC was highest in the HIV+/HAART- group with 16% and was reduced in the HIV+/HAART+ group to 5%, which was similar to the one in the HIV-negative group (4%). The prevalence of asymptomatic yeast-carriers (YnoS) was the same in HIV+/HAART- patients as in HIV-negative patients (14%) and slightly reduced in HIV+/HAART+ patients (9%) (Figure 16).

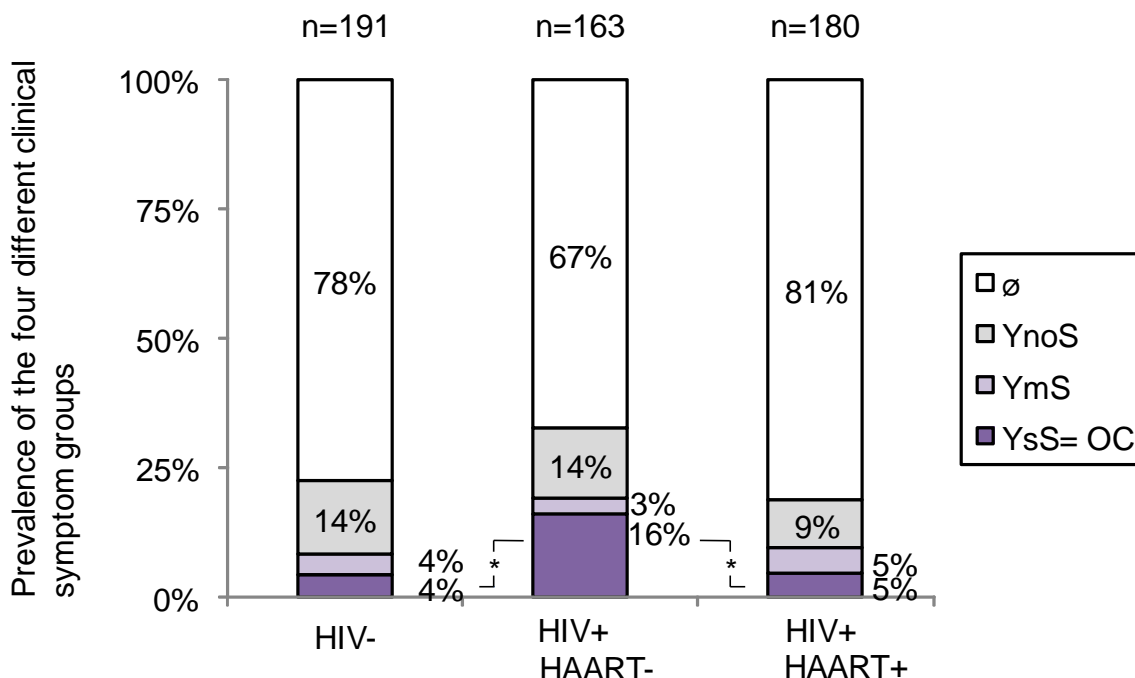


Figure 16: Prevalence of oral candidiasis. Yeast-positive patients with severe symptoms (YsS) were considered as patients with oral candidiasis (OC) and were significantly more prevalent in the HIV+/HAART- group with 16% (*= p≤0.01). ∅= yeast-negative patients; YnoS= asymptomatic yeast-carriers; YmS= yeast-carriers with mild symptoms; YsS= yeast-carriers with severe symptoms=patients with OC; n= number of patients.

3.8 Prevalence of the different forms of oral candidiasis in HIV-positive patients

Only yeast-carriers with severe symptoms (group YsS), as defined above in 3.4 and 3.7, were further considered and discussed for the diagnosis of OC. In this group, we observed different clinical presentations among our HIV-positive patients, which we classified according to the classification proposed by Axell et al. (Axell et al. 1997; Parihar 2011) (Table 3).

Table 3: Classification of oral candidiasis by Axell et al.

Primary oral candidosis (Group I)	Secondary oral candidoses (Group II)
<p>Acute Pseudomembranous Erythematous</p> <p>Chronic Erythematous Pseudomembranous Hyperplastic Nodular Plaque-like</p> <p>Candida-associated lesions Angular cheilitis Denture stomatitis Median rhomboid glossitis</p> <p>Keratinized primary lesions superinfected with Candida Leukoplakia Lichen planus Lupus erythematosus</p>	<p>Oral manifestations of Systemic mucocutaneous candidosis (due to diseases such as thymic aplasia and candidosis endocrinopathy syndrome)</p>

This table represents the revised classification of oral candidiasis by Axell et al. (Axell et al. 1997; Parihar 2011) on which we based our definition and classification of OC.

The majority of these patients presented with a coated (whitish) tongue affecting >50% of the area (48.6%; n=17/35) in HAART- as well as in HAART+ patients (Table 4). However, the white lesions were not easily removable in our patients and had no visible signs of inflamed erythematous area beneath. Therefore these cases were not classified as acute pseudomembranous candidiasis (PC). The chronic "plaque-like" OC (also called "hyperplastic" or "nodular" candidiasis) and keratinized primary oral

lesions superinfected with *Candida* as hairy leukoplakia or lichen planus were also taken into consideration. Due to lack of further investigations such as tissue biopsy or data about the onset of the symptoms this could, however, not be confirmed. As these variants were also clinically less likely, patients with the symptoms of white lesions as described above were classified as nonspecific OC.

The second most common clinical sign was the median rhomboid glossitis (MRG) (Figure 11D): white plaque on the tongue with central atrophy or erythematous area. Its prevalence was 25.7% (n=9/35). Nearly all of these cases appeared in the HIV+/HAART- group (n=8/9) and the median CD4 T cell count was 200 CD4 T cells/ μ l (Table 4). Furthermore, we had 5 cases (14.2%) of clinically diagnosable acute pseudomembranous candidiasis with additionally affected palate. These patients had a median CD4 T cell count <120 CD4 T cells/ μ l. Two cases had combined oral lesions (PC with MRG and PC with atrophy); their CD4 cell counts were <60 CD4 T cells/ μ l and two cases solely tongue atrophy (lingual papillary atrophy). The median CD4 T cell count of the latest was 338 CD4 T cells/ μ l.

Table 4: Distribution of the different clinical presentations of oral candidiasis in HIV-positive patients

	HIV+ HAART-	Median CD4 T cell count (cells/ μ l)	HIV+ HAART+	Median CD4 T cell count (cells/ μ l)	Total
Nonspecific OC	31.5%	149	17.1%	126	48.6%
Acute PC	8.6%	115	5.7%	60*	14.3%
MRG	22.9%	200	2.8%	n.d	25.7%
Atrophy	5.7%	338	0	-	5.7%
Mixture	5.7%	51	0	-	5.7%
Total	74.3% (n=26)		25.7% (n=9)		100% (n=35)

The majority presented with a coated whitish tongue affecting >50% of the tongues' surface classified as nonspecific OC. Second most frequent clinical presentation was the MRG, mainly in HIV+/HAART- patients. Acute PC= acute pseudomembranous candidiasis; MRG= median rhomboid glossitis; mixture= combination of acute PC with atrophy or MRG; n.d= not determined; * CD4 T cell count from one patient.

Except for the patients with symptoms of papillary tongue atrophy, all patients from group YsS (with OC) had a median CD4 T cell count ≤ 200 CD4 T cells/ μ l (Table 4 and Figure 17), reinforcing and supporting the diagnosis of OC in that group.

HIV-positive patients with severe symptoms who were yeast-negative had a median CD4 T cell count >250 CD4 T cells/ μ l (Figure 17) and the correlation between the absence of yeasts in the oral cavity of these patients and CD4 T cell counts >250 CD4 T cells/ μ l was significant ($p \leq 0.01$).

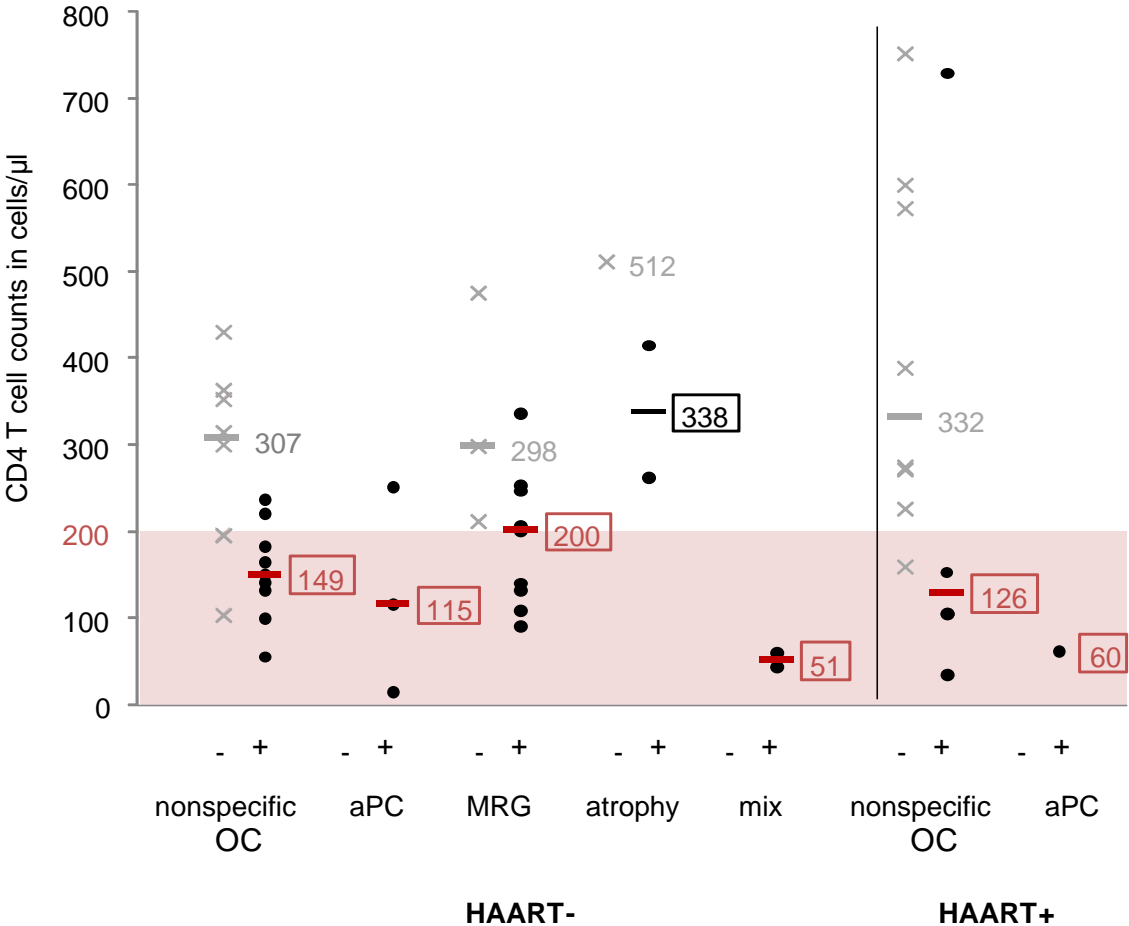


Figure 17: CD4 T cell count and clinical subtypes of oral candidiasis in HIV-positive patients. Patients with OC had a median CD4 T cell count ≤ 200 CD4 T cells/ μ l, except for those with papillary tongue atrophy. In cases of OC-like lesions but no presence of oral yeast growth, median CD4 T cell count was always >250 CD4 T cells/ μ l. - = yeast-negative, + = yeast-positive; nonspecific OC= nonspecific oral candidiasis, aPC= acute pseudomembranous candidiasis, MRG= median rhomboid glossitis, atrophy= papillary tongue atrophy, mix= combination of aPC with atrophy or MRG; horizontal bars indicate median CD4 T cell count; colored area indicates CD4 T cell counts ≤ 200 CD4 T cells/ μ l.

3.9 Oral colonization and age

There was no correlation or significance between age and colonization with yeasts in the different subgroups (Figure 18), although the median age of colonized HIV-negative patients (31 years) was slightly higher than the uncolonized ones (28 years).

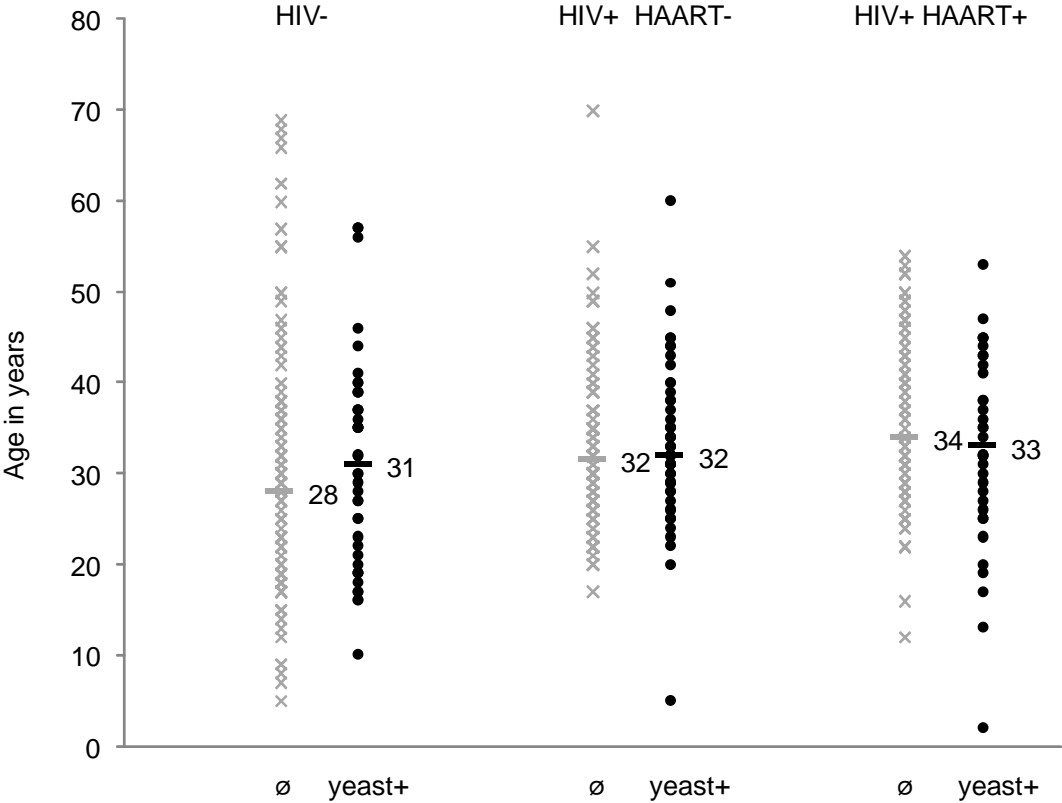


Figure 18: Age-dependent oral yeast colonization. In the control group median age of patients with oral yeast growth was slightly higher than those without yeasts. In HIV-positive patients no significant differences were observed. ø= yeast- . Horizontal bars indicate median age.

If we looked at the different age groups in the different subgroups (Figure 19), in HIV-negative, as well as in HIV+/HAART- patients, the prevalence of oral yeast colonization was highest in the age group of 36-45. In the HIV+/HAART+ group the prevalence was highest in the 19-25 age group and decreased with higher age which correlates with a longer time on HAART for the 19-45 years old. With increasing age, the patients of that group had an increased average of days of HAART: 19-25 (323 days on HAART); 26-35 (483 days); 36-45 (539 days).

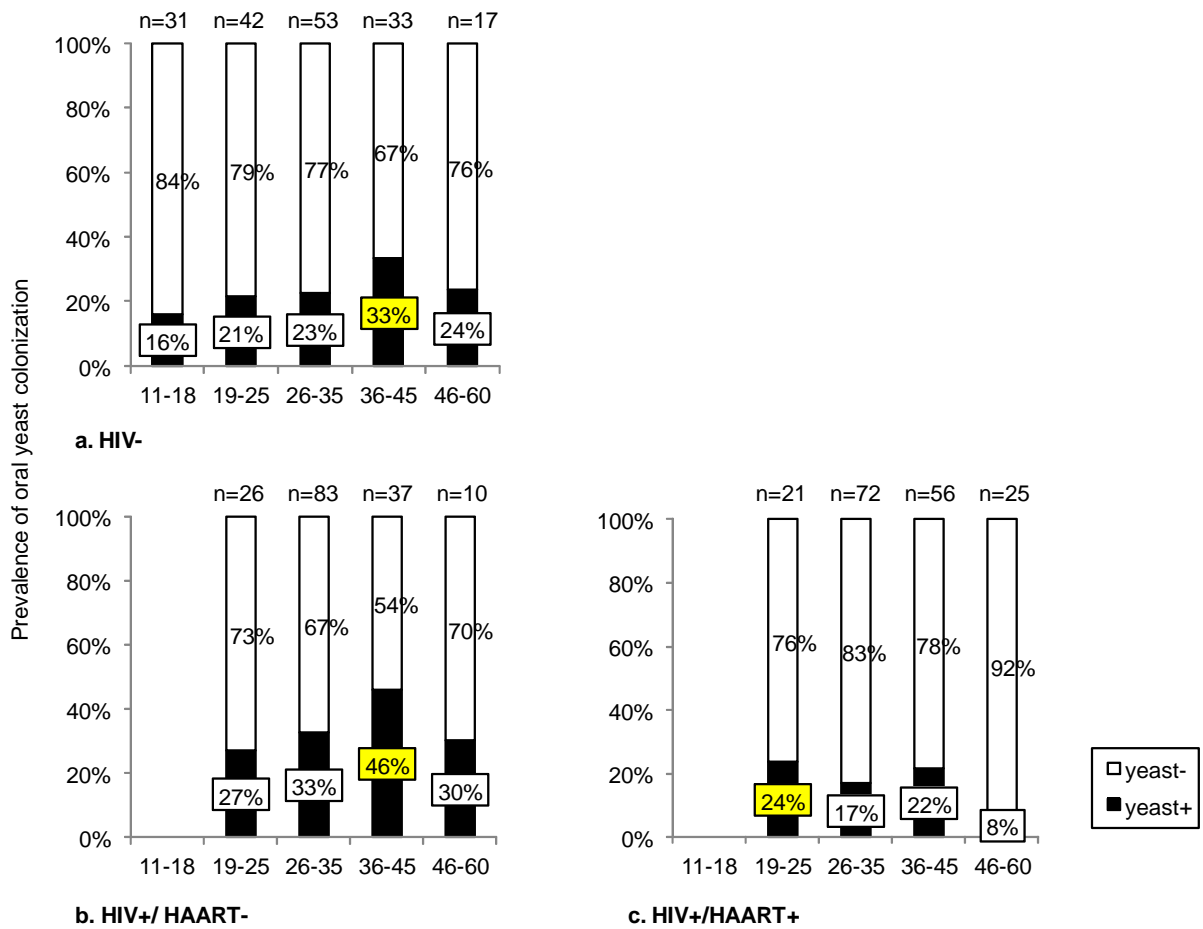


Figure 19: Prevalence of yeasts in the oral cavity according to age. In HIV- and HIV+/HAART- patients the highest prevalence of oral yeast growth was in the age group of 36-45, whereas in the HIV+/HAART+ group it was in the 19-25 age group. With rising age in HIV+/HAART+ patients (which correlated with a longer period of HAART), there was a decrease in oral colonization rate. n= number of patients.

3.10 Oral yeast colonization and fungal burden in HIV-negative and -positive female and male patients

There was no significant gender-dependent difference in the prevalence of oral yeast colonization in HIV-positive and -negative patients (Figure 20A). Although, yeast-positive male subjects had a higher oral fungal burden than female subjects in both groups; the difference was significant only in the HIV-negative group ($p=0.01$) (Figure 20B)

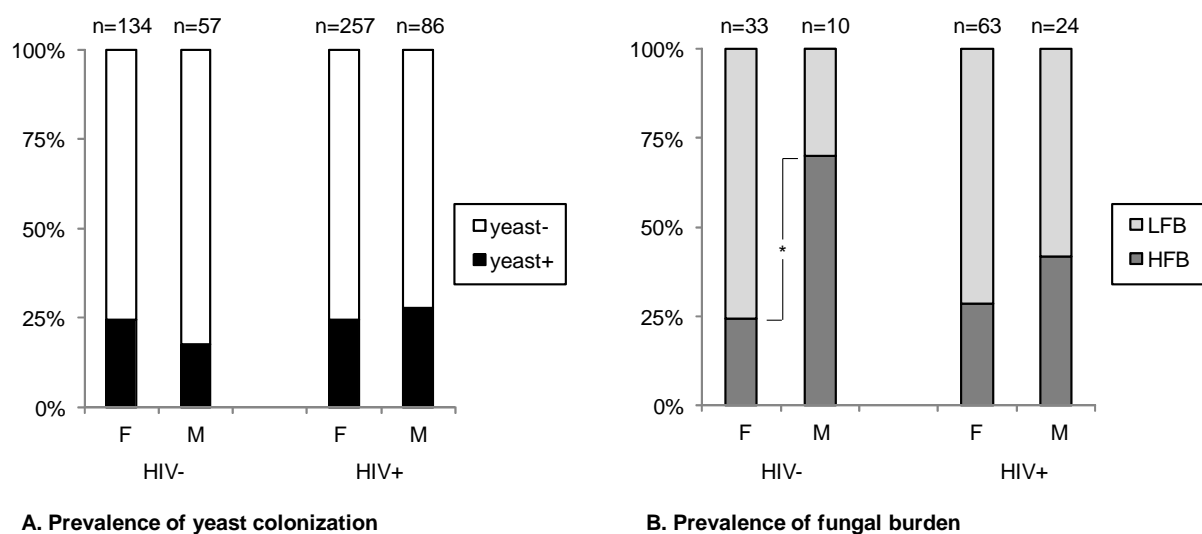


Figure 20: Prevalence of oral yeast colonization and fungal burden in HIV-negative and -positive female and male patients. In HIV-negative and HIV-positive patients there were no significant differences in the prevalence of oral yeast colonization between male and female. But in the HIV-negative group male patients had a significant higher oral fungal burden than female patients ($*= p=0.01$). n= number of patients; F= Female, M= Male; yeast- = yeast-negative; yeast+= yeast- positive; LFB= low fungal burden; HFB= high fungal burden.

3.11 Influence of antibiotics on oral colonization and infection with yeasts

We also analyzed the impact of antibiotics on fungal burden in the oral cavity among our patients. For this we excluded those patients which had taken antifungals at the time of or in the last three weeks before examination. Only drugs with antibacterial effectiveness at time of the first visit or in the last three weeks before were considered. Five different classes of antibiotics were in use: Co-trimoxazole, β -lactam antibiotics (Penicillin, Amoxicillin, Cloxacillin, Ampicillin), Ciprofloxacin, Metronidazole and Doxycyclin. Co-trimoxazole and β -lactam antibiotics were the most frequently prescribed. HIV+/HAART- patients more frequently received an antibiotic treatment. In total 26 (7.0%) out of 369 HIV-positive and 13 (8.8%) out of 148 HIV-negative patients had received an antibacterial treatment. Of these 26 HIV-positive patients, nine (34.6%) had a positive yeast culture, out of which eight (89%) were from the HIV+/HAART- group, and 17 (65.4%) had no presence of yeast growth. Although HIV+/HAART- patients were more frequently yeast-positive, in the different patients group there were no significant differences in the prevalence of oral colonization between patients with and without antibiotic treatment ($p=1.0$ (HIV-); $p=0.3$ (HIV+/HAART-) and $p=0.65$ (HIV+/HAART+) (Table 5).

Table 5: Prevalence of oral yeast colonization with and without antibiotics

	HIV-		HIV+/HAART-		HIV+/HAART+	
	no AB	AB	no AB	AB	no AB	AB
Yeast-	77%	77%	67%	56%	81%	87.0%
Yeast +	23%	23%	33%	44%	19%	13%
Significance	$p=1.0$		$p=0.3$		$p=0.65$	

HIV+/HAART- patients were more likely to be colonized with yeasts than HIV- or HIV+/HAART+ patients, but in general treatment with antibiotics had no significant influence on oral colonization with yeasts in all three subgroups.

3.12 Prevalence and distribution of yeast species

In total, 156 yeast isolates from 166 positive oral swabs of the HIV-positive and -negative patients were differentiated. 17 samples, due to longer storage, could not be recovered but 7 out of these could be differentiated by PCR.

In total, 171 yeasts were isolated and differentiated from the 156 yeast isolates. In all patient groups, *C. albicans* was the most prevalent species: 44.2% in HIV-negative (56.3% in the Maingara and 37.0% in the Bemouli HIV-negative groups; data not shown) 58.6% in HIV+/HAART+ and 87.1% in HIV+/HAART- patients (Figure 21). The HIV+/HAART+ and the HIV-negative patients had a significantly lower prevalence of *C. albicans* and a larger variety of non-*C. albicans* yeasts than the HIV+/HAART- patients ($p \leq 0.01$).

The next most prevalent yeast species were *I. orientalis* (12.1%; n=7) followed by *C. tropicalis* (8.6%; n=5) and *Saccharomyces cerevisiae* (*S. cerevisiae*) (6.9%; n=4) in the HIV+/ HAART+ group and *C. tropicalis* (4.3%; n=3) followed by *S. cerevisiae* (2.9%; n=2) in the HIV+/ HAART- group. In the combined HIV-negative group the second next most prevalent species was *S. cerevisiae* (14%; n=6) (Figure 21). Although, in the HIV-negative patients from Maingara *C. glabrata* was the second most frequent species. Among the HIV-negative subjects from Bemouli, no *C. glabrata* was found; instead, *S. cerevisiae* (18.5%; n=5) and *Pichia fabianii* (*P. fabianii*) (14.8%; n=4) were the next most prevalent species (data not shown).

Other isolated species in the HIV-positive group were: *C. parapsilosis*, *C. orthopsilosis*, *P. fabianii*, *P. farinosa* (= *C. cacaoi*), *P. guilliermondii*, *C. kefir* and *C. rugosa*. A *C. orthopsilosis*-like species together with *C. tropicalis* was isolated from the oral cavity of a HIV-positive patient undergoing HAART. In the HIV-negative groups further isolated species were *C. orthopsilosis*, *C. valida* and *C. pararugosa*. *C. dubliniensis* was not isolated from any of the patients, irrespectively of HIV status.

Across all subgroups, mixed colonization with \geq two yeast species was observed: seven patients (13.7%) in the HIV+/HAART+ group, three (4.4%) in the HIV+/HAART- group and four (10.5%) in the HIV-negative group.

In the HIV+/HAART- group, mixed colonization always included *C. albicans*, whereas in the HIV+/HAART+ group, mixed colonization predominantly included non-*C. albicans* species (Table 6).

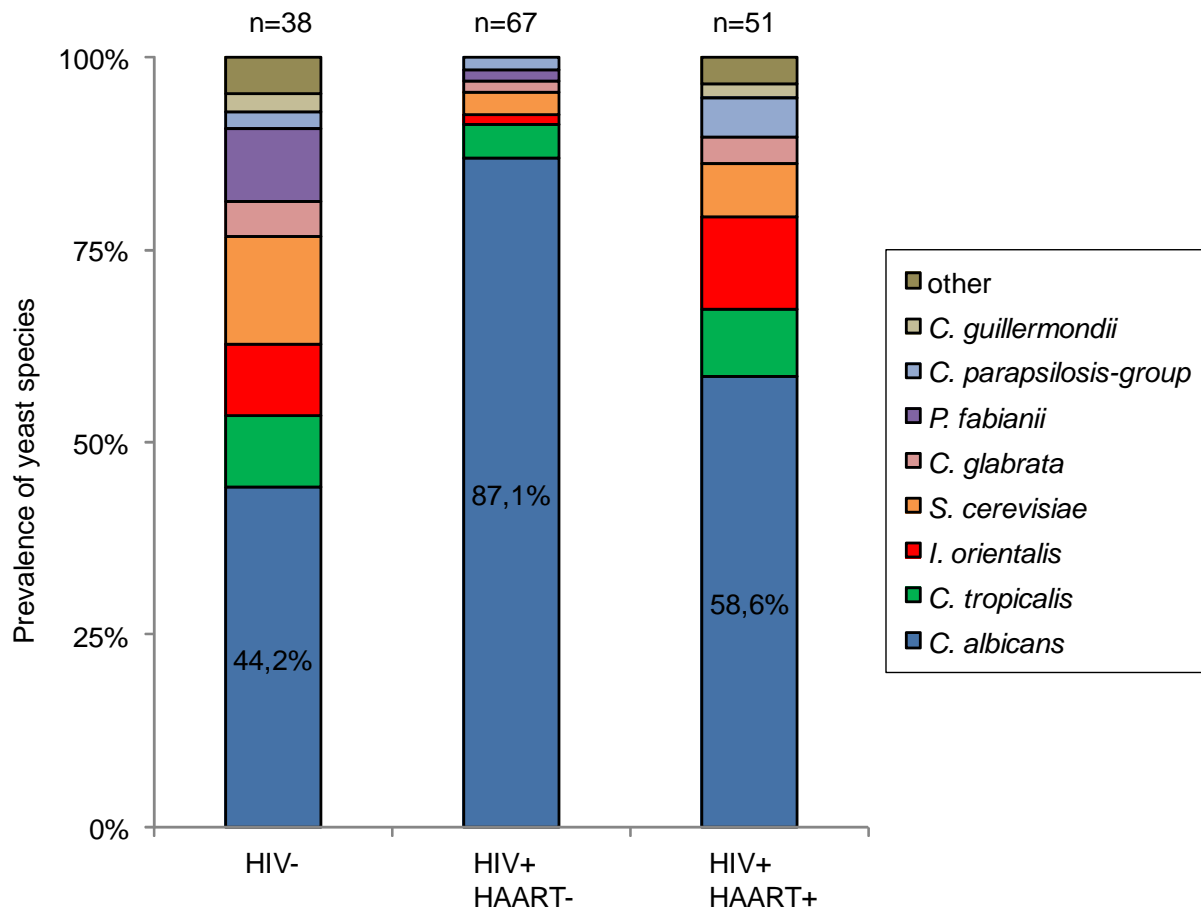


Figure 21: Species distribution. The number of patients with mixed colonization was: n=4 in the HIV- combined, n=3 in the HIV+/HAART- and n=7 in the HIV+/HAART+ group. The number of species which were not able to be recultivated from the transported agar slants was: n=5 in Bemouli, n=2 in the HIV+/HAART-, n=3 in the HIV+/HAART+ group. *C. albicans* (C.a) was the most prevalent species in all subgroups. HIV- and HIV+/HAART+ patients presented with a higher species diversity and lower prevalence of C.a. n= number of patients.

Table 6: Patients with simultaneous colonization of ≥ 2 species

Gender	Age	HIV status	HAART	Days of HAART	CD4 (cells/ μ l)	Swab I	Swab II
F	20	+	+	330	432	<i>C. tropicalis</i> <i>C. othopsilosis-like</i>	n.d.
F	31	+	+	996	703	<i>C. tropicalis</i> <i>I. orientalis</i>	Ø
F	27	+	+	n.a.	n.d.	<i>C. albicans</i> <i>C. tropicalis</i>	n.d.
M	2	+	+	n.a.	n.d.	<i>I. orientalis</i> <i>C. kefyr</i>	Ø
F	32	+	+	60 / 92	54	<i>C. albicans</i>	<i>C. albicans</i> <i>I. orientalis</i>
F	34	+	+	1036	566	Ø	<i>I. orientalis</i> <i>C. glabrata</i>
F	20	+	+	16 / 30	36	<i>C. albicans</i>	<i>C. albicans</i> <i>C. parapsilosis</i>
F	25	+	-		140	<i>C. albicans</i> <i>C. parapsilosis</i>	n.d.
F	23	+	-		131	<i>C. albicans</i> <i>I. orientalis</i>	n.d.
M	31	+	-		n.d.	<i>C. albicans</i> <i>C. tropicalis</i>	n.d.
F	40	-	n.a.		n.d.	<i>C. albicans</i> <i>S. cerevisiae</i>	n.d.
M	39	-	n.a.		n.d.	<i>C. albicans</i> <i>C. glabrata</i>	n.d.
M	56	-	n.a.		n.d.	<i>C. glabrata</i> <i>C. tropicalis</i> <i>I. orientalis</i>	n.d.
F	22	-	n.a.		n.d.	<i>C. albicans</i> <i>S. cerevisiae</i>	n.d.

In HIV+/HAART- patients mixed colonization was always found in combination with *C. albicans*. Ø= no yeast growth; n.a.= not applicable; n.d.= not determined; in green= mixed colonization.

3.13 Association of CD4 T cell number with HAART, oral fungal burden, symptoms and yeast species

For each HIV-positive patient, the initial CD4 T cell counts were routinely determined. Although patients were supposed to have their CD4 T cells regularly counted for the follow-up, a current CD4 T cell count was not available for all of the patients in the study due to limits in compliance. Therefore, only the subset of patients with recent CD4 T cell counts (within the last three months) could be considered for this evaluation.

3.13.1 CD4 T cell counts and oral fungal burden

Among the patients with an available recent CD4 T cell count (less than 90 days before sampling), its relationship with HAART and fungal burden (measured by the number of fungal CFU from the direct oral smear) was analyzed. In both HIV-positive subgroups the median average CD4 T cell count of the patients with a high fungal burden (HFB) was <150 CD4 T cells/ μ l. In contrast, there was a significant difference ($p=0.04$) in the median CD4 T cell count between HAART-treated and untreated patients which only had a low fungal burden (LFB). The median CD4 T cell count was 214 CD4 T cells/ μ l in the HIV+/HAART- group as compared to 336 CD4 T cells/ μ l in the HIV+/HAART+ group (Figure 22). For those patients where no yeasts were found in the oral cavity, there was no difference between both subgroups. The mean average CD4 T cell count was 321 and 313 CD4 T cells/ μ l in the HIV+/HAART- and HIV+/HAART+ groups, respectively.

The majority of the HIV+/HAART+ patients without yeasts in the oral cavity had been under treatment for over one year (median: 520 days). For those patients on HAART with low fungal burden, the median time of treatment was 346 days and for those with high oral fungal burden 51 days. Therefore, there was a significant quantitative reduction of colonizing yeasts depending on the duration of HAART.

In summary, there was a correlation between low CD4 T cell counts, no or only short time of HAART and high oral fungal burden.

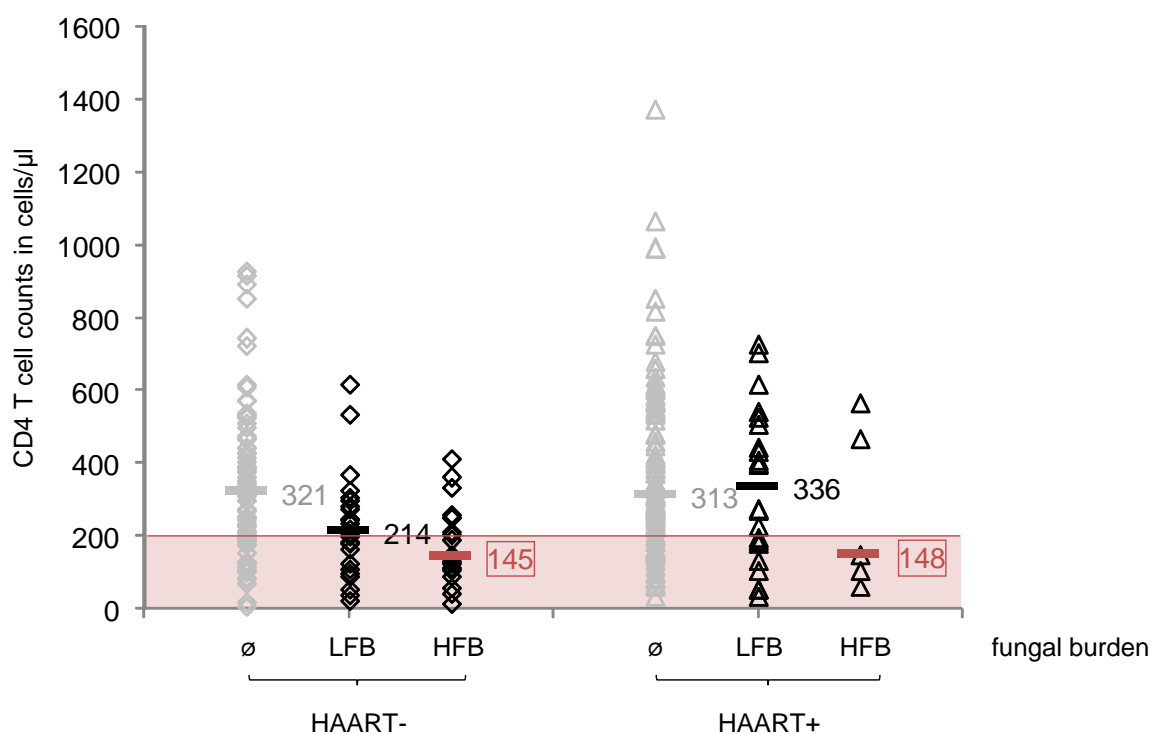


Figure 22: Relationship between CD4 T cell counts and fungal burden in HIV-positive patients with and without HAART. High fungal burden was associated with CD4 T cell counts <200 CD4 T cells/μl in HIV+/HAART- as well as in HIV+/HAART+ patients. ∅= no fungal growth, LFB= low fungal burden, HFB= high fungal burden. Horizontal bars indicate the median CD4 T cell counts per subgroup

3.13.2 CD4 T cell counts in asymptomatic and symptomatic HIV-positive patients

There was no significant difference seen in the median CD4 T cell count between asymptomatic and symptomatic yeast-negative HIV-positive patients. Median CD4 T cell count of yeast-negative HIV-positive patients was 345 and 349 CD4 T cells/μl in asymptomatic, 289 and 314 CD4 T cells/μl in patients with mild symptoms and 307 and 293 CD4 T cells/μl in patients with severe symptoms in the HAART- and HAART+ groups, respectively (Figure 23). In both subgroups, asymptomatic and symptomatic yeast-carriers with only mild symptoms had a median CD4 T cell count >200 CD4 T cells/μl. However, HIV+/HAART+ yeast-carriers without or with only mild symptoms had significantly ($p=0.02$) higher median CD4 T cell counts (432 and 397 CD4 T cells/μl) than HIV+/HAART- yeast-carriers (212 and 207 CD4 T cells/μl) with the same symptoms. Only yeast-positive patients with severe symptoms had a median CD4 T cell count <200 CD4 T cells/μl (Figure 23). Therefore OC was highly

associated with CD4 T cell counts <200 CD4 T cells/μl. Nevertheless, 47% (n=8/17) of asymptomatic yeast-carriers in the HIV+/HAART- group and 36% (n=4/11) in the HIV+/HAART+ group had CD4 T cell counts <200 CD4 T cells/μl.

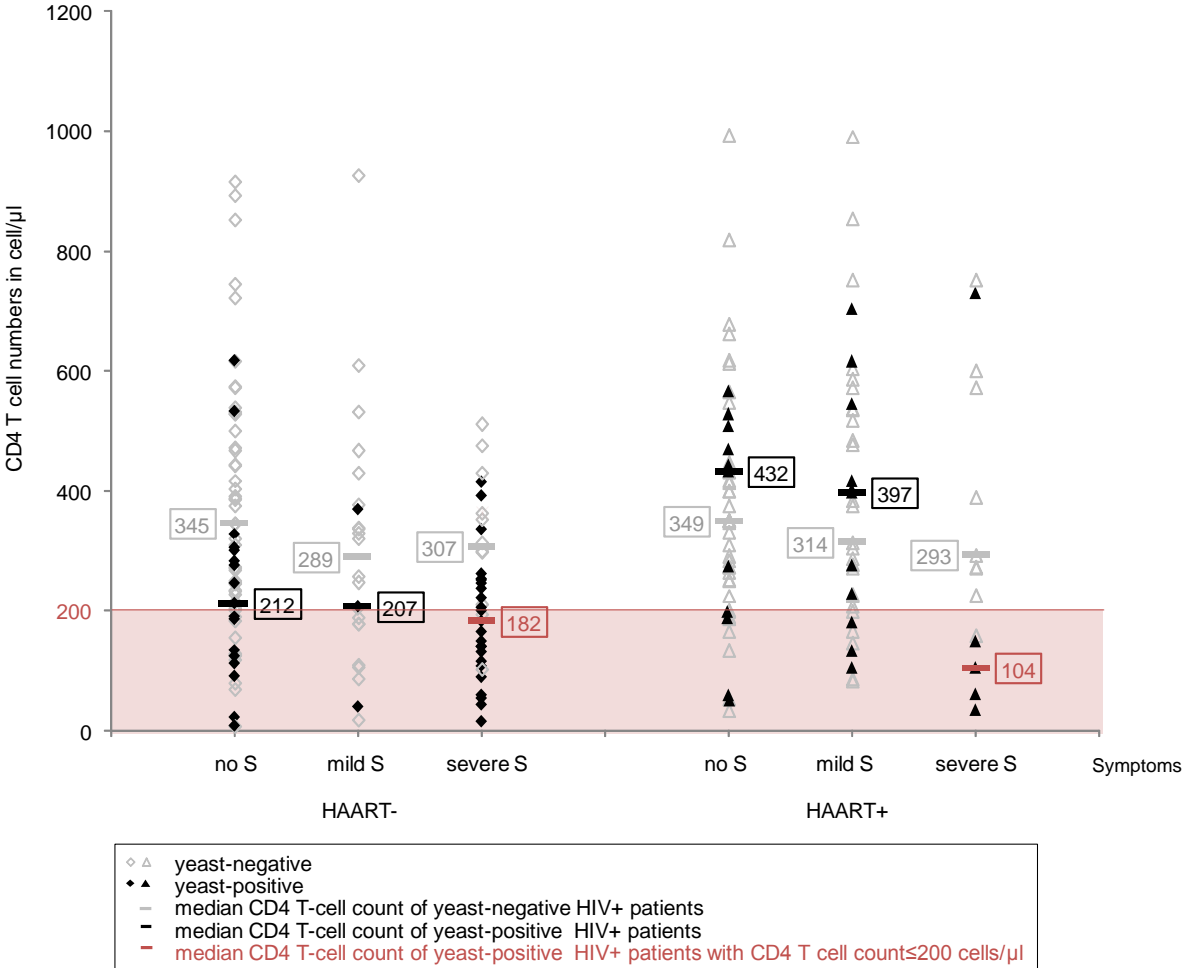


Figure 23: Asymptomatic and symptomatic HIV-positive yeast-carriers and their CD4 T cell counts. Only yeast-positive patients with severe symptoms had a median CD4 T cell counts <200 CD4 T cells/μl in HIV+/HAART- as in HIV+/HAART+ patients. S= symptoms.

3.13.3 CD4 T cell counts, yeast species distribution and fungal burden

C. albicans was the most frequently isolated yeast species in HIV-positive patients and associated with a median CD4 T cell count <200 CD4 T cells/μl especially in patients with high oral fungal burden (Table 7 and Figure 24). HIV+/HAART+ patients were significantly ($p \leq 0.01$) more often colonized with non-*C. albicans* yeast spp. (50%, n=13) than HIV+/HAART- patients (12%,n=5) (Table 8). Median CD4 T cell

count of HIV+/HAART+ patients harboring non-*C. albicans* yeast spp. was 432 CD4 T cells/ μ l and was significantly ($p=0.01$) higher than patients colonized by *C. albicans* (148 CD4 T cells/ μ l) in the same group (Table 7).

Table 7: Median CD4 T cell count for *C. albicans* and non-*C. albicans* yeast species

HAART-				HAART+		
Fungal burden	Low	High	combined	Low	High	combined
<i>C. albicans</i>	214	140	188	230	104	148
Non- <i>C. albicans</i>	125	274	212	409	517	432
Significance			$p=0.6$			$p=0.01$

High oral fungal burden and colonization with *C. albicans* was associated with CD4 T cell counts <200 CD4 T cells/ μ l irrespectively of HAART. In HIV+/HAART+ patients colonization with non-*C. albicans* yeast spp. was significantly associated with CD4 T cell counts >400 CD4 T cells/ μ l ($p=0.01$).

Table 8: Distribution of *C. albicans* and non-*C. albicans* yeast species

HAART-				HAART+		
Growth category	Low	High	total	Low	High	total
<i>C. albicans</i>	20 (87%)	18 (90%)	38 (88%)	10 (47%)	3 (60%)	13 (50%)
Non- <i>C. albicans</i>	3 (13%)	2 (10%)	5 (12%)	11 (53%)	2 (40%)	13 (50%)
Significance			$p\leq 0.01$			

There was a significantly higher prevalence ($p \leq 0.01$) of non-*C. albicans* yeast spp. in the HIV+/HAART+ group. Colonization with non-*C. albicans* yeast spp. in that group was more associated with a low fungal burden.

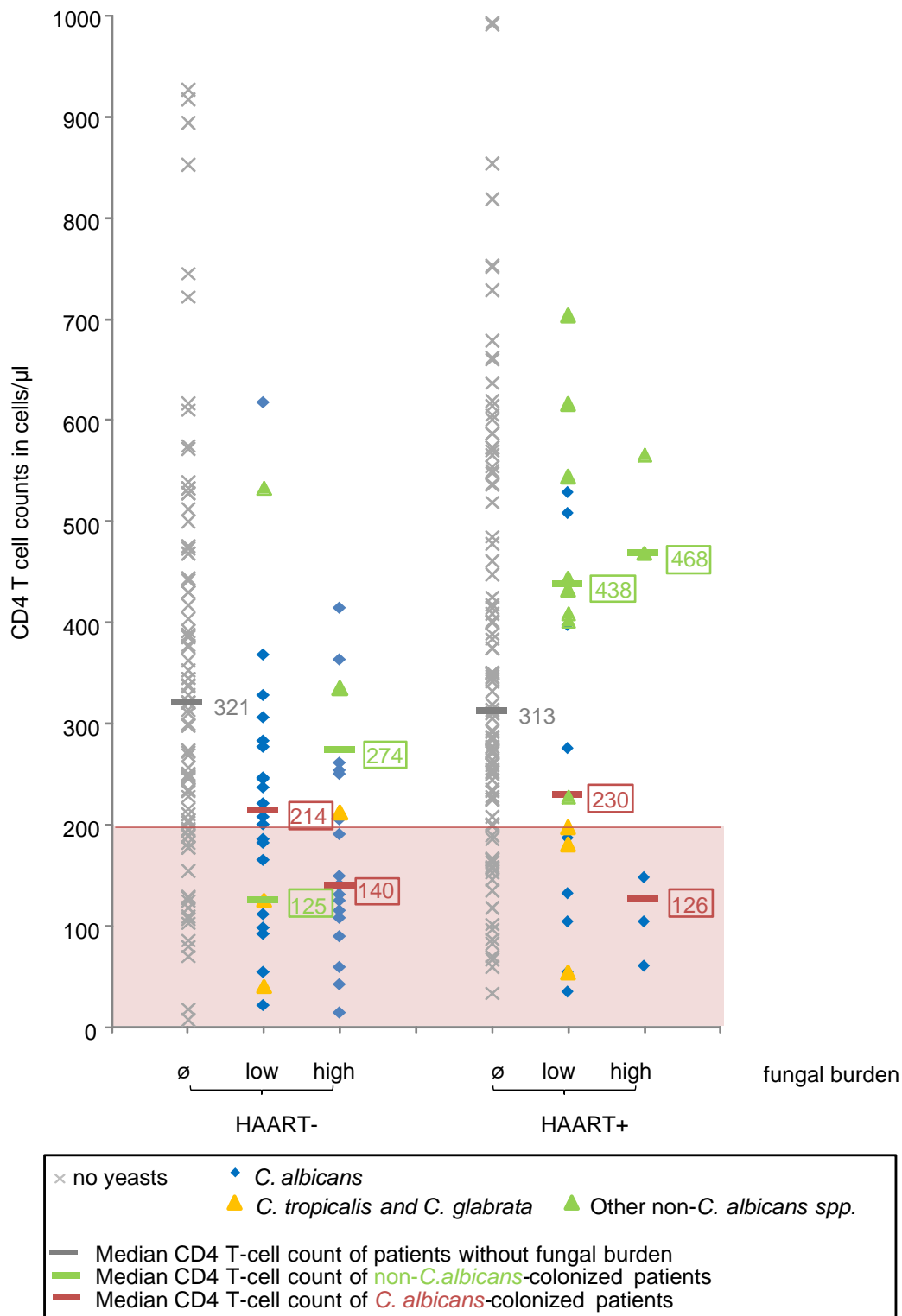


Figure 24: Species distribution in the oral specimen of HIV-positive patients according to their CD4 T cell count and oral fungal burden. Irrespectively of HAART, *C. albicans* was the most frequently isolated species and associated with CD4 T cell counts <200 CD4 T cells/ μ l in patients with a high oral fungal burden. Colonization with non-*C. albicans* species was more frequent in HIV+/HAART+ patients and in the majority of the cases associated with CD4 T cell counts >350 CD4 T cells/ μ l and a low oral fungal burden. Only the non-*C. albicans* species *C. tropicalis* and *C. glabrata* were associated with CD4 T cell counts <200 CD4 T cells/ μ l in both subgroups.

3.13.4 CD4 T cell counts, yeast species distribution and clinical symptoms

There was a high association between severe symptoms, colonization with *C. albicans* and CD4 T cell counts <200 CD4 T cells/ μ l irrespectively of HAART (Figure 25A). Almost all patients with severe symptoms harbored *C. albicans* alone or *C. albicans* combined with a non-*C. albicans* yeast spp. In contrast, colonization with non-*C. albicans* yeast spp. was more often associated with mild or no symptoms, a low fungal burden, as well as with a high CD4 T cell count in HIV+/HAART+ patients (Figure 25A+B). Interestingly, colonization with *C. glabrata* or *C. tropicalis* was associated with CD4 T cell counts <200 CD4 T cells/ μ l in HIV+/HAART- and HIV+/HAART+ patients, but not with severe symptoms (Figure 25). Therefore colonization with these species was not associated with OC. There was only one case with severe symptoms and high fungal burden with a non-*C. albicans* yeast spp. This patient was without HAART, had a CD4 T cell count of 335 CD4 T cells/ μ l and was colonized by *P. fabianii*.

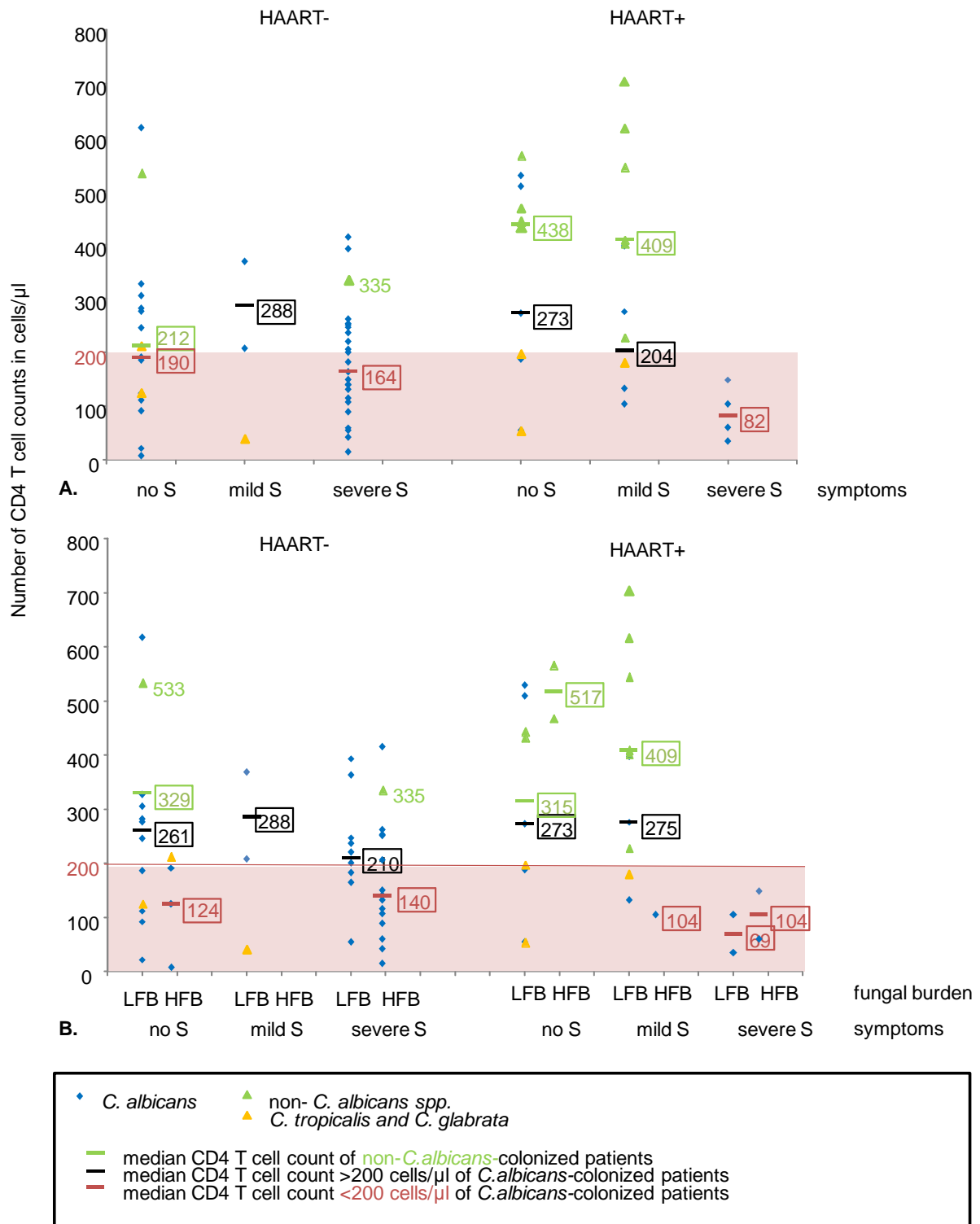


Figure 25: Distribution of the species in asymptomatic and symptomatic HIV-positive patients. Severe symptoms were highly associated with CD4 T cell counts <200 CD4 T cells/ μ l and colonization with *C. albicans* irrespectively of HAART (A). Irrespectively of symptoms, patients with a high oral fungal burden and colonization with *C. albicans* mainly had CD4 T cell counts <200 CD4 T cells/ μ l (B). Colonization with non-*C. albicans* spp. in HIV-positive patients was more associated with mild or no symptoms, low fungal burden and CD4 T cell counts >200 CD4 T cells/ μ l, except for the species *C. glabrata* and *C. tropicalis*. S= symptoms; LFB= low fungal burden; HFB= high fungal burden.

3.13.5 Oral fungal burden, symptoms and species distribution with time of HAART

Division of the patients into subgroups according to their length of HAART showed that the prevalence of colonization and symptoms changed over time. It was characterized by a general quantitative reduction of yeasts in the oral cavity (Figure 26A), reduced occurrence of severe symptoms (Figure 26B), as well as reduced colonization specifically with *C. albicans* with a shift towards colonization with non-*C. albicans* yeast spp. correlating with the increase in CD4 T cell number (Figure 27). High fungal burden and severe symptoms were highest in HIV-positive patients during their first month of therapy and decreased significantly ($p=0.01$) during the first six months of therapy from 36.4% ($n=4/11$) to 3% ($n=1/33$). The prevalence of yeast-positive patients with severe symptoms (=patients with OC) is not completely eradicated after this period of time, few cases are still seen even with a treatment exceeding this time. Similarly asymptomatic yeast-carriers and patients with high oral fungal burden are still present (Figure 26A+B).

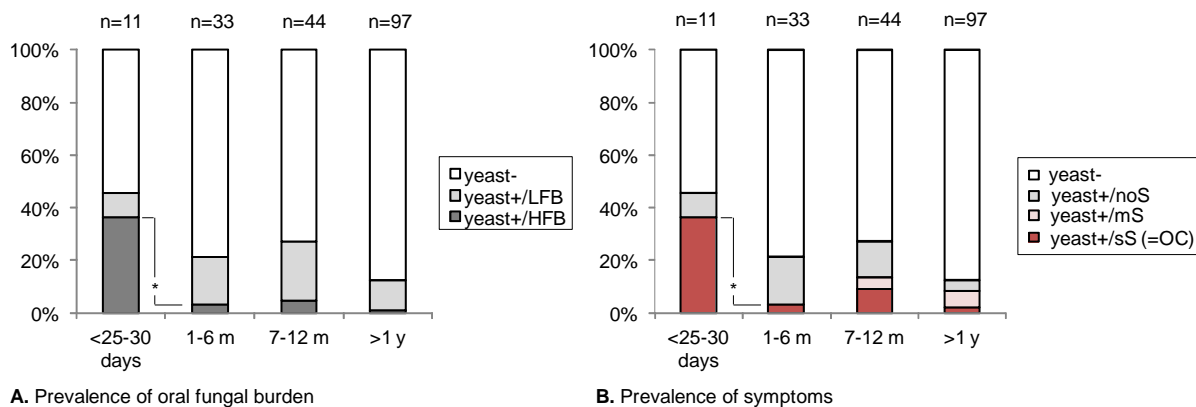


Figure 26: Influence of the duration of HAART on oral fungal burden and symptoms. There is a diminution in oral fungal burden (A) and symptoms (B) with rising time of HAART which was significant in the first six months of HAART (*= $p=0.01$). yeast-= yeast-negative; yeast+= yeast-positive; LFB= low fungal burden; HFB= high fungal burden; noS= no symptoms; mS= mild symptoms; sS= severe symptoms; OC= oral candidiasis; m= months; y= year.

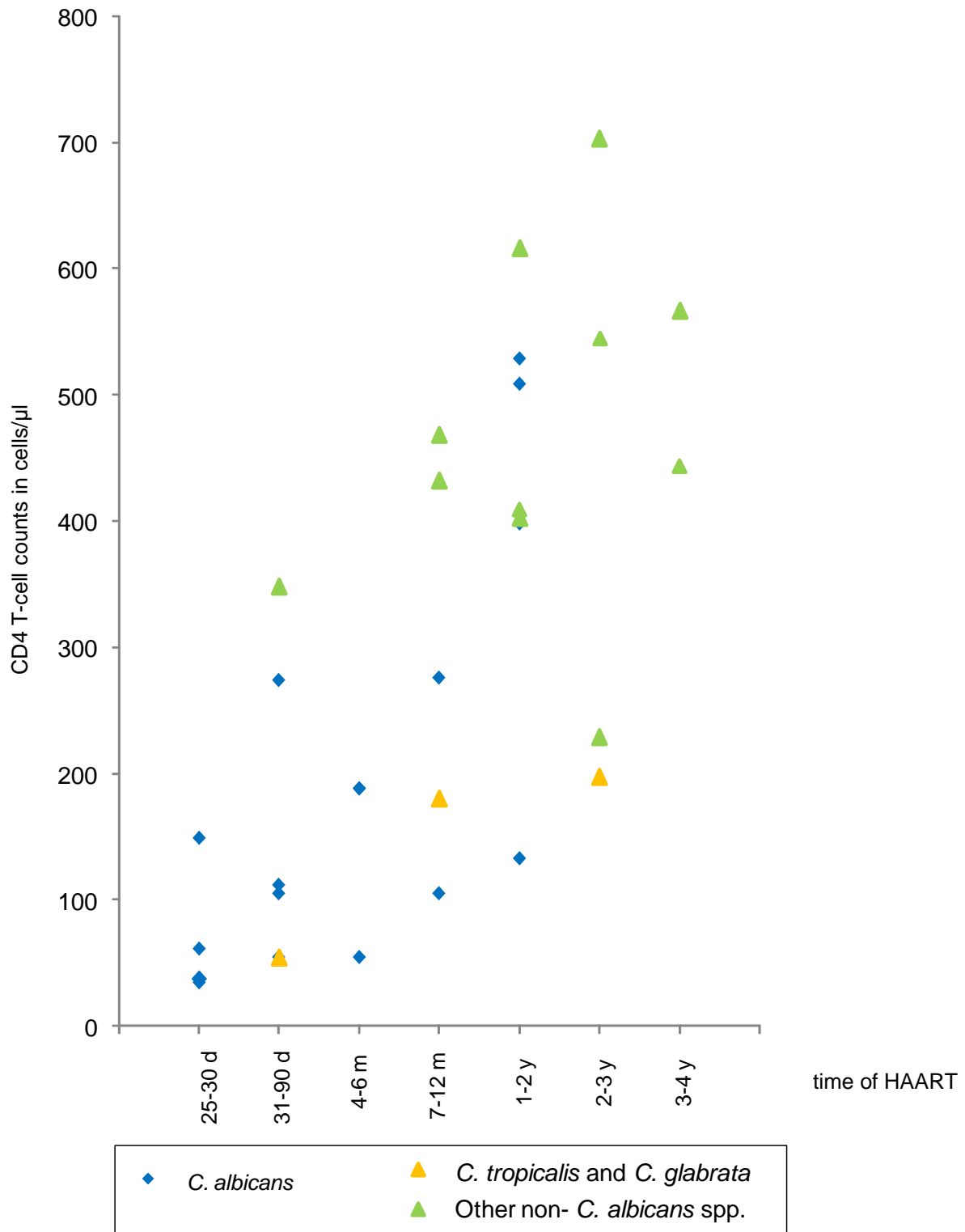


Figure 27: Influence of the duration of HAART on species distribution. There is a shift from colonization with *C. albicans* towards colonization with non-*C. albicans* yeast spp. with time of HAART, correlating with a rise in CD4 T cell number. Colonization with *C. tropicalis* and *C. glabrata* stayed associated with CD4 T cells <200 CD4 T cells/μl. d= days; m= months; y= years.

3.14 Efficacy of antimycotic treatment and antifungal drug susceptibility testing

3.14.1 Efficacy of antimycotic therapy

At time of the first visit or in the last three weeks before, 5 (1.4%) out of 348 HIV-positive patients were under antimycotic treatment, at second visit 12 out of 134 (8.9%) and 6 out of 25 (24.0%) at third visit. Patients had received either treatment with azoles (oral Fluconazole or Ketoconazole), polyenes (mouthwash with Amphotericin B or Nystatin), or topical Clotrimazole, which was administered when patients presented with dermatomycoses and/or oral thrush.

We analyzed the effect of antifungal treatment and HAART on oral fungal burden as well as on the oral clinical symptoms. A patient was considered to respond to treatment when there was an improvement in oral fungal burden (from yeast-positive to yeast-negative, or from HFB to LFB) and/or in symptoms (symptomatic to asymptomatic or severe to mild symptoms). The reverse cases were considered mild (yeast-negative to LFB or asymptomatic to mild symptoms) or severe worsening (HFB or severe symptoms) of the condition, respectively, and thus treatment failure.

All colonized HIV+/HAART- patients treated with only an antifungal (n=11) responded with clearance of oral yeast or reduction in oral fungal burden (Figure 28A). Further two HIV+/HAART+ with severe symptoms and a high fungal burden (CD4 T cell counts were $<200 \text{ cells} \cdot \mu\text{l}^{-1}$) were treated with an antifungal and were cured (with no presence of yeast) (Figure 28).

In patients with no therapy at all the oral fungal burden got severely worse in 12% (n=6/52) of the cases, stayed unchanged in 69% (n=36/52) of the cases and in 12% (n=6/52) there was an improvement with clearance of yeast in the oral cavity. HIV+/HAART+ patients showed almost the same outcome (Figure 28A). Although, the majority of the cases with unchanged oral fungal burden were patients with no fungal burden.

The analysis of the symptomatic outcome showed similar results except for the observed patients treated only with an antimycotic. There only 33 % (n=5/15) had a clinically visible improvement of symptoms under antifungal treatment (Figure 28B). Seven patients out of ten presented with the severe symptoms before and after

treatment. Anyhow, in six out of these seven cases there was an improvement in fungal burden with clearance of yeast. Antifungal treatment was therefore very effective against colonization with *Candida* even when not always clinically visible.

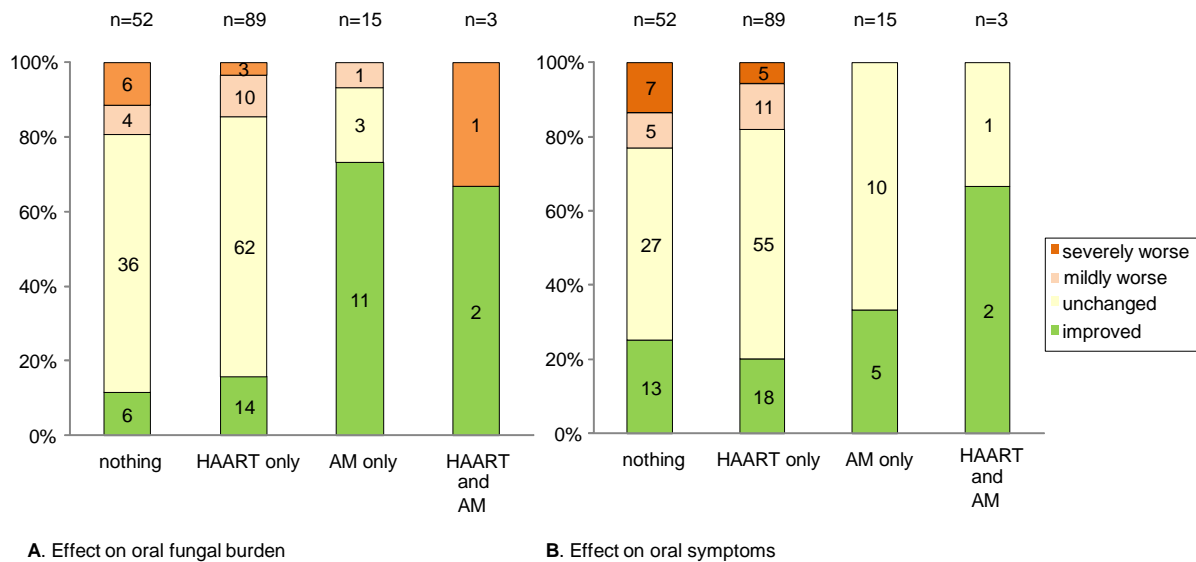


Figure 28: Effect of HAART and antimycotic therapy on oral fungal burden. Oral fungal burden clearly improved under antimycotic treatment (**A**). All yeast-positive patients responded to solely antimycotic treatment with clearance of yeast, but not always with clinically visible change of symptoms (**B**). The majority of the patients with unchanged oral fungal burden (**A**) were yeast non-carriers. Severely worse= shift from yeast-negative or LFB to HFB (**A**), or asymptomatic or mild symptoms to severe symptoms (**B**); mildly worse= yeast-negative to LFB (**A**), or asymptomatic to mild symptoms (**B**).

A species shift under antimycotic treatment could neither be seen in HIV+/HAART+ nor in HIV+/HAART- patients (data not shown). Nevertheless, a shift from *C. albicans* or a mixed culture with *C. albicans* towards colonization with only non-*C. albicans* yeast spp. was seen in four (out of nine colonized) HIV+/HAART+ patients without any antifungal treatment (data not shown).

If we looked at the efficacy of antifungal treatment focusing on patients with OC, there was a statistically significant difference between treated and non-treated patients ($p \leq 0.01$). Patients with OC profited from HAART, antifungal therapy or the combination of both (Table 9).

Table 9: Effect of treatment in patients with oral candidiasis

	nothing	HAART only	AM only	both
Improvement of visible symptoms or/and fungal burden	1	3 (75%)	8 (100%)	2 (100%)
No improvement	5 (83.3%)	1	0	0
Total	6	4	8	2
Significance	$p \leq 0.01$			

Oral fungal burden of patients with OC was significantly improved in treated patients with HAART, antimycotic or the combination of both (Fisher's exact test $p \leq 0.01$).

3.14.2 *In vitro* antifungal susceptibility of the isolated yeast species

To determine the susceptibility towards common antifungal substances, all yeast strains isolated were tested according to the NCCLS Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast, Approved Standard M27-A2. Substances tested were Amphotericin B, Nystatin, Fluconazole, Itraconazole, and Caspofungin. Yeast species were considered resistant when their MICs were above the clinical breakpoints set by the clinical laboratory standard Institute (CLSI). All isolates in this study were susceptible to Amphotericin B with MIC ranges between 0.031 - 0.5 µg/ml. *C. albicans* had mainly MICs of 0.125 - 0.250 µg/ml. All *C. albicans* isolates were fully susceptible to Fluconazole and Itraconazole. 15 *I. orientalis* isolates (79.0%) were resistant to Fluconazole, eleven with a MIC of 64 µg/ml and four with a MIC of 96 µg/ml. All the other non- *C. albicans* species were fully susceptible to Fluconazole. The nine isolated *C. glabrata* were fully resistant to Itraconazole, with MICs of mainly 2 µg/ml. 17 (89.5%) out of 19 isolated *I. orientalis*, four (19%) out of 21 *C. tropicalis*, all six *S. cerevisiae*, all two *P. guilliermondii* and the only isolated *C. parapsilosis* were resistant to Itraconazole with MICs ≥ 1 µg/ml. The MIC ranges of all isolates for Nystatin and Caspofungin were 1 - 8 µg/ml and 0.063 - 0.5 µg/ml respectively. Only one *C. parapsilosis* isolate had a MIC of 1 µg/ml for Caspofungin (Figure 29).

Non- *C. albicans* yeast isolates with MICs above the clinical breakpoint were not more prevalent in patients with recorded history of one or more exposure to antimycotic therapy and there were no cases of clinical OC caused by species other than *C. albicans*. Furthermore all patients with OC responded to antifungal therapy (Table 9).

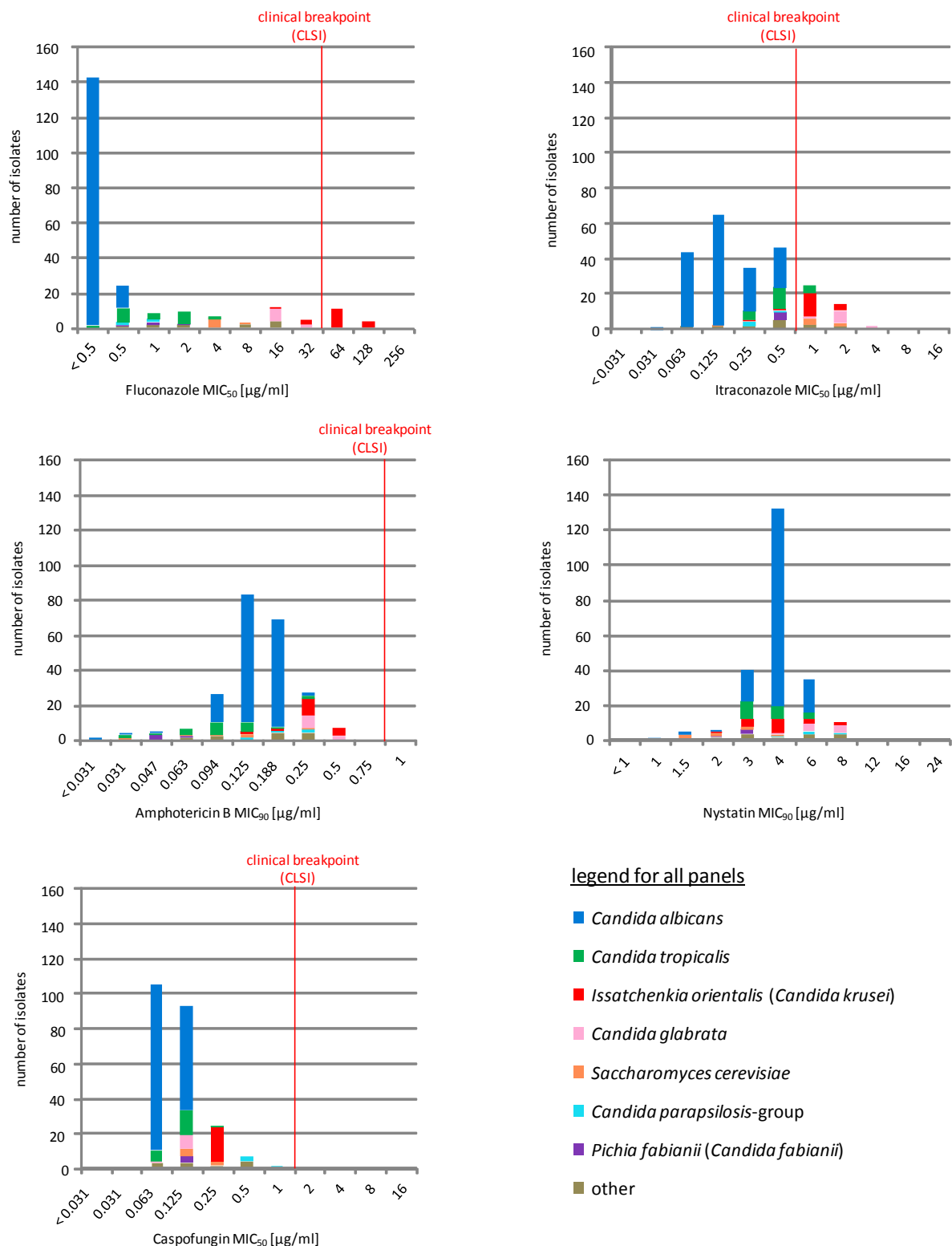


Figure 29: *In vitro* susceptibility of the isolated yeast species to the different antifungal agents. A clinical breakpoint was not yet defined for Nystatin. All species with MICs above the clinical breakpoint were considered resistant (*in vitro*). All *C. glabrata* isolates were resistant to Itraconazole but were susceptible to Fluconazole; resistance to Itraconazole was further seen for the species *I. orientalis*, *C. tropicalis*, all *S. cerevisiae*, *P. guilliermondii* and *C. pararugosa* spp. *I. orientalis* were the only species resistant to Fluconazole. All species were susceptible to Amphotericin B and Caspofungin. MIC= minimal inhibiting concentration.

4. Discussion

Despite the introduction of antiretroviral therapy, under which the prevalence of opportunistic infections has decreased (Hood et al. 1998; Schmidt-Westhausen et al. 2000; Greenspan et al. 2004), OC still remains a significant and common opportunistic infection in HIV-infected individuals (Thompson et al. 2010; Mataftsi et al. 2011). This is especially true in Sub-Saharan Africa, where HAART is still not widely available (Hamza et al. 2006; Tirwomwe et al. 2007; Hamza et al. 2008). The prevalence of OC and the yeast species involved vary throughout the world, as well as within the countries themselves due to many confounding variables, such as the availability of HAART, access to health care, nutrition, present oral and environmental yeast flora. *Candida albicans* has been reported to be the most frequently isolated yeast species throughout the world. Also, the incidence of other *Candida* species less susceptible or resistant to the antifungal drugs in use has been increasing and has become a strong concern for clinicians in the management of affected individuals (Pfaller et al. 2007). Yeast identification and antifungal susceptibility testing have therefore become of high importance. As oral and invasive yeast infections are mostly caused by the endogenous colonizing yeasts of the mucosal surfaces (Fetter et al. 1993; Pfaller 1995; Vargas and Joly 2002; Grimoud et al. 2003) and the distribution as well as the pathogenicity and antifungal drug susceptibility of the species vary in the different geographical regions, it is important to investigate their prevalence and distribution throughout the world. Although numerous epidemiological studies in Europe and the Americas on the prevalence of yeasts causing candidiasis have been published, little is known about the yeast flora and etiology of candidiasis in people living in developing countries. In Sub-Saharan Africa, where the majority of HIV-infected individuals live, only few studies on the prevalence of yeasts in the oral cavity of HIV-positive patients (Hodgson and Rachanis 2002) have been performed. For Chad, even no data are available at all. We have therefore studied the prevalence and epidemiology of oral asymptomatic and symptomatic yeast carriage of HIV-positive vs. HIV-negative individuals from Southern Chad, identified the yeast species involved and analyzed the impact of age, sex, HAART, CD4 T cell numbers as well as the use of different antimicrobials on

oral yeast colonization and infection. Susceptibility of the differentiated yeast isolates to common antifungals was determined.

Prevalence of oral candidiasis

The overall oral *Candida* carriage rate (25%) as well as the prevalence of OC (10.2%) of HIV-positive patients found in Chad was surprisingly low as compared to other African countries where numbers ranged from 41.2% in Cameroon (Lohoue Petmy et al. 2004) to 81.5% in Ghana (Kwamin et al. 2013) and 81.3% in South Africa (Patel et al. 2006). These differences may be due to the selected study groups (e.g. HIV disease stage and availability of antiretroviral treatment). However, chosen diagnostic tools and criteria and experience level of the investigators may also play a role. In the clinic of Maingara, as it is a specialized center for HIV/AIDS patients with rigorous follow up and availability of HAART, cases with advanced stages of immune deterioration were rare.

Many previous studies have demonstrated that potent antiretroviral treatment like HAART containing a combination of antiretrovirals significantly reduces opportunistic infections such as OC (Powderly et al. 1998; Yang et al. 2006; Lourenco et al. 2011). This has been found in Cameroun, where the prevalence of OC was 30% in HIV+/HAART+ versus 70% in HIV+/HAART- patients, as well as in Tanzania where values were 15% and 38%, respectively (Hamza et al. 2006; Njunda 2011). This improvement of oral fungal infection by HAART is as well supported by our findings. Although our numbers of OC prevalence (5% in HIV+/HAART+ and 16% in HIV+/HAART-) were far lower, they were closer to those of studies performed in South India (Umadevi et al. 2007) and Taiwan (Yang et al. 2006), where the prevalence of OC reached 8% and 24% in South India and 2.1% and 10.6% in Taiwan in HIV+/HAART+ and HIV+/HAART- patients, respectively. A possible explanation for the very low prevalence of 5% OC found in our cohort of HIV+/HAART+ patients might be the treatment duration. The prevalence of OC was significantly reduced with HAART over a period of six to twelve months and beyond. The same was found in a cohort of 532 HIV-positive patients recruited from the Muhimbili National Hospital HIV Clinic in Dar es Salaam, Tanzania (Hamza et al. 2006) and the prospective longitudinal study of 142 HAART-treated HIV-positive patients in Taiwan (Yang et al. 2006). In a cohort of 92 HIV-positive patients from Germany, the prevalence of OC was even reduced to zero (Jordan 2007). In our

study, 53% of the patients were under HAART, 78% (n=141/180) for at least six months, and 54% (n=97/180) for 12 months or more.

Fungal infection is usually caused by commensal yeast species of the mucosa (Fetter et al. 1993; Pfaller 1995; Vargas and Joly 2002). Therefore, another factor explaining our results could be the general lower prevalence of colonizing yeasts in the Chadian population, represented by our HIV-negative control group (22.5%).

It may also have been possible, that among the HIV+/HAART- patients, the HIV infection had been only recent and therefore at an early stage, where opportunistic infections like OC are not yet apparent (Wanzala et al. 1989; Owotade et al. 2008).

In many developing countries, traditional herbal medicine is still widely used, some of which have been shown to have an anticandidal effect (Rukayadi et al. 2008; Marzouk et al. 2009). Due to the poor and marginally developed health care system, the use of traditional herbal medicine is widely spread in Chad. However, precise data concerning its use were not available for this study.

Saccharomyces cerevisiae was found to be the second (in Bemouli) and third (in Maingara) most common yeast species isolated among our Chadian patients without being a cause of infection. Potentially, these may originate from wild *S. cerevisiae* yeast strains used for the local home-brewed and widely consumed alcoholic beverage in Chad, known as *bili-bili*. *Bili-bili* is made by fermenting locally grown sorghum and millet (Maoura 2005). Oral uptake of *S. cerevisiae* is known to have an inhibitory effect on OC (Premanathan 2011).

Although the introduction of HAART has led to a significant decrease or even absence of oral lesions such as OC (Powderly et al. 1998; Yang et al. 2006; Jordan 2007; Lourenco et al. 2011), OC continues to be a significant oral lesion highly associated with HIV infection in both developed and developing countries (Ranganathan et al. 2004; Hodgson et al. 2006). Oral colonization with *Candida*, even though observed to decrease, was also still present: 9 % of our HIV+/HAART+ patients were asymptomatic yeast carriers, including those with mild symptoms the prevalence rose up to 14%. This correlated with the observations made in a study in Italy (Cauda et al. 1999) and in Taiwan (Yang et al. 2006).

Classification and epidemiology of the different clinical features of oral candidiasis (OC)

Very few studies investigating on the prevalence of OC describe the observed oral lesions in a defined way; and most rely on presumptive rather than definite diagnosis. The most common used classification for oral lesions in HIV-infected patients, especially in developing countries, is the ECC classification (EC-Clearinghouse 1993). In this revised classification from 1993 only two forms of OC are distinguished: the pseudomembranous (PC) and the erythematous candidiasis (EC). The description of the PC: "white or yellow spot or plaques that can be located in any part of the oral cavity and can be wiped off to reveal an erythematous surface which may bleed" (EC-Clearinghouse 1993, page 289) only fitted to five (14.3%; 5/35) of our HIV-positive patients with OC. The majority of our HIV-positive patients with severe symptoms (n=41/64; 64%; n=17/35= 48.6%) suffered from a whitish or yellowish coating affecting >50% of the dorsum of the tongue, which was not easily removable. Only 39% (n=16/41) of these patients were yeast-positive and in 94% (n=16/17) of the cases colonized by *C. albicans*. The median CD4 T cell count of these yeast-positive patients was <150 CD4 T cells/ μ l, so that the manifestation of oral lesions strongly associated with HIV was highly suspect. Although the diagnosis of OC still stays essentially clinical (Coronado-Castellote and Jimenez-Soriano 2013), none of the criteria of the described oral lesions of the ECC classification corresponded to the oral manifestations seen here. The proposed classification by Axell et al. (1997) differentiates between an acute and a chronic type of OC, *Candida*-associated lesions and keratinized primary lesions superinfected with *Candida*. Of these variants, superinfected keratinized primary lesions like oral hairy leukoplakia or lichen planus with *Candida* could be taken into consideration, as the lesions were not easily to remove. Anyhow, the coating in our patients affected the dorsum of the tongue, which is neither usual for the oral hairy leukoplakia nor the lichen planus. Oral hairy leukoplakia is usually described to be found on the lateral tongue, with eventually vertical corrugations or a flat appearance which cannot be wiped off (EC-Clearinghouse 1993). Lichen planus of the mouth appears as lace-like fine white lines usually at the inside of the cheeks (AAOMP 2005). These variants clinically seemed less likely. Another possible variant could be the hyperplastic plaque-like candidiasis. Tissue biopsy to confirm the hyperplastic form was not available and clinically, the hyperplastic candidiasis is described as lesions at the mouth

commisures (Akpan and Morgan 2002; Williams and Lewis 2011; Madhu 2013), which did also not fit to our cases. Pseudomembranous candidiasis is highly associated with CD4 T cells dropping <200 CD4 T cells/ μ l, which is in accordance with our study (Ranganathan et al. 2004; Mercante et al. 2006; Witzel et al. 2008; Bodhade et al. 2011). Our yeast-positive patients with observed whitish or yellowish coating of more than 50% of the tongue area, classified as nonspecific OC, had a median CD4 T cell count <150 cells, making a presumptive classification as pseudomembranous candidiasis reliable.

The second most common clinical sign in our patients was the median rhomboid glossitis which affected 20.3% (n=13/64) of the patients with severe symptoms. Out of these, 69% (n=9/13) were yeast-positive, in eight out of nine *C. albicans* was isolated. The median rhomboid glossitis presents as a central red area with papillary atrophy of the dorsum of the tongue or palate. Median rhomboid glossitis has infrequently been described as a lesion also seen in HIV-positive patients (Gazzard and Smith 1990; Flaitz and Hicks 1999; Barasch et al. 2000; Okunseri et al. 2003). In the classification of Axell et al. it has been included and mentioned as a distinct form of "*Candida*-associated lesions" (Axell et al. 1997). In the ECC classification it is not mentioned. Some authors suggest it is "a form of erythematous candidiasis" (Kolokotronis et al. 1994; Lalla et al. 2013). Therefore, it may have been misclassified as erythematous candidiasis in previous studies. As outlined above, the most common types mentioned and discussed are the pseudomembranous and the erythematous candidiasis. In accordance with the description of the erythematous candidiasis in the ECC classification ("red areas located on the palate or dorsum of the tongue"), the patients in our study group which presented with the features of the median rhomboid glossitis could be classified as erythematous candidiasis.

Since the erythematous candidiasis is as well described as a red atrophic area with loss of filiform papillae when affecting the tongue (Lalla et al. 2010), our patients with symptoms of tongue atrophy and positive yeast growth could as well be classified as erythematous candidiasis resulting in a total prevalence of 38.4% (n=10/26) of erythematous candidiasis in HIV+/HAART- patients. This was similar to the findings in a study performed among HIV+/HAART- patients in India where the prevalence was 39.3% (Bodhade et al. 2011).

The epidemiology (relationship with HAART and CD4 T cell counts or other factors) of the different forms of candidiasis in HIV-positive patients has rarely been

investigated. In a cohort from Germany the erythematous candidiasis was only found in patients not undergoing any antiretroviral treatment (Jordan 2007). It is not clear, if the patients classified as erythematous candidiasis included the features of the median rhomboid glossitis. Anyhow, if the median rhomboid glossitis is a form of erythematous candidiasis, our findings would be similar to the German and Indian study (Jordan 2007; Bodhade et al. 2011). In studies from Kenya and South Africa performed before the HAART era, the erythematous candidiasis was significantly more prevalent than the pseudomembranous candidiasis (Hodgson and Rachanis 2002) and in a Tanzanian study including HIV+/HAART+ patients, the prevalence of erythematous candidiasis was only 1.4% (Hamza et al. 2008). Therefore, a correlation between the appearance of erythematous candidiasis (and possibly median rhomboid glossitis) and the absence of HAART seems reliable, and is supported by our findings here, and the German and Indian study (Jordan 2007; Bodhade et al. 2011).

A correlation between erythematous candidiasis and CD4 T cell counts dropping <400 CD4 T cells/ μ l has been observed (Mercante et al. 2006; Witzel et al. 2008), which would correlate with our findings: patients with median rhomboid glossitis had a median CD4 T cell count of 200 CD4 T cells/ μ l and those with atrophy 338 CD4 T cells/ μ l. In a study in Zambia, erythematous candidiasis was even found to be associated with CD4 T cell counts <200 CD4 T cells/ μ l (Hodgson 1997).

According to Bodhade et al. (2011), erythematous candidiasis may as well be a good marker of immunosuppression, anyhow, not as sensitive and specific as the pseudomembranous candidiasis.

The distribution of pseudomembranous and erythematous candidiasis among our patients with OC (n=34) would then be 68% and 32% respectively, which matches the results in a study published in India (2008), where 83.3% of the cases with OC had pseudomembranous type lesions followed by erythematous candidiasis with 16.6% (Nadagir et al. 2008). The higher rate of pseudomembranous candidiasis in the Indian study can be explained by the fact, that the cohort consisted of terminally ill patients, here again underlining the high predictive value and correlation of pseudomembranous candidiasis with a severely deteriorated immune system (Bodhade et al. 2011).

Species prevalence and association with OC

Worldwide, *C. albicans* has frequently been found to be the most prevalent yeast species found in the oral cavity of HIV-positive and healthy subjects ranging from 50% in Iran (Badiee et al. 2010) to 91.5% in South Africa (Blignaut 2007). This species was also predominant in our study performed in Chad; with 44.2% in the HIV-negative control group, 58.6% in the HIV+/HAART+ group, and 87.1% in HIV+/HAART- patients, which matches with the findings of Nweze and Ogbonnaya (2011) in Nigeria.

I. orientalis and *C. tropicalis* were the second most common yeast species isolated in the oral cavity of our HIV-positive patients, which correlates with the findings of a study in an HIV cohort from Nigeria (Enwuru et al. 2008) and partly with those from Uganda (Agwu et al. 2011). Other studies found *C. glabrata* at second position (Sanchez-Vargas et al. 2005a; Sanchez-Vargas et al. 2005b; Badiee et al. 2010). *C. glabrata* ranked only fifth in our HIV-positive patients.

As *C. glabrata* has often been co-isolated with *C. albicans* in patients with OC its clinical relevance has been questioned (Dronda et al. 1996; Ally et al. 2001; Redding 2001). In our study groups of HIV-positive patients *C. glabrata* and *C. tropicalis* were more frequently present as sole colonizing yeasts of the oral cavity when CD4 T cell counts were <200 CD4 T cells/ μ l. But these patients did not have any clinical signs of infection. Furthermore all the other non-*C. albicans* spp. were only associated with a low fungal burden, mild or no symptoms, as well as high CD4 T cell counts. This would underline the fact, that *Candida* species other than *C. albicans* are considered to be less pathogenic and may simply act as commensals (Dronda et al. 1996; Fidel et al. 1999).

Although isolation of non-*C. albicans* yeast spp. has been reported in HIV-positive patients, in almost all reports, OC has been reported to be mainly linked with the presence of *C. albicans* or *C. albicans* mixed with a non-*C. albicans* yeast spp. (Redding 2001; Patel et al. 2006; Agwu et al. 2011). Similarly in our cases of OC, *C. albicans* was also the most prevalent yeast. Consequently, *C. albicans* is considered to be more pathogenic than other *Candida* species. Its putative virulence factors seem to be expressed in higher ratios in HIV-positive patients when the hosts immune system fails (De Bernardis et al. 1996; Wu et al. 1996; Fidel 2011; Cassone and Cauda 2012).

Nevertheless, *C. glabrata* should not be ignored as mixed infections of *C. glabrata* with *C. albicans* are more severe in patients with HIV infection (Redding 2001). Furthermore, the intrinsic resistance of *C. glabrata* to many azoles (Bagg et al. 2003), especially Fluconazole, makes co-infection due to this species more difficult to treat (Redding 2001).

The increasing reports of the emergence of non-*C. albicans* species associated with OC and HIV infection (Schoofs et al. 1998; Melo et al. 2004; Enwuru et al. 2008) has been explained by the repeated exposure to azoles, which results in the selection of less susceptible species like *C. glabrata* or *I. orientalis* (Cartledge et al. 1999; Hope et al. 2002; Snyderman 2003; Hamza et al. 2008; Agwu et al. 2011). Antifungal exposure was rare in our study group and patients harboring non-*C. albicans* yeast spp. did not have a history of previous fungal therapy. Therefore, the emergence of non-*C. albicans* yeast spp. in our study cannot be explained by this hypothesis.

Emergence and etiology of non-*C. albicans* yeast species

Besides the overall reduced rate of yeast prevalence in the oral cavity and clinical manifestation of OC, our study group of HIV+/HAART+ patients also displayed an increased species diversity, as similarly seen in a study in Brazil (Melo et al. 2004). Non-*C. albicans* yeast spp. isolated from our patients were *I. orientalis*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. orthopsilosis*, *P. fabianii*, *P. farinosa*, *P. guilliermondii*, *C. kefyr*, *C. pelliculosa*, *C. rugosa*, *C. pararugosa* and one isolate of a novel *C. orthopsilosis*-like species. It is well established that the protease inhibitors (PI) that may be included in HAART have an effect on colonization at least with *C. albicans*, possibly through reduction in adhesion to epithelial cells by inhibiting the secretory aspartic proteases (SAP) of *C. albicans* (Borg-von Zepelin et al. 1999; Cassone et al. 1999; Witzel et al. 2008). The National Guidelines of the Republic of Chad, however, specify treatment of HIV-infected patients with a combination of the three reverse transcriptase inhibitors (RTI) stavudine, lamivudine, and nevirapine and patients were treated accordingly. Nucleoside reverse transcriptase inhibitors (NRTI) appear to have little *in vitro* effect on virulence traits of *C. albicans* (Ahmadou Ahidjo et al. 2008). Although the genome of *C. albicans* contains several transcriptionally active genes with similarities to reverse transcriptase (e.g. the “zorro element” family) which appear to be involved in the process of filamentous growth and are lacking from most non-*C. albicans* yeast spp. (Goodwin et al. 2001). This could explain the rise in non-

C. albicans yeast *spp.* under this particular treatment, although no particular data are available on interaction of these *C. albicans* proteins with NRTIs. Therefore, in the absence of PIs and antifungal treatment, the improvement of the immune function under HAART with increased CD4 T cell counts and decreased viral loads is likely to be responsible for the decrease of OC (Fethi et al. 2005; Sanchez-Vargas et al. 2005a; Fidel 2006; Yang et al. 2006; Ortega et al. 2009; Wu et al. 2011) and the emergence of non-*C. albicans* yeast *spp.* colonizing the oral cavity of HIV-infected patients (Nweze and Ogbonnaya 2011).

HIV-negative subjects also presented with lower prevalence of *C. albicans* and higher diversity of non-*C. albicans* yeast *spp.* which is similar to the reports from Hauman, Mc Collough, Xu and Mitchell and Pomarico (Hauman et al. 1993; McCullough 2001; Xu and Mitchell 2003; Pomarico et al. 2009). A review giving an overview over oral fungal infection in Africa showed that the resident oral yeast flora of HIV-negative individuals in resource-poor countries is markedly different from that in developed countries with non-*C. albicans* yeast *spp.* being more prevalent (Hodgson and Rachanis 2002). This is in agreement with our study and a report from China in 2002 (Xu and Mitchell 2003): in healthy HIV-negative individuals from villages in China without antifungal history, the prevalence of *C. albicans* was only 9.4% compared to 77-84% in healthy HIV-negative individuals from Hong Kong (Sedgley and Samaranayake 1994). Poor oral hygiene and no access to dental health care or even malnutrition in the studied regions may favor colonization by non-*C. albicans* yeast *spp.* (Jabra-Rizk et al. 2001). It has been further suggested that the wider spectrum of yeast species observed in antifungal naive HIV-negative individuals may reflect "an ancestral human-yeast association" and that development factors as industrialization, lifestyle and regular dental health care "may have favored the selection of *C. albicans* over other species" (Xu and Mitchell 2003). This is supported by the results of a study performed among an indigenous population in a remote area of French South Guiana, where it was found that the prevalence of colonizing non-*C. albicans* yeast *spp.* was far higher than *C. albicans* (Angebault et al. 2013). The fact that *C. albicans* is more prevalent in HIV-positive patients in Africa, may be due to the fact, that *C. albicans* is more pathogenic than other *Candida* species and highly associated with infection by the HI virus (Redding 2001; Agwu et al. 2011). Furthermore as extensive antifungal therapy and the availability of HAART are often rare in Africa, the shift

towards non-*C. albicans* yeast spp. is less marked in African countries and *C. albicans* colonization more prevalent.

***Candida dubliniensis*: an HIV-associated species?**

C. dubliniensis has repeatedly been reported to be highly prevalent in HIV-infected patients (Paugam et al. 2008). We did not find a single isolate of this species among our patients. However, most of the studies in which *C. dubliniensis* has been found have been conducted outside of the African continent (Sullivan et al. 1995; Binolfi et al. 2005; Loreto et al. 2010; Wu et al. 2011) and studies performed on the African continent in Tanzania, Uganda and Nigeria showed the same phenomenon like in our investigation (Enwuru et al. 2008; Hamza et al. 2008; Agwu et al. 2011). Technically, the absence of *C. dubliniensis* may have been due to the media we used for initial culture, where *C. dubliniensis* could not be discriminated from *C. albicans* and a lower number of *C. dubliniensis* colonies may have gone unnoticed. However, the absence of *C. dubliniensis* is in line with the hypothesis, that Africans may be less susceptible to colonization or infection with this species as shown in an South African cohort (Blignaut et al. 2003).

Under Fluconazole therapy, *C. albicans* may be replaced by *C. dubliniensis* (Martinez et al. 2002) as a colonizer of the oral cavity. This is supported by a study performed in India among terminally ill and patients not responding to Fluconazole therapy, where *C. dubliniensis* was the second most prevalent fungal species after *C. albicans* (Nadagir et al. 2008). Most of these subjects had been exposed to prolonged Fluconazole therapy. A rare practice of antifungal drug prophylaxis or extensive exposure to azoles in developing countries might be another explanation for the absence or very low prevalence (Hamza et al. 2008; Kwamin et al. 2013) of *C. dubliniensis* in low-resource settings.

In further contrast, *C. dubliniensis* has also been found to be more prevalent among HIV-negative subjects in studies in Tunisia (Khlif et al. 2009) and Brazil (Back-Brito et al. 2009) and in South African White healthy individuals (Blignaut et al. 2003) than in HIV-positive subjects. The epidemiology of this new species is therefore so far not yet well understood and needs further investigations.

Oral fungal burden and OC

A high fungal burden was highly associated with the presentation of severe symptoms, colonization with the species *C. albicans* and CD4 T cell counts <200 CD4 T cells/ μ l irrespectively of HAART. These findings were comparable with the ones found in a study evaluating the clinical oral state of patients with the quantitative growth of *C. albicans*; patients with OC had higher CFU counts than *Candida* carriers although CFU counts in that study were counted from saliva (Epstein et al. 1980). Similarly, in a recent study from South Africa, high oral *Candida* CFU correlated with low CD4 T cell counts in HAART+ patients (Owotade et al. 2013). Patients with mild symptoms, defined as whitish or yellowish coating of the dorsum of the tongue <50% of the area, were less likely to be yeast-positive and if yeast-positive, the number of CFU counts was very low. This would emphasize that our patients classified with mild symptoms and a positive yeast culture were less suspect to suffer from OC, but were more likely to be colonized only.

Independently of the CFU counts and irrespectively of HAART, OC, especially with the features of the pseudomembranous candidiasis, was highly associated with CD4 T cell counts <200 CD4 T cells/ μ l and the isolation of *C. albicans*. This was not always as conclusive in patients with mild symptoms. As shown in several studies conducted in Tanzania (Matee et al. 2000), Ghana (Kwamin et al. 2013) and India (Lattif et al. 2004; Mane et al. 2010; Anwar Khan 2012), OC was highly associated with CD4 T cell counts <200 CD4 T cells/ μ l and as shown in a study in Brazil (Witzel et al. 2008) with isolation of *C. albicans*.

All our patients with severe symptoms and positive yeast growth were colonized by *C. albicans*, except for one case: here, the patient was colonized by *P. fabianii* and had the clinical features of the median rhomboid glossitis. The patient was HIV+/HAART- and had a CD4 T cell count of 495 CD4 T cells/ μ l. Median rhomboid glossitis is an HIV associated lesion and the patient had high oral fungal burden; nevertheless, we would rather suggest an oral yeast colonization in that case as *P. fabianii* is not common in causing infection (Dabas 2013). Although erythematous candidiasis may as well be a good marker of immunosuppression as postulated by Bodhade et al. (2011), it is not as sensitive and specific as the pseudomembranous candidiasis (Patton 2000). Nevertheless, when HIV-positive subjects present with severe oral lesions (as e.g. OC) a progression of immune failure or a signal for

therapy failure in HIV+/HAART+ patients with a dropping CD4 T cell count <200 CD4 T cells/ μ l should be suspected, especially when CD4 T cell counts are not available. In the majority, OC can be diagnosed by the clinical appearance itself, but in HIV+/HAART+ patients, the clinical impression of white coating on the tongue giving suspicion of OC may be misleading. In our study, in almost 60% of our HIV+/HAART+ patients with severe symptoms no yeasts were found in the oral cavity, indicating, that antifungal treatment was not necessary. Therefore, analysis of further symptoms, like burning sensation, cotton taste, swallowing pain as well as the microbiologic diagnosis leading to the identification of *Candida* can help to identify patients in need of antifungal or antiretroviral treatment.

For HIV+/HAART- patients with severe symptoms, the probability of OC was higher. Here, 60 % of our HIV+/HAART- patients with severe symptoms were yeast-positive, had a mean CD4 T cell count <200 CD4 T cells/ μ l and were mainly colonized with *C. albicans*. Anyhow, HIV+/HAART- patients may be severely immunocompromised without presenting any clinically visible lesions. Here, 14% were asymptomatic yeast carriers of which more than 50% had CD4 T cell counts <200 CD4 T cells/ μ l.

Impact of age and gender

Although a significant age influence on oral yeast colonization and infection could not be shown here, a tendency towards higher colonization rate with rising age and drop again after the age of 45 could be observed in both, HIV+/HAART- and HIV-negative subjects. This phenomenon has also been reported in Jordanian patients (Rawashdeh et al. 2011). In HIV+/HAART+ patients the observation was reverse. The younger group of patients aged from 18-25, which also was the group being sexually more active at highest risk for the HIV infection, had the highest prevalence of positive yeast growth. With rising age the yeast prevalence was decreasing, also correlating with the duration of HAART.

We did not find any significant differences according to the gender, only that the oral fungal burden was higher in male than in female patients. Alcohol consumption and smoking, which may influence oral candidal colonization and overgrowth (Nittayananta et al. 2001; Petruzzi et al. 2013), is more common among men; this fact and that HIV-infected male patients would only present themselves at the HIV health care center in a more advanced stage of the disease, thus have more severe symptoms and a higher oral fungal burden, could explain the difference observed. In

a study among HIV-positive patients in Thailand, men were also significantly more likely to have oral lesions than women (Nittayananta et al. 2001).

Susceptibility and effectivity of antifungal treatment

In our setting, a basic selection of drugs was available to treat and prevent the most common AIDS-related opportunistic diseases, including a limited supply of antimycotics. During the study period, several patients were treated with antimycotics (oral Nystatin, Ketoconazole, or Fluconazole). All patients with severe symptoms and positive yeast carriage treated during the study period and seen for a follow-up were yeast free after treatment, including those under Fluconazole prophylaxis, confirming the absence of clinically resistant species. The observed effectivity of antifungal treatment seen in patients with severe symptoms treated during the study period was reflected in the *in vitro* susceptibility testing. All *C. albicans* isolates were susceptible to the antifungals tested. Decreased susceptibility was seen within the epidemiologic cut-offs for intrinsic resistance to azoles in *C. glabrata*, *C. tropicalis* and *I. orientalis* especially for Itraconazole (Pfaller et al. 2006). Otherwise, all the species were susceptible for Fluconazole, except for *I. orientalis*. That more species were resistant to Itraconazole may be due to the fact that Ketokonazole, which has a closer structural topology to Itraconazole, had been more available than Fluconazole in the clinic. Increased MIC values towards Caspofungin were determined for species of the *C. parapsilosis* complex, as also described previously (Badiie et al. 2010). Azole exposure has previously been described to lead to a shift in the pattern of colonizing yeast species towards less susceptible species like *C. glabrata* or *I. orientalis* (Cartledge et al. 1999; Hope et al. 2002; Snyderman 2003; Hamza et al. 2008; Agwu et al. 2011). However, all of our patients harboring *C. glabrata* or *I. orientalis* had no previous history of antifungal treatment. A relationship between antifungal treatment and emergence of non-*C. albicans* yeast spp. was therefore not apparent among the patients from Chad, but rather a correlation with antiretroviral therapy and higher CD4 T cell counts.

5. Summary

The study was performed to determine the prevalence and epidemiology of oral candidiasis among HIV-positive patients in Chad and evaluate the susceptibility of the yeasts found in the oral cavity to five antifungals.

The prevalence of oral candidiasis (10.2%) and colonization (25.4%) among HIV-positive patients in the studied area of Chad was surprisingly low. Several factors may have contributed to this result: (i) the fact that it was a specialized center with a high coverage of HAART and eventually a high prevalence of patients in early stages of HIV infection, (ii) a general low colonization rate among the population and (iii) use of medicinal plants or beverages which may have some antimicrobial effect.

The main types of oral candidiasis seen were an atypical type of pseudomembranous candidiasis and the median rhomboid glossitis. Last type was mainly seen in HIV+/HAART- patients. High fungal burden was highly associated with oral candidiasis, CD4 T cell counts <200 CD4 T cells/ μ l and the presence of *C. albicans*. There were no cases of oral candidiasis caused by non-*C. albicans* yeast spp.

Candida albicans was the predominant species found in all subgroups with the highest prevalence in HIV+/HAART- patients. HIV-negative as well as HIV+/HAART+ patients had a higher yeast species diversity, but no *C. dubliniensis* was found. HAART significantly reduced the rate of oral candidiasis and colonization with yeasts, and was associated with a shift towards non-*C. albicans* yeast spp. correlating with the duration of HAART.

Clinically, neither antifungal drug resistance nor therapy refractory cases were found, but higher MICs for azoles in the species *C. glabrata*, *I. orientalis* and *C. tropicalis* were present. The emergence of non-*C. albicans* yeast spp. was not associated with antifungal treatment, but was rather the result of a recovered immune system under extended HAART with a rise in CD4 T cell counts.

In conclusion, our results suggest that (i) severe oral lesions as oral candidiasis are strong markers of immunodeficiency and may be used to evaluate the patient's immune status and guide therapy, (ii) oral candidiasis is associated with the species *Candida albicans*, (iii) antifungal resistance and infection caused by non-*C. albicans* yeast spp. is not yet a point of concern in Chad; Fluconazole may be continued to be used as first-line treatment if available.

6. Appendix

6.1 Ethical committee of Chad (AILS)

CONFERENCE EPISCOPALE DU TCHAD

ASSOCIATION INTERDIOCESAINE DE LUTTE CONTRE LE SIDA (AILS)

COORDINATION

BP 454 NDJAMENA - TCHAD

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TO WHOM IT MAY CONCERN

This is to confirm that MRS LILIANE TAVERNE, a medical student from the University of Göttingen, Germany, undertook a research study in our facility, Medical Center of Maingara, Sarh, Chad, to determine the epidemiology of candidosis in HIV infected patients in Chad.

The study was conducted in collaboration with the University Medical Centre of Göttingen.

The research which was done during the period of April – June 2007, was undertaken in accordance with the ethical and medical requirements of the Medical Center.

During the research period, MRS LILIANE TAVERNE was under the tutorship and guidance of DR LYDIA KERSCH, the medical doctor in charge of the Medical Center.

À QUI DE DROIT

Par la présente il est confirmé que Madame LILIANE TAVERNE, étudiante à la Faculté de Médecine de l'Université de Göttingen/ Allemagne, a réalisé l'étude de recherche scientifique dans notre structure médicale, le Centre de Santé de Maingara à Sarh/Tchad en vue d'examiner la situation épidémiologique des candidoses buccales chez les malades vivant avec le VIH/SIDA.

L'étude a été menée en collaboration avec la Faculté de Médecine de l'Université de Göttingen.

Les recherches ont été effectuées durant la période d'avril – juin 2007 et elles correspondent aux exigences éthiques et médicales du Centre de Santé.

Durant la période de l'étude, Madame LILIANE TAVERNE a travaillé sous la responsabilité du Dr. LYDIA KERSCH, médecin responsable du Centre de Santé de Maingara.

Le VICE PRESIDENT DE L'AILS


DR MBATOLOUM WEINA

6.2 Informed consent document

Ville / Jour / Mois / Année

..... / / /

OBJET : Accord écrit pour le projet « Infection de la muqueuse orale chez des patients atteints par le virus du SIDA »

Je soussigné (e), Nom:

Prénom:

Sexe :

Age:

Nationalité :

Adresse :

déclare participer de mon plein gré (sans contrainte d'une tierce personne) à *l'étude concernant l'apparition de la colonisation de la muqueuse orale par le champignon de levure « Candida albicans » et l'évaluation de sa prévalence en rapport avec l'infection par le virus du SIDA.*

Je suis bel et bien informé (e) que cette étude à laquelle je participe est une étude simplement descriptive qui n'intervient en aucun cas dans le traitement standard pratiqué à l'hôpital.

D'autre part, il m'a été certifié que le médecin en charge me fera un prélèvement buccal avec un bâtonnet enroulé du coton. Quant au traitement standard appliqué à l'hôpital, il restera le même. Après une période d'observation, dont la durée sera fixée par le médecin, le (la) patient (e) que je suis, devra se présenter de nouveau pour un deuxième prélèvement buccal.

Signé le / / 2007 à

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6.5 List of abbreviations

AIDS	Acquired immunodeficiency syndrome
AILS	Association Interdiocesaine de Lutte contre le SIDA
API	Analytical profile index
ATCC	American Type Culture Collection
C.	<i>Candida</i>
<i>C. a; C. albicans</i>	<i>Candida albicans</i>
CBS	Center for Biological Sequence analysis
CCUG	Culture Collection University of Göteborg (Sweden)
CD4	Cluster of differentiation 4
CDC	Center of Disease Control
CFU	Colony-forming units
CIA	Central Intelligence Agency
CLSI	Clinical and Laboratory Standards Institute
CNLS	Conseil National de Lutte contre le SIDA
DE	Deutschland (Germany)
DNA	Deoxyribonucleic acid
EC	Erythematous candidiasis
ECC	EC-Clearinghouse
ECG	Electrocardiogramm
et al.	et alia (latin: and others)
HAART	Highly active antiretroviral therapy
HCCA	Alpha-cyano-4-hydroxy cinamic acid
HFB	High fungal burden

HIV	Human immunodeficiency virus
Hz	Hertz
IL	Israel
<i>I. orientalis</i>	<i>Issatchenkia orientalis</i> (=C. krusei)
IT	Italy
ITS2	Internal transcribed spacer region 2
kDa	kilo Dalton
LCBC	Lake Chad Bassin Commission
LFB	Low fungal burden
MALDI-TOF	Matrix assisted laser desorption/ionization in time of flight
MIC	Minimal inhibiting concentration
MRG	Median rhomboid glossitis
NAM	National AIDS Manual
NCCLS	National Committee for Clinical Laboratory Standards
nm	nanometer
NRTI	Nucleosid reverse transcriptase inhibitor
OC	Oral candidiasis
<i>P.</i>	<i>Pichia</i>
PC	Pseudomembranous candidiasis
PCR	Polymerase chain reaction
PI	Protease inhibitor
rDNA	Ribosomal deoxyribonucleic acid
RPMI	Roswell Park Memorial Institute
SARAMIS	Spectral Archive and Microbial Identification System
<i>Spp.</i>	Species

UNAIDS	United Nations Programme on HIV/AIDS
UNDP	United Nations Development Programme
UNGASS	United Nations General Assembly
UK	United Kingdom
US	United States
WHO	World Health Organization
WHOPAR	World Health Organization Public Assessment Report
WMA	World Medical Association
YnoS	Yeast carriers without symptoms
YmS	Yeast-carriers with mild symptoms
YsS	Yeast-carriers with severe symptoms

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